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Emilia Pauline Liao *Editor*

Extraskeletal Effects of Vitamin D

A Clinical Guide

Humana Press

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Series Editor

Leonid Poretsky
Division of Endocrinology
Lenox Hill Hospital
New York, NY, USA

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Series Editor Foreword

Vitamin D is both a vitamin and a hormone that is well known to play an important role in bone and calcium metabolism. Recently, however, vitamin D deficiency has been associated with a number of diseases, including such extremely common and important conditions as cardiovascular disease, diabetes, and cancer. Association, however, does not imply causation. A controversy therefore exists regarding the effectiveness of supplementing vitamin D in extraskeletal abnormalities associated with vitamin D deficiency and whether normalizing vitamin D levels improves outcomes in extraskeletal disorders.

Dr. Emilia Pauline Liao has edited a volume that explores this still new and developing area. The authors of chapters that comprise this monograph are international authorities on the subjects of their chapters. Although not all questions have definitive answers at this point, the monograph summarizes this emerging field extensively and provides the reader with up-to-date, evidence-based information.

The book is recommended to all those interested in vitamin D physiology and pathophysiology. The editor and all contributors are to be congratulated on their excellent effort in reviewing this developing field comprehensively and with commendable objectivity.

New York, NY — Leonid Poretsky, MD

Preface

In 2003, I was starting my academic career at a time when research in vitamin D was exploding. Vitamin D had long been known for its vital role in mineralizing bone, but low levels of vitamin D were now being reported in healthy populations, not just nursing home residents and the home bound, and vitamin D deficiency was associated with diseases involving every organ system. Was vitamin D just a marker of health, or was it truly implicated in the pathogenesis in diseases as varied as tuberculosis, multiple sclerosis, hypertension, and cancers? And could vitamin D ameliorate all these diseases?

Over 10 years later, there is still compelling evidence for vitamin D's role in many complex diseases, but there are also many unanswered questions. In this book, we review the current data on the role of vitamin D in autoimmune disease, infectious disease, various cancers, reproductive function, diabetes, cardiovascular disease, and neurologic disease. This book is a clinical review, with discussion on the mechanistic actions vitamin D may effect in these conditions.

I am grateful to all the authors for their time and contributions and for making the daunting task of assembling this book (my first) easier and also enjoyable. I also thank Len Poretsky for his mentorship and friendship.

New York, NY — Emilia Pauline Liao

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Contributors

Jungmi Ahn, PhD Department of Molecular Medicine/Institute of Biotechnology, School of Medicine, The University of Texas Health San Antonio, San Antonio, TX, USA

Dimiter Avtanski, PhD Endocrine Research Laboratory, Department of Medicine, Lenox Hill Hospital, New York, NY, USA

Shir Azrielant, MD Sheba Medical Center, The Zabludowicz Center for Autoimmune Diseases Ramat-Gan, Israel

Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

Daniel D. Bikle, MD, PhD Medicine and Dermatology, VA Medical Center and University of California San Francisco, San Francisco, CA, USA

Geoffrey Brown, BSc, PhD Institute of Clinical Sciences, Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham Edgbaston, Birmingham, UK

Rupinder S. Buttar, MD Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

Bandana Chatterjee, PhD Research, South Texas Veterans Health Care System (STVHCS), San Antonio, TX, USA

Foundation for Advancing Veterans' Health Research, Audie Murphy VA Hospital, San Antonio, TX, USA

Department of Molecular Medicine/Institute of Biotechnology, School of Medicine, The University of Texas Health San Antonio, San Antonio, TX, USA

Albert Do, MD, MPH Department of Medicine, Yale University School of Medicine, New Haven, CT, USA

Anabel Garcia, MD Division of Endocrinology, Lenox Hill Hospital, New York, NY, USA

Francesca Gimigliano, MD, PhD Department of Mental and Physical Health and Preventive Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy

Victoria Hensley, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

Giovanni Iolascon, MD Department of Medical and Surgical Specialties and Dentistry, University of Campania "Luigi Vanvitelli", Naples, Italy

Caleb Killer, BS Research, South Texas Veterans Health Care System (STVHCS), San Antonio, TX, USA

Foundation for Advancing Veterans' Health Research, Audie Murphy VA Hospital, San Antonio, TX, USA

Andrzej Kutner, BSc, PhD, DSc Department of Pharmacology, Pharmaceutical Research Institute, Warsaw, Poland

Emilia Pauline Liao, MD Division of Endocrinology, Department of Medicine, Hofstra Northwell School of Medicine, Lenox Hill Hospital, Northwell Health, New York, NY, USA

Ewa Marcinkowska, MD, PhD Laboratory of Protein Biochemistry, Faculty of Biotechnology, University of Wroclaw, Poland

Michal L. Melamed, MD, MHS Department of Medicine (Nephrology), Albert Einstein College of Medicine/Montefiore Medical Center, Bronx, NY, USA

Antimo Moretti, MD Department of Medical and Surgical Specialties and Dentistry, University of Campania "Luigi Vanvitelli", Naples, Italy

Ellen M. Mowry, MD, MCR Johns Hopkins School of Medicine, Baltimore, MD, USA

Anindita Nandi, MD Icahn School of Medicine, Graduate School of Biomedical Sciences, New York, NY, USA

Sulgi Park, MS Research, South Texas Veterans Health Care System (STVHCS), San Antonio, TX, USA

Department of Molecular Medicine/Institute of Biotechnology, School of Medicine, The University of Texas Health San Antonio, San Antonio, TX, USA

Petr Protiva, MD, MPH Department of Medicine, Yale University School of Medicine, New Haven, CT, USA

Department of Medicine, VA Connecticut's Healthcare System, West Haven, CT, USA

Samantha Roman, MD Johns Hopkins School of Medicine, Baltimore, MD, USA

Yehuda Shoenfeld, MD, FRCP, MaACR Sheba Medical Center, The Zabludowicz Center for Autoimmune Diseases, Ramat-Gan, Israel

Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

Incumbent of the Laura Schwarz-Kip Chair for Research of Autoimmune Diseases, Tel-Aviv University, Tel-Aviv, Israel

Kaushal Sondarwa, MD Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

Letizia Stefano, MD Department of Medical and Surgical Specialties and Dentistry, University of Campania "Luigi Vanvitelli", Naples, Italy

Christian Wejse, MD, PhD Department of Infectious Diseases, Aarhus University Hospital, Aarhus, Denmark

Center for Global Health, Dept of Public Health, Aarhus University, Aarhus, Denmark

Cecilie Blenstrup Patsche, BSc Department of Infectious Diseases, Aarhus University Hospital, Aarhus, Denmark

Center for Global Health, Dept of Public Health, Aarhus University, Aarhus, Denmark

Chapter 1
Vitamin D Biochemistry and Physiology

Daniel D. Bikle

Introduction

With the findings that both the vitamin D receptor (VDR) and the enzyme (CYP27B1, the 1α-hydroxylase) required to convert the major circulating metabolite of vitamin D (25-hydroxyvitamin D (25(OH)D)) to the most biologically active metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), are found in many if not all cells, interest in vitamin D metabolism and mechanisms of action has exploded. Much of this interest is attributed to the potential for vitamin D metabolites and analogs to affect not only the regulation by the classic tissues—the intestine, bone, and kidney—of bone and mineral metabolism but also that of most tissues and their functions not necessarily related to bone and mineral metabolism. This interest is further piqued by the observations from advanced genomic techniques such as RNA-seq and ChIP-seq that the VDR has thousands of binding sites throughout the genome affecting the transcription of hundreds of different genes, and the profile of the affected genes shows substantial diversity among the different cell types. In this chapter, I will first discuss vitamin D production and metabolism, then focus on the mechanism of action of $1,25(OH)_2D$, and conclude with a discussion of the impact of the vitamin D metabolites on a representative sampling of different tissues, both the classic and nonclassic.

D.D. Bikle, MD, PhD
Medicine and Dermatology, VA Medical Center and University of California San Francisco, San Francisco, CA, USA
e-mail: Daniel.bikle@ucsf.edu

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Vitamin D Production

The skin contains substantial amounts of 7-dehydrocholesterol (7-DHC, provitamin D_3), which when irradiated by UV light (UVB spectrum 280–320), typically from the sun, undergoes a two-step process to form vitamin D_3 (D_3) (cholecalciferol) (Fig. 1.1). In the first step, UVB breaks open the B ring of 7-DHC forming pre-D_3 that isomerizes to D_3 in a thermosensitive but noncatalytic process. Both UVB intensity and skin pigmentation level contribute to the extent of D_3 formation [1]. Skin pigmentation and other chromogenic agents that can absorb UVB block D_3 production, as do clothing and sunscreen. Moreover, both season and latitude affect the intensity of UVB from sunlight so that those living at higher latitudes have a shorter period of the year in which sunlight is capable of producing D_3 [2]. Vitamin D can also be obtained from the diet. Most foods with the exception of fatty fish contain little vitamin D unless fortified, whereas fish contain only D_3, which they obtain from plankton (or from the ingestion of other fish). Plants and fungi (e.g., mushrooms) contain ergosterol, which, similar to D_3 production from 7-DHC, is

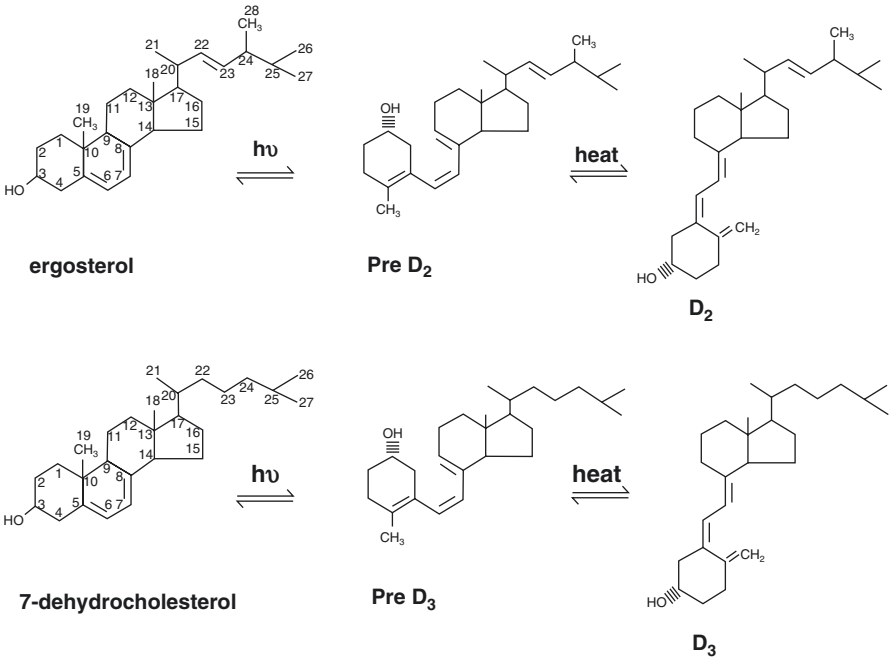

Fig. 1.1 Vitamin D Production. Vitamin D_3 is produced in the skin from 7-dehydrocholesterol (7-DHC). The B ring of 7-DHC is broken by ultraviolet light B (UVB) to form previtamin D_3 which isomerizes in a temperature-dependent process to form vitamin D_3. In the same way, ergosterol in plants and fungi when exposed to UVB forms previtamin D_2 that isomerizes to vitamin D_2. Vitamin D_2 differs from vitamin D_3 only in the side chain in having a double bond between C22 and C23 and a methyl group at C24

converted to D_2 (ergocalciferol) by UVB via the same two-step process. D_2 differs from D_3 in having a double bond between C22 and C23 and a methyl group at C24 in the side chain (Fig. 1.1). Many foods such as milk and orange juice are fortified by D_2. Knowledge of which vitamin D is being consumed either as supplements or in fortified foods is important because their pharmacokinetics, metabolism, and measurement of their metabolites by various immunoassays differ. The structural differences between D_2 and D_3 in the side chain affect their affinity for DBP resulting in faster clearance of D_2 from the circulation, limit the conversion of D_2 to 25(OH)D_2 by at least some of the 25-hydroxylases to be described subsequently, and alter its catabolism by the 24-hydroxyase (CYP24A1) [3–5]. As such, equivalent amounts of D_2 do not give as high or as long-lasting level of 25(OH)D as does D_3 [6]. That said, the active metabolites of D_2 and D_3, namely, 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$, have comparable affinities for the VDR [4] and are thus expected to have comparable biologic activity. Therefore, in this review, if no subscript is employed, both D_2 and D_3 and their metabolites are being considered.

Vitamin D Metabolism

The three main steps in vitamin D metabolism, 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation, are all performed by cytochrome P450 mixed function oxidases (CYPs). These enzymes are located either in the endoplasmic reticulum (ER) (e.g., CYP2R1) or in the mitochondria (e.g., CYP27A1, CYP27B1, and CYP24A1). The ER enzymes utilize nicotinamide adenine dinucleotide phosphate (NADPH)-dependent P450 reductase as their electron donor, whereas the electron donor for mitochondrial enzymes is a complex of ferredoxin and ferredoxin reductase. Only the cytochrome P450 of the enzyme complex is specific for the substrate being hydroxylated. At this point, only CYP2R1 and CYP24A1 have been crystallized, but their homology with the other vitamin D-metabolizing enzymes suggests common structural features. These include 12 helices (A–L) and loops and a common prosthetic group, namely, the iron-containing protoporphyrin IX (heme) linked to the thiolate of cysteine. The I helix traverses the center of the enzyme above the heme where a threonine or serine and aspartic or glutamic acid pairing is essential for catalytic activity [7]. The ER enzyme CYP2R1 contains additional two helices thought to form a substrate channel in the bilayer of the ER [7] with the B′ helix serving as a gate closing on substrate. It is not known if such structures exist in the mitochondrial enzymes. With this overview, consideration of the individual enzymes follows.

25-Hydroxylase. The liver is the major but not the sole source of 25OHD production from vitamin D. A number of enzymes (all CYPs) have been shown to have 25-hydroxylase activity. CYP27A1 is the only known mitochondrial 25-hydroxylase, initially identified as a sterol 27-hydroxylase involved in bile acid synthesis. Its tissue distribution is wide, not limited to the liver. A number of studies have cast doubt on its being the main 25-hydroxyase. First of all, CYP27A1 does

not 25-hydroxylate D_2. Secondly, at least in the mouse, deletion of CYP27A1 actually increases 25OHD levels [8]. Finally, mutations of CYP27A1 in humans result in cerebrotendinous xanthomatosis with abnormal bile and cholesterol metabolism, but not rickets [9]. More recently, a microsomal 25-hydroxylase, CYP2R1, was identified in mouse liver [10]. CYP2R1 25-hydroxylates both D_2 and D_3 with comparable kinetics. Its expression is more tissue limited (primarily liver and testes), and this expression is increased in mice in which CYP27A1 is deleted, probably accounting for the rise in 25(OH)D in the CYP27A1-null mouse. In contrast to the CYP27A1 deletion, deletion of CYP2R1 does reduce 25(OH) (by 50%), but not to zero [8]. Even if both CYP2R1 and CYP27A1 are deleted, the blood level of 25OHD does not fall to zero. Moreover, these deletions do not significantly affect circulating levels of calcium and phosphate [8] indicating compensation by other enzymes with 25-hydroxylase activity. That said, a human mutation in CYP2R1 (leu99pro) was found in a Nigerian with severe bone disease associated with biochemical evidence of rickets [11], and in vitro testing determined that this mutation had a significant effect on CYP2R1 activity. Other enzymes including the drug-metabolizing enzyme CYP3A4 have 25-hydroxylase activity [12], but CYP2R1 appears to be the major 25-hydroxylase.

1α-Hydroxylase. The kidney is not the only tissue capable of producing $1,25(OH)_2D$, although it is the major source of circulating levels of $1,25(OH)_2D$. CYP27B1 is the only enzyme recognized to have 25-OHD 1α-hydroxylase activity as proven by its cloning by several laboratories from different tissues [13–16]. Mutations in CYP27B1 cause a hereditary form of rickets known as pseudo-vitamin D deficiency due to inadequate $1,25(OH)_2D$ production. These individuals respond to $1,25(OH)_2D$ but not to vitamin D itself [13–16]. CYP27B1 is highly homologous with the mitochondrial CYPs involved with vitamin D metabolism: CYP27A1 and CYP24A1. As mentioned above, the kidney is not the only tissue expressing CYP27B1, and regulation of this critical enzyme for vitamin D metabolism differs among the tissues in which it is expressed [17]. Examples of extrarenal CYP27B1 expression include the epithelial cells in the skin, lungs, breast, intestine, and prostate; endocrine glands including the parathyroid gland, pancreatic islets, thyroid, testes, ovary, and placenta; cells of the immune system including macrophages, T and B lymphocytes, and dendritic cells; osteoblasts and chondrocytes; and a variety of tumors derived from these cells. In the kidney, CYP27B1 is tightly regulated primarily by three hormones, PTH, FGF23, and $1,25(OH)_2D$ itself (Fig. 1.2). PTH stimulates, whereas FGF23 inhibits CYP27B1. Increased levels of calcium and phosphate suppress CYP27B1 activity primarily by inhibiting PTH secretion (calcium) and stimulating FGF23 secretion from bone (phosphate), respectively, although these ions can have direct effects on renal CYP27B1 on their own [18, 19]. Whether $1,25(OH)_2D$ has a direct inhibitory effect on CYP27B1 in the kidney or regulates $1,25(OH)_2D$ levels indirectly remains unclear. $1,25(OH)_2D$ has been reported to inhibit CYP27B1 expression directly through a complex mechanism involving VDR and a vitamin D inhibitory receptor (VDIR) that brings both histone deacetylases (HDACs) and DNA methyl transferases to the promoter of CYP27B1 inhibiting its transcription [20]. This observation has not been confirmed by other

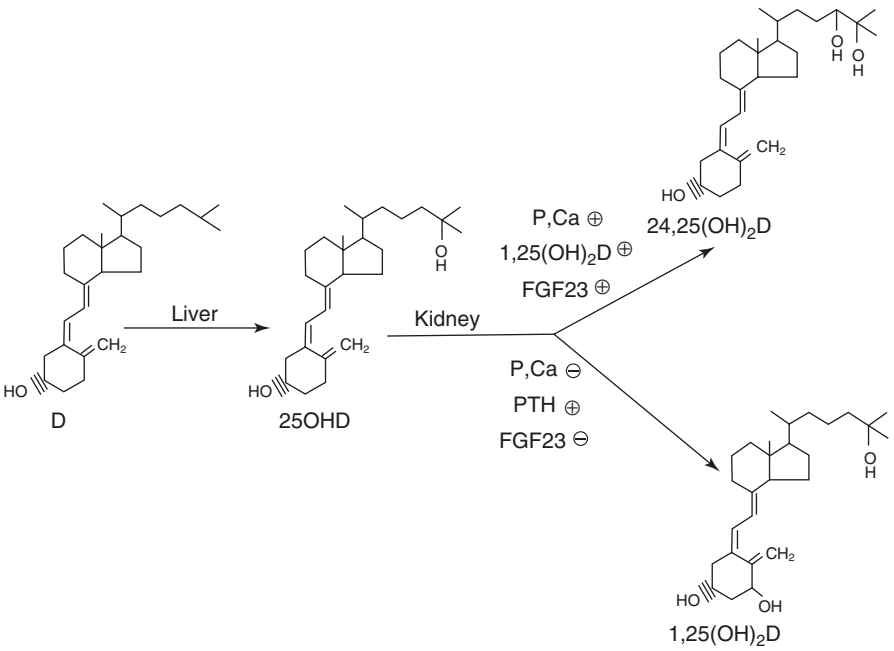

Fig. 1.2 Vitamin D metabolism. The liver converts vitamin D to 25-OHD. The kidney converts 25-OHD to 1,25-$(OH)_2$D and 24,25-$(OH)_2$D. Control of metabolism is exerted primarily at the level of the kidney, where low levels of serum phosphorus, calcium, and fibroblast growth factor 23 (FGF23) and high levels of parathyroid hormone (PTH) favor production of 1,25-$(OH)_2$D, whereas high serum levels of phosphorus, calcium, FGF23, and 1,25-$(OH)_2$D and low levels of PTH favor 24,25-$(OH)_2$D production

investigators. 1,25$(OH)_2$D acts indirectly by inhibiting PTH and increasing FGF23 secretion. Moreover, 1,25$(OH)_2$D induces CYP24A1 (see below) that metabolizes and thus reduces 1,25$(OH)_2$D levels as well as its precursor 25(OH)D.

Regulation of extrarenal CYP27B1 differs from that in the kidney. This has been best studied in keratinocytes and macrophages. In keratinocytes, neither PTH nor FGF23 seem to play a role. Moreover, 1,25$(OH)_2$D does not have a direct effect on CYP27B1 expression. Rather, 1,25$(OH)_2$D regulates its own levels in the keratinocyte by inducing CYP24A1 [16]. However, CYP24A1 induction by 1,25$(OH)_2$D and/or its function in macrophages is blunted [21]. The mechanism appears to involve the expression of a truncated form of CYP24, which includes the substrate-binding domain but not the mitochondrial targeting sequence [22]. Cytokines such as tumor necrosis factor-α (TNFα) [23] and interferon-γ (IGFγ) [24] appear to be the major regulators of CYP27B1 activity in the keratinocyte and macrophage [21, 25–27], although FGF23 has been shown to be inhibitory in monocytes [28]. In parathyroid cells, FGF23 is reported to stimulate CYP27B1 expression, opposite of its actions in the kidney [29].

24-Hydroxylase. Like CYP27B1, CYP24A1 is the only known 24-hydroxylase. This enzyme has both 24-hydroxylase and 23-hydroxylase activity, the ratio of

which is species dependent [30]. The human enzyme has both, but the rat enzyme is primarily 24-hydroxylase [31]. Single base pair mutations can shift the ratio of 23- to 24-hydroxylase activity [32]. The 24-hydroxylase pathway leads to the production of calcitroic acid, a biologically inert end product, whereas the 23-hydroxylase pathway leads to the biologically active 1,25–26,23 lactone. All steps are performed by one enzyme [31]. $1,25(OH)_2D$ and 25(OH)D are both substrates for CYP24A1. The initial product of CY24A1 metabolism of $1,25(OH)_2D$, $1,24,25(OH)_3D$, has approximately 1/10th the affinity of $1,25(OH)_2D$ for the VDR and has biologic activity. $24,25(OH)_2D$ may have biologic activity in the growth plate [33], although such a role is controversial. The biologic impact of deleting CYP24A1 results in defective mineralization of intramembranous (not endochondral) bone [34], but this appears to be due to large increases in $1,25(OH)_2D$ and not to a deficiency of $24,25(OH)_2D$ [34]. Inactivating mutations in CYP24A1 are one cause of idiopathic infantile hypercalcemia, which presents with severe hypercalcemia, hypercalciuria, and nephrocalcinosis, decreased PTH, low $24,25(OH)_2D$, and inappropriately normal to high $1,25(OH)_2D$ [35]. In this syndrome, the failure of CYP24A1 to control $1,25(OH)_2D$ levels appears to account for the phenotype.

Most tissues express CYP24A1, and increased expression is a nearly universal marker of $1,25(OH)_2D$ action on that tissue. The promoter of CYP24A1 contains two vitamin D response elements (VDREs) upstream of the transcriptional start site to which VDR/RXR bind along with other transcription factors [36]. More distant sites downstream of the human CYP24A1 gene to which histone acetyl transferases and RNA polymerase II are recruited have been shown to play a role in CYP24A1 induction [37]. In the kidney, CYP24A1 regulation is the reciprocal of that of CYP27B1 in that PTH limits the induction of CYP24A1 by $1,25(OH)_2D$ [38, 39], whereas FGF23 increases its expression [40]. FGF23 has a similar role in the uterus [41], but this has not been studied in other tissues. On the other hand, PTH enhances $1,25(OH)_2D$ induction of CYP24A1 transcription in osteoblasts through the same apparent mechanism, namely, the cAMP/PKA pathway, by which it reduces CYP24A1 induction in the kidney [42]. Thus, like the regulation of CYP27B1, the regulation of CYP24A1 can be tissue specific.

3-Epimerase. The C-3 epimers of the vitamin D metabolites have recently gained widespread attention mainly as contaminants in LC-MS/MS assays of these metabolites. This issue is particularly important in assessing 25OHD levels in infants where levels of the C-3 epimer of 25OHD can equal or exceed that of 25OHD [43]. However, these epimers have been recognized for decades. 3-Epimerase activity was first identified in keratinocytes where it produces the 3-epi form of $1,25(OH)_2D$ [44] but has also been found in colon cancer cells (Caco2), parathyroid cells, osteoblasts, and hepatocyte-derived cells (HepG2). Surprisingly, this epimer has not been found in renal preparations [43]. The enzyme has not yet been purified and so remains an activity that could be due to several enzymes. The 3-epimerase isomerizes the C-3 hydroxy group of the A ring from the alpha to beta orientation of all natural vitamin D metabolites. This does not affect subsequent metabolism but does reduce binding to DBP of the 3-epi form of 25(OH)D relative to 25OHD and binding to VDR of the 3-epi form of $1,25(OH)_2D$ relative to $1,25(OH)_2D$ [45]. Thus, the

C-3 epimers have reduced biologic activity in general [45], but, surprisingly, the 3-epi form of $1,25(OH)_2D$ appears to be equipotent to $1,25(OH)_2D$ with respect to PTH suppression [46]. The extra effort required to measure the C-3 epimers separately from the classic metabolites may prove necessary especially in children to accurately determine vitamin D status.

CYP11A1. CYP11A1, known also as the side chain cleavage enzyme, is a key enzyme essential for steroidogenesis. Recently, CYP11A1 has been shown to provide an alternative pathway for vitamin D activation converting vitamin D to 20(OH)D [47]. 20(OH)D, or its metabolite $20,23(OH)_2D$, appear to have activity similar to $1,25(OH)_2D$ at least for some functions. It is unclear whether these metabolites require further metabolism by CYP27B1 to be active. The biologic significance of this pathway remains unclear, as it does not compensate for animals lacking CYP27B1.

Transport of Vitamin D Metabolites in the Blood and Their Cellular Uptake

The vitamin D metabolites are transported in blood bound primarily to vitamin D-binding protein (DBP) (85–88%) and albumin (12–15%) [48–50]. The normal range of DBP concentrations is 4-8 μM, such that DBP is only about 1–2% saturated by normal levels of the vitamin D metabolites. DBP has high affinity for these metabolites ($Ka = 5 \times 10^8 M^{-1}$ for 25OHD and $24,25(OH)_2D$, $4 \times 10^7 M^{-1}$ for $1,25(OH)_2D$ and vitamin D). Thus, under normal circumstances, only approximately 0.03% 25OHD and $24,25(OH)_2D$ and 0.4% $1,25(OH)_2D$ are free [49–51]. Conditions such as liver disease, nephrotic syndrome, and acute illness resulting in reduced DBP and albumin levels will lead to a reduction in total 25OHD and $1,25(OH)_2D$ levels without necessarily affecting the free concentrations [52–55]. On the other hand, oral (not transdermal) estrogens and pregnancy [49] increase DBP levels and so may increase total levels of the vitamin D metabolites without increasing (and may even decrease) the free concentrations [49, 56]. High levels of 25(OH)D in cases of vitamin D intoxication can increase the degree of DBP saturation such that despite the normal levels of total $1,25(OH)_2D$, the free concentrations of $1,25(OH)_2D$ are increased [57] contributing to the hypercalcemia/hypercalciuria observed in these cases. DBP is a 58 kDa protein with 458 amino acids that is homologous to albumin and α-fetoprotein (αFP) (40% homology at the nucleotide level, 23% at the amino acid level) [58]. DBP like albumin and αFP is made primarily but not exclusively in the liver. Other sites include the kidney, testes, and fat.

Direct measurement of the free levels of the vitamin D metabolites becomes important if most cells take up only the free concentration, a hypothesis known as the free hormone hypothesis. An early articulation of this hypothesis comes from observations that patients with nephrotic syndrome had low levels of circulating thyroid hormone (assessed as PBI) and increased urinary losses of PBI but yet appeared clinically euthyroid [59]. This suggested to the authors that the supply of

hormone to the tissues in these patients was normal. Similar observations have recently been made in patients with nephrotic syndrome with regard to lack of changes in serum calcium, phosphate, PTH, and bone mineral density measurements despite lower vitamin D metabolite levels and increased urinary losses of DBP [53]. Similar conclusions regarding the importance of the free levels of vitamin D metabolites come from observations that the increase in $1,25(OH)_2D$ levels with administration of oral contraceptives or during the third trimester of pregnancy is associated with a parallel increase in DBP but not with changes in calcium metabolism, at least until the latter stages of pregnancy when the measured free levels of $1,25(OH)_2D$ increase despite the increase in DBP [49, 60]. The concept that the major role of DBP is as a blood transporter of the vitamin D metabolites is further demonstrated in mice in which the DBP gene was deleted. Although these mice lost substantial amounts of the vitamin D metabolites in the urine and their circulating levels of 25(OH)D were very low, they did not develop evidence of rickets until put on a low-vitamin D diet [61].

However, the free hormone hypothesis does not apply to all tissues. The renal tubule differs from most other tissues in its mechanism for at least 25(OH)D uptake and likely for all vitamin D metabolites. DBP and its bound 25(OH)D are filtered in the glomerulus and reabsorbed in the proximal tubule through endocytosis mediated by the megalin/cubilin complex. This provides 25(OH)D for further metabolism in the kidney tubule [62, 63]. The megalin/cubilin complex is not specific for DBP, but when megalin is deleted, the major protein lost in the urine is DBP, bone growth is slowed, and the skeleton is osteopenic [62]. Similar if less severe results were obtained with cubilin deletion [63]. The parathyroid gland and placenta also express megalin/cubilin [64], but at this point, experiments to determine the impact of either megalin or cubilin deletion from these tissues have not been reported.

Vitamin D Mechanism of Action

The best-known and most widely studied actions of vitamin D involve genomic actions regulated by $1,25(OH)_2D$ interacting with its receptor VDR. However, a growing body of literature is concerned also with the nongenomic actions of $1,25(OH)_2D$, some mediated also by VDR and others by a nonnuclear receptor variously named membrane-associated rapid response steroid (MARRS)-binding protein, ERp57/GRp58/ERp60, and protein disulfide isomerase family A member 3 (Pdia3). In this section, the genomic and nongenomic actions will be described separately.

Genomic actions. All genomic actions of $1,25(OH)_2D$ are mediated by the VDR. VDR is a transcription factor and member of the steroid hormone nuclear receptor family with which it has substantial homology especially in the DNA-binding domain. Based on the original cloning of the estrogen and glucocorticoid receptors, these nuclear hormone receptors were recognized to have six distinct domains: A–F. The A/B domain is the N-terminal region, known in other receptors as the

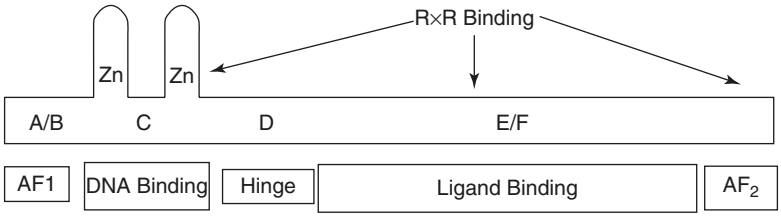

Fig. 1.3 Domains of the VDR. The N terminus of VDR, domains A/B, forms the short AF1 domain. The C domain contains the DNA domain containing the two zinc fingers. The D domain includes the hinge region. The E/F domains include the ligand-binding domain and the C terminal AF2 domain to which coactivators bind following ligand binding

activation domain 1. In VDR, this domain is quite short (24 amino acids), and in the f allele of the FokI polymorphism, it is further shortened by 3 amino acids [65]. The C domain is the DNA-binding domain with 65 amino acids containing 2 zinc fingers that bind to the grooves of the DNA at discrete sites called vitamin D response elements (VDREs). The highly flexible hinge region (domain D) with 143 amino acids separates the DNA-binding domain from the E/F domain (195 amino acids) that contains the ligand-binding domain and terminal activation domain (AF2). This domain also serves the function of dimerization with VDR partners (e.g., RXR) and binding of corepressors as well as coactivators (in AF2). These domains are illustrated in Fig. 1.3. The structure of the ligand-binding domain has been solved by X-ray crystallography [66]. It is comprised of 12 helices. Helix 12 serves as a gating mechanism closing around the incorporated ligand (i.e., $1,25(OH)_2D$) and forming an interface for coactivators and the nuclear hormone dimerization partners such as RXR. As mentioned above, the VDR binds to select regions in the genome called VDREs. The sequence of VDREs is highly variable, but those with the highest affinity for VDR are direct repeats of hexanucleotides with a spacing of three nucleotides between the half sites. This motif is called a DR3. VDR binding to its VDRE then recruits coregulatory complexes required for its genomic activity. These coregulatory complexes are required to remodel the chromatin, altering the condensation state by histone modifications to create binding sites for additional coregulatory complexes and facilitating the link to the RNA polymerase II to initiate transcription. The complexes that participate in these functions are the ATPase-containing SWI/SNF complex involved with remodeling the chromosome [67], complexes with activities that modify the histones via histone acetyl transferases (HATs) such as the coactivator CBP/P300 complex containing the steroid receptor coactivator family (SRC 1–3), histone methyl transferases (HMTs), and histone deacetylases (HDACs), which are part of the corepressor complexes of SMRT and NCoR, histone demethylases (DMTs) [68], and the mediator complex that is thought to link the RNA polymerase to the transcription start site [69]. The SRC and mediator complexes include a subunit that directly binds to the VDR generally through an LXXLL motif. Corepressors such as SMRT and NCoR, on the other hand, bind through a L*XXX*I*XXX*(I/L) motif. These complexes can be both gene and cell specific, enabling the selectivity of $1,25(OH)_2D$ action among cell types on which it acts.

The newer techniques of RNA-seq and ChIP-seq [70, 71] have markedly expanded our understanding of vitamin D mechanism of action at the genomic level. Moreover, the development of CRISPR/Cas9 to specifically and relatively quickly delete regions of the genome has enabled testing of the various putative regulatory regions of the genome for functional significance [72]. For example, in the mouse osteoblast, 1200 VDR binding sites were found under basal (i.e., no $1,25(OH)_2D$) conditions, whereas 8000 sites were observed following $1,25(OH)_2D$ administration [73]. In a separate study with human lymphoblastoid cell lines treated with $1,25(OH)_2D$, 2776 VDR binding sites were found altering the expression of 229 genes [74]. Although there is some overlap among different cell types, the profile of VDR binding sites and genes activated varies substantially from cell to cell as well as at different times after exposure to $1,25(OH)_2D$ in the same cell [75]. These VDR binding sites can be anywhere in the genome, often quite distant from the coding region of the gene being regulated, and just because VDR binds to a site does not mean that the site is functional with respect to regulation of the expression of that gene in that cell. Other transcription factors and their binding sites are generally found in association with VDR at its binding site. In osteoblasts, for example, these include RUNX2 and C/EBP α and β, among others [76, 77]. These sites often demonstrate a distinct epigenetic histone signature involving methylation and/or acetylation of lysines within H3 and H4 [78]. In general, a gene is regulated by more than one enhancer element [71], and the adjacent transcription factors may vary altering the regulation of that gene. An interesting example of this is the gene, *Tnfsf11*, that encodes RANKL. This gene is regulated by parathyroid hormone (PTH), a number of cytokines in addition to $1,25(OH)_2D$. It plays a role not only in osteoclast activation but in immune regulation and other cellular functions. Five strong VDR binding sites (D1–D5) were identified by ChIP-seq up to 75 kb upstream of the transcription start site [79]. PTH-induced CREB binding was found at some of these sites, and IL6-induced STAT3 binding was found at another. These sites in combination with additional sites even further upstream seem to regulate which cell (e.g., osteoblast or hematopoietic cell) and/or which hormone (PTH, cytokine, $1,25(OH)_2D$) regulates the expression of *Tnfsf1* [80, 81] in that cell. Thus, the key aspects of genomic regulation by VDR and its ligand $1,25(OH)_2D$ can be summed up as follows:

1. The profile of VDR binding sites in the genome varies from cell to cell and with time after $1,25(OH)_2D$ administration.
2. Most but not all binding sites require $1,25(OH)_2D$ for VDR binding.
3. The VDR binding sites are generally DR3 in which VDR binds in combination with RXR.
4. VDR binding sites can be located nearly anywhere in the gene and may be close to or thousands of base pairs away from the transcription start site.
5. The VDR binding sites are generally part of a cluster containing binding sites for a number of other transcription factors, which like the profile of the VDR binding sites themselves are cell specific.

Nongenomic actions. $1,25(OH)_2D$ also exerts effects that are too rapid to involve a genomic action. The earliest description of this nongenomic action involved rapid

stimulation of intestinal calcium transport in a vitamin D replete chick, called transcaltachia [82]. Of interest is that this phenomenon did not occur in a vitamin D-deficient chick indicating that the vitamin D-induced mechanisms for calcium transport need to be in place. Analogs of $1,25(OH)_2D$ that had no apparent genomic activity were as effective as $1,25(OH)_2D$ itself. Other examples emerged including effects on the chondrocytes in the growth plate [83] and keratinocytes in the skin [84]. Two receptors have been identified. One is the VDR itself albeit in a different configuration to enable binding by nongenomic VDR agonists [85]. The second is a novel receptor for $1,25(OH)_2D$ variably known as membrane-associated rapid response steroid (MARRS)-binding protein, ERp57/GRp58/ERp60, and protein disulfide isomerase family A member 3 (Pdia3) as mentioned earlier [86]. These receptors are located in the membrane within caveolae/lipid rafts [87] where they are poised to activate kinases, phosphatases, and ion channels. This latter receptor has not been crystallized so the binding of $1,25(OH)_2D$ to it is not known. On the other hand, the VDR has been crystallized, and the structure deduced indicated that the binding pocket in VDR would accommodate only agonists with a 6s-trans configuration. However, analogs with the 6s-cis configuration are active in inducing these nongenomic actions. Mizwicki and Norman [85] proposed an alternative model in which the 6s-cis analogs could fit into an alternative pocket in the VDR, although crystallographic evidence for this configuration has not been obtained. At this point, the physiologic significance of the nongenomic actions of $1,25(OH)_2D$ remains unclear, although deletion of the MARRS (Pdia3) gene from the intestine in vivo [88], from osteoblasts in vitro [89], or in heterozygotes (global knockouts are embryonic lethal) [90] does disrupt the rapid actions of $1,25(OH)_2D$ in those cells with altered intestinal calcium transport and bone and cartilage abnormalities in the relevant in vivo models.

Vitamin D Regulation of Cellular Function

In this section, I will first discuss the "classic" target tissues of vitamin D involved with bone mineral homeostasis, following which I will discuss the "nonclassic" tissues which although influenced by calcium regulation are not in themselves central to the regulation of calcium homeostasis.

Classic Vitamin D Target Tissues

*Intestine***.** Intestinal calcium absorption, in particular the active component of transcellular calcium absorption, is one of the oldest and best-known actions of vitamin D. Absorption of calcium from the luminal contents of the intestine involves both transcellular and paracellular pathways. The transcellular pathway dominates in the duodenum, and this is the pathway primarily regulated by $1,25(OH)_2D$ [91,

92]. Calcium entry across the brush border membrane (BBM) occurs down a steep electrical-chemical gradient and requires no input of energy. This is achieved by changes in membrane fluidity, a $1,25(OH)_2D$ induced calcium channel TRPV6 in the BBM, and a $1,25(OH)_2D$ induced translocation of calmodulin to the BBM. Calcium movement through the cell occurs with minimal elevation of the intracellular free calcium concentration [93] by packaging the calcium in calbindin-containing vesicles [94–96] that form in the terminal web following $1,25(OH)_2D$ administration. Removal of calcium at the basolateral membrane must work against this gradient, and energy is required. This is achieved by the CaATPase (PMCA1b) and the sodium/calcium exchanger NCX. The first step, calcium entry across the BBM, is accompanied by changes in the lipid composition of the membrane including an increase in linoleic and arachidonic acid [97, 98] and an increase in the phosphatidylcholine/phosphatidylethanolamine ratio [99]. These changes are associated with increased membrane fluidity [98] and are rapid and nongenomic [99]. The epithelial-specific calcium channel, TRPV6, is homologous to TRPV5, a calcium channel originally identified in the kidney [100, 101]. Mice null for TRPV6 have a partial reduction in intestinal calcium transport [102], although the reduction is modest [103]. Calmodulin also participates in intestinal calcium transport. It is the major calcium-binding protein in the microvillus [104], and its concentration in the microvillus is increased by $1,25(OH)_2D$ but not its overall levels in the cell and does not require new protein synthesis [105]. Inhibitors of calmodulin block $1,25(OH)_2D$-stimulated calcium uptake by BBMV [106]. Calmodulin has been shown to regulate TRPV6 activity [107]. Calmodulin is bound to myosin 1A (myo1A), binding that is increased by $1,25(OH)_2D$ [105]. This complex increases with differentiation of the intestinal epithelial cell as does the capacity for calcium transport [108]. However, its exact role in calcium transport is unclear in that mice null for myo1A do not show reduced intestinal calcium transport (Bikle and Munson, unpublished observations). Calcium entering the cytoplasm across the BBM must then be moved into and through the cytoplasm without disrupting the function of the cell. In the vitamin D-deficient animal, calcium accumulates along the inner surface of the plasma membrane of the microvilli [109, 110], from which calcium is released following vitamin D or $1,25(OH)_2D$ administration to enter the cytoplasm where it is found in mitochondria and calbindin-containing vesicles within the terminal web [94, 95, 109, 110]. The vesicles appear to shuttle the calcium to the lateral membrane, where it is pumped out of the cell. Calbindin is the dominant calcium-binding protein in the cytoplasm [104, 111]. However, the role of calbindin in intestinal calcium transport does not appear to be critical in that mice null for calbindin9k grow normally and have normal intestinal calcium transport and their serum calcium levels and bone mineral content are equivalent to wild-type mice regardless of the calcium content of the diet [112]. Moreover, even the double deletion of both TRPV6 and calbindin does not completely block $1,25(OH)_2D$-stimulated calcium transport [113]. The PMCA1b and NCX at the basolateral membrane are responsible for removing calcium from the cell against the same steep electrochemical gradient as it facilitates calcium entry at the BBM [114]. PMCA1b is induced by $1,25(OH)_2D$ [115]. Calmodulin activates the pump, but calbindin may do likewise [116]. The

effect of PMCA1b deletion on calcium transport has not been evaluated. The role of NCX is not considered to be as important as PMCA1b for intestinal calcium transport [117]. It is clear that both genomic and nongenomic actions of $1,25(OH)_2D$ are involved in regulating intestinal calcium transport, but much remains to be learned regarding their relative importance.

Although less studied, intestinal phosphate transport is also under the control of vitamin D. Active phosphate transport is greatest in the jejunum, in contrast to active calcium transport that is greatest in the duodenum. NaPIIb, a sodium phosphate transporter in the small intestine homologous to the type IIa sodium phosphate transporter in the kidney, has been cloned and sequenced [118]. It is induced by $1,25(OH)_2D$ [119], but the impact of deleting this transporter has not been reported. Moreover, it may not be the only or even the major phosphate transporter in the intestine [120]. Transport of phosphate through the cytoplasm is not well understood but, like calcium, may occur in vesicles [121].

Bone. Nutritional vitamin D deficiency, altered vitamin D responsiveness such as vitamin D receptor mutations (hereditary vitamin D-resistant rickets), and decreased $1,25(OH)_2D$ production as in mutations in the CYP27B1 gene (pseudovitamin D deficiency) all have rickets as their main phenotype indicating the critical role of vitamin D and in particular $1,25(OH)_2D$ in bone development and turnover. Like most other cells, VDR is found in bone cells [122, 123], and vitamin D metabolites have been shown to regulate many processes in bone. The VDR makes its first appearance in the fetal rat at day 13 of gestation with expression in osteoblasts and the proliferating and hypertrophic chondrocytes by day 17 [124]. However, fetal development is quite normal in vitamin D-deficient rats [125] and VDR knockout mice [126] suggesting that vitamin D and the VDR are not critical for skeletal formation. Rickets develops postnatally, becoming most manifest after weaning. Even at this point, the rickets resulting from vitamin D deficiency or VDR mutations (or knockouts) can be corrected by supplying adequate amounts of calcium and phosphate either by infusions or orally [127, 128]. Moreover, expressing the VDR in the intestine is sufficient to reverse the skeletal changes observed in the VDR-null mouse [129]. These observations suggest that the role of vitamin D on bone is primarily or totally indirect. However, arguing for a physiologically nonredundant direct action of vitamin D on bone is the development of osteoporosis and decreased bone formation in VDR- or CYP27B1-null mice that is not corrected by the high-calcium/high-phosphate diet [130]. In vivo studies of the impact of vitamin D on bone are complicated by the impact of vitamin D on systemic calcium homeostatic mechanisms such as PTH and FGF23. Furthermore, within bone, the vitamin D metabolites can alter the expression and/or secretion of a large number of skeletally derived factors including insulin-like growth factor-1 (IGF-I), transforming growth factor β (TGFβ) [131], vascular endothelial growth factor (VEGF) [132], and a number of cytokines all of which can exert effects on bone of their own as well as modulate the actions of the vitamin D metabolites on bone. Similarly, species differences, differences in responsiveness of bone and cartilage cells according to their states of differentiation, and differences in responsiveness in terms of the vitamin D metabolite being examined all contribute

to the complexity and uncertainty in distinguishing the direct and indirect roles of the vitamin D metabolites on bone formation and turnover.

The impairment of endochondral bone formation observed in vitamin D deficiency is associated with decreased alkaline phosphatase activity of the hypertrophic chondrocytes [133], alterations in the lipid composition of the matrix [134] perhaps secondary to reduced phospholipase activity [135], and altered proteoglycan degradation [136] due to changes in metalloproteinase activity [136, 137]. Both $1,25(OH)_2D$ and $24,25(OH)_2D$ appear to be required for optimal endochondral bone formation [33]. Some of these actions of $1,25(OH)_2D$ and $24,25(OH)_2D$ on endochondral bone formation are nongenomic in that they take place with isolated matrix vesicles and membrane preparations from these cells [138]. On the other hand, deletion of the VDR or CYP27B1 specifically from chondrocytes does not have a direct impact on chondrocyte development and maturation but does affect bone through FGF23 regulation of phosphate [139, 140]. As mentioned above, osteoblasts at different stages of differentiation differ in their response to $1,25(OH)_2D$ [141]. In the latter stages of differentiation, rat osteoblasts respond to $1,25(OH)_2D$ with an increase in osteocalcin production [142], but do not respond to $1,25(OH)_2D$ in the early stages. Mice, however, differ from rats in that $1,25(OH)_2D$ inhibits osteocalcin expression [142]. Similar species differences are found for other proteins. Osteocalcin and osteopontin in human and rat cells have well-described VDREs in their promoters [143–145], but these genes in mouse cells do not [146]. These maturation-dependent effects of $1,25(OH)_2D$ on bone cell function may explain the surprising ability of excess $1,25(OH)_2D$ to block mineralization leading to hyperosteoidosis [147–149] as such doses may prevent the normal maturation of osteoblasts. That said, the phenotype of mice in which the VDR has been deleted in osteoblasts is modest and suggests more of an impact on bone resorption (decreased) than on bone formation [150].

$1,25(OH)_2D$ also promotes bone resorption by increasing the number and activity of osteoclasts [151]. It is unclear whether mature osteoclasts contain the VDR [152, 153], but the stimulation of osteoclastogenesis by $1,25(OH)_2D$ is mediated by osteoblasts [154, 155]. $1,25(OH)_2D$ induces a membrane-associated protein known as RANKL (receptor activator of nuclear factor (NF)-kB ligand) in osteoblasts that in combination with mCSF also induced by $1,25(OH)_2D$ in osteoblasts activates RANK on osteoclasts and their hematopoietic precursors to stimulate the differentiation of osteoclast precursors and promote their activity [156]. As discussed earlier, the regulation of RANKL expression involves a number of different hormones working in conjunction with or independent of $1,25(OH)_2D$.

Kidney. The regulation of calcium and phosphate transport by vitamin D metabolites in the kidney has received less study than that in the intestine, but the two tissues have similar although not identical mechanisms. Most of the calcium in the glomerular filtrate is reabsorbed in the proximal tubule. This is a paracellular, sodium-dependent process with little or no regulation by PTH and $1,25(OH)_2D$. Regulation of calcium reabsorption by vitamin D takes place in the distal nephron where calcium moves against an electrochemical gradient (presumably transcellular) in a sodium-independent fashion [157]. The majority of phosphate reabsorption also

takes place in the proximal tubule but in this case is closely regulated by PTH and FGF23 [158, 159]. In parathyroidectomized (PTX) animals, Puschett et al. [160–162] demonstrated acute effects of 25OHD and 1,25$(OH)_2$D on calcium and phosphate reabsorption. Subsequent studies indicated that PTH could enhance or was required for the stimulation of calcium and phosphate reabsorption by vitamin D metabolites [163, 164].

The molecules critical for calcium reabsorption in the distal tubule include the VDR, calbindin, TRPV5, and BLM calcium pump (PMCA1b as in the intestine), a situation similar to the mechanism for calcium transport in the intestine [165]. The calbindin in the kidney in most species is 28 kDa, whereas the 9 kDa form is found in the intestine in most species. The kidney has mostly TRPV5, whereas the intestine is primarily TRPV6. Calmodulin and a brush border myosin I like protein are also found in the kidney brush border, but their role in renal calcium transport has not been explored. Not all distal tubules express these proteins [100, 101, 166, 167] suggesting that not all distal tubules are involved in calcium transport. 1,25$(OH)_2$D upregulates the VDR [168], calbindin [169, 170], and TRPV5 expression [171]. Deletion studies of these proteins are limited.

Phosphate reabsorption in the proximal tubule is mediated at the brush border by sodium-dependent phosphate transporters (NaP2a and NaP2c) that rely on the basolateral membrane Na,K-ATPase to maintain the sodium gradient that drives the transport process [172]. It is not clear whether 1,25$(OH)_2$D regulates the expression or activities of these transporters as it does the homologous NaP2b in the intestine.

Nonclassic Vitamin D Target Tissues

Vitamin D signaling in nonclassic target tissues can be categorized into three different not mutually exclusive actions:

1. Regulation of proliferation and differentiation
2. Regulation of hormone secretion
3. Regulation of immune function

Examples of these mechanisms of action will be discussed in turn.

Regulation of Proliferation and Differentiation

In this section, I will discuss a normal tissue, the skin, as representing a good example of the regulation of proliferation and differentiation by VDR and 1,25$(OH)_2$D, followed by cancer in which such regulation is lost.

Skin. Epidermal keratinocytes express the entire vitamin D metabolic pathway from the production of vitamin D_3 from 7-DHC, its conversion to 25(OH)D by CYP27A1 [173] (expression of CYP2R1 has been described in fibroblasts [174] but

not in keratinocytes), and its further conversion to $1,25(OH)_2D$ by CYP27B1 [175]. Moreover, the skin also expresses CYP24A1, limiting the levels of $1,25(OH)_2D$ in keratinocytes under vitamin D replete conditions [176, 177]. CYP27B1 is expressed primarily in the basal cells of the epidermis [178], as the cells differentiate the mRNA and protein levels of CYP27B1and its activity decline [179].

$1,25(OH)_2D$ regulates keratinocyte differentiation in partnership with calcium [180]. The keratinocytes express the calcium-sensing receptor (CaSR) critical for their response to calcium, and CaSR is induced by $1,25(OH)_2D$ [181]. Keratinocytes grown at calcium concentrations below 0.07 mM continue to proliferate but fail to differentiate. Acutely increasing the extracellular calcium concentration (Cao) above 0.1 mM (calcium switch) initiates the differentiation process. Within hours of the calcium switch, keratinocytes switch from making the basal keratins K5 and K14 and begin making keratins K1 and K10 [182] followed, subsequently, by increased levels of profilaggrin (the precursor of filaggrin, an intermediate filament-associated protein), involucrin, and loricrin (precursors for the cornified envelope) [183, 184]. Loricrin, involucrin, and other proteins [185] are cross-linked into the insoluble cornified envelope (CE) by the calcium-sensitive, membrane-bound form of transglutaminase [186, 187], which like involucrin and loricrin increases within 24 h after the calcium switch [188]. $1,25(OH)_2D$ increases the mRNA and protein levels for involucrin and transglutaminase and promotes CE formation at subnanomolar concentrations in preconfluent keratinocytes [189–192]. Deletion of either the VDR or CaSR from keratinocytes in vivo [193, 194] also blocks the formation of the lipids that are secreted into the cornified envelope by the lamellar bodies in the stratum granulosum to waterproof the permeability barrier. Moreover, deletion of CYP27B1 from keratinocytes in vitro blocks the induction of antimicrobial peptides that are likewise incorporated into the lamellar bodies and secreted into the cornified envelope as part of the barrier [195]. This will be discussed further in the section on innate immunity.

Calcium affects the ability of $1,25(OH)_2D$ to stimulate keratinocyte differentiation and vice versa [196]. The calcium switch also leads to the rapid redistribution of a number of proteins from the cytosol to the membrane where they participate in the formation of intercellular contacts. These include the calcium-sensing receptor (CaSR), phospholipase C-γ1 (PLC-γ1), src kinases, and E-cadherin/catenin complex. This complex plays a critical role in calcium and vitamin D signaling in the keratinocyte. Besides E-cadherin, it contains phosphatidyl inositol 3 kinase (PI3K), phosphatidyl inositol 4-phosphate 5-kinase 1α (PIP5K1α), and the catenins Ctnna1, Ctnnb1, and Ctnnd1 (α- and β-catenin, p120). These all play important roles in calcium- and vitamin D-induced differentiation [197–202]. PI3K and PIP5K1α sequentially phosphorylate PIP and PIP2 to PIP3 that activates PLC-γ1. PLC-γ1 cleaves PIP2 to form IP3 and diacylglycerol. IP3 releases calcium from intracellular stores, important for the sustained increase in intracellular calcium (Cai) required for the differentiation process [203]. Diacylglycerol along with calcium activates protein kinase C alpha (PKCα) that also promotes differentiation [204]. $1,25(OH)_2D$ is required for the formation of the E-cadherin/catenin complex and induces some of its constituents such as PLC-γ1 [205]. Deletion of the CaSR from keratinocytes

reduces their stores of calcium and like the deletion of VDR blocks their response to extracellular calcium (Cao) including the formation of the E-cadherin/Ctnn complex and the permeability barrier [199, 206]. Thus, calcium and vitamin D signaling are essential partners for keratinocyte differentiation.

Cancer. The antiproliferative, prodifferentiating effects of vitamin D signaling on many, if not all, cell types have raised the hope that vitamin D, $1,25(OH)_2D$, or one or more of its analogs would prove useful in the prevention and/or treatment of cancer. This section will focus on the antiproliferative/prodifferentiating actions as shown in a number of cellular and animal studies, but a large number of other mechanisms have been invoked as recently reviewed [207].

Cellular mechanisms. Most tumors express the VDR and often express CYP27B1, but their expression is often lost as the tumor undergoes progressive dedifferentiation [208–210]. On the other hand, CYP24A1 expression is often increased in tumors and is associated with resistance to $1,25(OH)_2D$ [210, 211]. These changes in vitamin D metabolism and responsiveness reduce the ability of $1,25(OH)_2D$ to control the proliferation and differentiation of these tumors. Moreover, a number of miRNAs have been identified to be regulated by $1,25(OH)_2D$/VDR relevant to their antiproliferative actions [212]. These include increased expression of miR145, which blocks the expression of E2F3, a key regulator of proliferation [213] or miR-32 that blocks the proapoptotic protein Bim that somewhat paradoxically actually protects the cell (human myeloid leukemia) from AraC-induced apoptosis [214].

$1,25(OH)_2D$ typically causes arrest at the Go/G1 and/or G1/S transitions in the cell cycle associated with a decrease in cyclins and an increase in the inhibitors of the cyclin-dependent kinases (CDK) such as $p21^{cip1}$ and $p27^{kip1}$ [215, 216]. One class of transcription factors that have been shown to be involved in suppression of proliferation and increased apoptosis is the family of Forkhead box O (FoxO) proteins. $1,25(OH)_2D$ promotes their interaction with VDR as well as their regulation by Sirt1 and protein phosphatase 1 maintaining these proteins in the transcriptionally active dephosphorylated state [217]. $1,25(OH)_2D$ reduces the expression of proproliferative genes such as Myc, Fos, and Jun [77] while stimulating the expression of IGF-binding protein 3 (IGFBP3) in prostate and breast cancer cells, thus blocking the proproliferative actions of IGFs 1 and 2 [218, 219]. In epithelial cells, $1,25(OH)_2D$ stimulates the expression of TGFβ2, which is antiproliferative in these cells [220–222], and suppresses components of the hedgehog pathway (HH), which when overexpressed result in basal cell carcinomas (BCC) [223, 224]. $1,25(OH)_2D$ inhibits EGF stimulation of proliferation by inhibiting the expression of its receptor in breast cell lines [225]. Constitutive activation of the wnt/β-catenin pathway is the cause of most colorectal cancers (CRC). When activated, β-catenin enters the nucleus where it binds to TCF/LEF sites in genes promoting proliferation (e.g., cyclin D1). $1,25(OH)_2D$/VDR blocks this pathway both by binding to β-catenin, restricting its proproliferative actions in the nucleus, and by stimulating the formation of the E-cadherin/catenin complex in the cell membrane to which β-catenin binds restricting its translocation to the nucleus [226]. Moreover, $1,25(OH)_2D$ can increase the expression of the wnt inhibitor dickkopf (DKK)-1 [227] while inhibiting that of the wnt activator DKK-4 [228] in colon cancer cells.

$1,25(OH)_2D$ promotes the apoptosis of a number of cell types [229, 230] by stimulating the expression of proapoptotic genes such as GOS2 (Go/G1 switch gene 2) [216], Bax [231], DAP (death-associated protein)-3, CFKAR (caspase 8 apoptosis-related cysteine peptidase), FADD (Fas-associated death domain), and caspases (e.g., caspase 3, 4, 6, and 8) [221] in a variety of cell lines, while suppressing the expression of proapoptotic genes such as Bcl2 and Bcl-X_L in these and others [231–233].

Animal studies. Animal studies demonstrating the efficacy of $1,25(OH)_2D$ in preventing or slowing the progression of different tumors are numerous with those of the colorectum (CRC), breast, prostate, and skin being most studied in both animal and human studies. A Western diet low in calcium and vitamin D fed to mice increases their risk of CRC, a risk that can be reversed with a diet supplemented with calcium and vitamin D [234]. Tumors induced by the combination of azoxymethane and dextran sulfate can be at least partially prevented with the administration of vitamin D metabolites [235]. Activation of the wnt/β-catenin pathway caused by mutations in adenomatous polyposis coli (APC^{min}) develops tumors much faster on a Western diet [236], on a vitamin D-deficient diet [237], or when bred with VDR-null mice [238]. As for CRC, the number of breast cancers induced in this case by dimethylbenzanthracene (DMBA) is increased when the rats are fed a Western diet [239] or when DMBA is given to VDR-null mice [240]. VDR agonists prevent the growth of breast cancer xenografts [241]. Vitamin D analogs can also inhibit the growth of prostate cancer regardless of androgen receptor status [242]. PC3 prostate cancer cells in bone grow more rapidly when the mice are fed a vitamin D-deficient diet [243]. Similarly, breeding the transgenic prostate tumor model, LPB-Tag with VDR-null mice, stimulates the growth of these tumors [244], whereas high doses of $1,25(OH)_2D$ suppress the development of tumors in the TRAMP model of prostate cancer [245]. The most common skin cancers are squamous cell carcinomas (SCC) and basal cell carcinomas (BCC). In animals, these tumors are typically induced by DMBA topically or orally often followed by repeated topical application of phorbol esters or by chronic exposure to UVB. Nearly all VDR-null mice treated with DMBA or chronic UVB exposure develop skin tumors, but not their controls [224, 246]. Topical $1,25(OH)_2D$ is protective at least of the early effects of UVB on markers of DNA damage such as cyclobutane pyrimidine dimers [247].

Clinical studies. Most of the evidence supporting a role for vitamin D in tumor prevention in humans is epidemiologic. The evidence for a link between vitamin D and CRC is reasonably strong [248, 249]. One such study found a risk reduction of 0.88 (CI 0.8–0.96) comparing the highest to lowest levels of vitamin D intake [248] and 0.67 (CI 0.54–0.80) comparing the highest to the lowest serum 25OHD levels [248]. In breast cancer, the largest cohort studies [250, 251] (Nurses' Health Study with 88,891 participants and Women's Health Study with 31,487 participants) showed a relative risk (RR) of 0.72 (CI 0.55–0.94) and 0.65 (CI 0.42–1.00), respectively, but only in premenopausal women. However, several meta-analyses did not make this distinction regarding menopause status. One such study demonstrated a risk reduction of 0.55 (CI 0.38–0.80) comparing the highest quintile of 25OHD levels to the lowest [252]. Another meta-analysis showed a RR of 0.89

(0.82–0.98) for a 10 ng/mL increase in 25OHD when all studies were included and 0.83 (0.79–0.87) when only case control studies were pooled [253]. In contrast to CRC and breast cancer, the role of vitamin D in prostate cancer is decidedly mixed. In a recent summary of 14 studies examining the association between 25OHD levels and the development of prostate cancer, 11 showed no association [254]. Similarly, studies examining the association of dietary vitamin D intake to prostate cancer did not show benefit [253, 255]. Studies examining the link between vitamin D and nonmelanoma skin cancer (NMSC) are difficult to interpret because UVB is the common etiologic agent for both cancer development and vitamin D production. Those studies that have been reported are mixed. NMSC incidence in the osteoporotic fractures in men (MrOS) study indicated that those with the highest baseline serum 25OHD levels (30 ng/mL) had a relative risk of 0.53 (CI 0.3–0.93) compared to those with the lowest baseline 25OHD levels [256]. However, other studies found that higher 25(OH)D levels were associated with an increased risk of BCC [257, 258].

Regulation of Hormone Secretion

Parathyroid hormone secretion. The promoter of the parathyroid hormone (PTH) gene contains a negative VDRE by which $1,25(OH)_2D$ acting through its receptor is thought to control PTH synthesis [259]. More recent studies identified an E-box element in the PTH gene similar to that found in the CYP27B1 gene. VDR/RXR binds to this element but through the vitamin D inhibitory receptor complex similar to the inhibition by $1,25(OH)_2D$ of CYP27B1 in the kidney [260, 261]. This leads to suppression of transcription via the same mechanisms (HDAC recruitment) as in the CYP27B1 gene. Of interest is that PTH levels are more highly correlated with circulating 25OHD levels than with circulating $1,25(OH)_2D$ levels [262]. As noted earlier, the parathyroid gland (PTG) expresses the megalin/cubilin complex likely enabling uptake of the 25(OH)D/DBP complex into the gland providing the substrate for the CYP27B1 in the PTG to produce its own $1,25(OH)_2D$ [263]. It was initially presumed that the locally produced $1,25(OH)_2D$ utilized the PTG VDR to suppress PTH production, but when the VDR was specifically deleted in PTG, the effect on PTH secretion was modest, and hyperplasia of the gland was not observed [264]. However, deletion of CYP27B1 in the PTG had a much greater impact on PTH secretion (250% increase vs. 80% increase in the PTG VDR deletion) and a surprising drop in serum calcium and $1,25(OH)_2D$ levels suggesting that the CYP27B1 in the PTG was also providing $1,25(OH)_2D$ to the circulation [265]. The drop in serum calcium may have contributed to the greater increase in PTH secretion when CYP27B1 was deleted from the PTG than when the VDR was deleted. In addition to suppression of PTH secretion, $1,25(OH)_2D$ inhibits the proliferation of parathyroid cells (PTC) in vivo [266] and in vitro [267]. In chronic kidney disease, epidermal growth factor receptor (EGFR) and its ligand transforming growth factor α (TGFα) are increased and thought to drive the PTG hyperplasia [268, 269].

$1,25(OH)_2D$ decreases TGFα and EGFR expression [268] and increases the expression of the cell cycle inhibitors p21 and p27 to block the hyperplasia [270].

$1,25(OH)_2D$ interacts with other signaling mechanisms to enhance its regulation of PTH secretion and PTG proliferation. The promoter of the CaSR has two functional VDREs through which $1,25(OH)_2D$/VDR stimulates the expression of CaSR [271]. The CaSR may, in turn, increase VDR levels as suggested by the observation that low-calcium diets decrease the VDR in PTG but high-calcium diets increase the VDR in PTG [272]. $1,25(OH)_2D$ also stimulates Klotho expression in PTC, which, along with FGF receptors, enables FGF23 responsiveness and in turn FGF23 stimulates CYP27B1 [29]. This effect of FGF23 on CYP27B1 in the PTC is opposite to the effect of FGF23 on CYP27B1 in the kidney for unclear reasons. The ability of $1,25(OH)_2D$ to inhibit PTH production and secretion has been exploited clinically in that $1,25(OH)_2D$ and several of its analogs are used to prevent and/or treat secondary hyperparathyroidism associated with renal failure.

Insulin secretion. $1,25(OH)_2D$ stimulates insulin secretion, although the mechanism is not well defined [273, 274]. Moreover, insulin secretion is reduced in vitamin D deficiency [275] and in VDRKO mice [276]. However, calcium is important for insulin secretion, and low calcium levels can be suppressive [277]. Therefore, the early results with vitamin D deficiency may also have reflected the low calcium levels in this condition. When VDRKO mice were placed on a rescue diet to maintain normal calcium levels, insulin secretion was not different from wild type [278]. On the other hand, VDR, CYP27B1, and calbindin-D_{28k} are found in pancreatic beta cells [279–281] suggesting a direct role of VDR and $1,25(OH)_2D$ in insulin secretion. Moreover, studies using calbindin-D_{28k}-null mice have suggested that calbindin-D_{28k}, by regulating intracellular calcium, can modulate depolarization-stimulated insulin release [282]. Furthermore, calbindin-D_{28k}, by buffering calcium, can protect against cytokine-mediated destruction of beta cells [283]. The renin/angiotensin system (RAS) may also play a role by impairing beta cell function and insulin sensitivity. $1,25(OH)_2D$ suppresses the RAS in VDRKO mice, and this property in mouse islets may contribute to the ability of $1,25(OH)_2D$ to stimulate insulin secretion [284].

Fibroblast growth factor (FGF23). FGF23 is produced primarily by bone, and in particular by osteoblasts and osteocytes. $1,25(OH)_2D_3$ stimulates this process, but the mechanism is not clear [285]. As noted earlier, FGF23 in turn inhibits $1,25(OH)_2D$ production by the kidney resulting in a feedback loop similar to that for PTH secretion to maintain a balance in the levels of these hormones. Diseases in which FGF23 is overexpressed or not catabolized properly result in decreased $1,25(OH)_2D$ levels, whereas the opposite is true when FGF23 fails to be secreted or in conditions such as Klotho gene deletion when its target tissues are unresponsive [286].

Renin. VDR- and CYP27B1-null mice have increased levels of renin [287, 288]. Renin converts angiotensinogen to angiotensin I, which is further converted to angiotensin II, a powerful vasoconstrictor as well as stimulator of aldosterone production. In mice lacking VDR or CYP27B1, blood pressure is increased, with increased cardiac hypertrophy, impaired systolic and diastolic function, and increased arterial stiffness [287–289]. In the global VDR knockout, renin expres-

sion is reduced both in the kidney and the heart as is the cardiac expression of atrial natriuretic factor (ANP) [290]. However, in the cardiomyocyte-specific VDRKO, the increased renin expression is found, but cardiomyopathy is not, suggesting that the effect of renin on the heart is indirect.

Regulation of the Immune System

The immune system is comprised of two interacting forms of immunity: adaptive and innate. Adaptive immunity refers to the process by which cells specialized in antigen presentation, dendritic cells (DC) primarily, and the cells responsible for antigen recognition, T and B lymphocytes, are activated by foreign antigens to initiate a series of functions such as cytokine production, antibody production, and cell killing. The major classes of T helper cells differentiating from the parent CD4 lymphocyte include Th1, Th2, Th9, Th17, and Treg. These responses adapt to the antigen presented. The innate immune response involves the activation of toll-like receptors (TLRs), of which there are ten in the human genome. These TLRs are established during cell development (innate) [291]. TLRs are found in a number of cells including polymorphonuclear cells (PMNs), monocytes, macrophages, and a wide variety of epithelial cells including keratinocytes of the skin, gingiva, intestine, vagina, bladder, and lungs. TLRs are pathogen-recognition receptors that recognize various products of infectious agents including bacteria and viruses and trigger the cell to produce various antimicrobial peptides (AMPs), the best studied of which is cathelicidin. In general, vitamin D signaling suppresses adaptive immunity but promotes innate immunity.

The VDR and CYP27B1 are expressed in most, if not all, cells of the immune system including the epithelial cells, at least when activated [292–294]. Moreover, several of these cells express CYP2R1 and so in combination with CYP27B1 can produce $1,25(OH)_2D$ from circulating vitamin D as well as 25(OH)D [294]. As noted earlier, the regulation of CYP27B1 in these cells differs substantially from that in the kidney, being insensitive to hormonal regulators such as PTH and FGF23, its product $1,25(OH)_2D$, and calcium and phosphate levels. In these immune cells, CYP27B1 is stimulated by cytokines such as tumor necrosis-α (TNFα) and interferon-γ (IGFγ) [23–26]. Thus, activation of these immune cells in diseases such as sarcoidosis or lymphomas can lead to hypercalcemia with elevated $1,25(OH)_2D$ levels.

Adaptive immunity. $1,25(OH)_2D$ decreases the maturation of DC, thus decreasing their ability to present antigen [295]. Furthermore, $1,25(OH)_2D$ suppresses the proliferation and differentiation of the T and B cells by suppressing IL-12 production, important for Th1 development, IL-23 and IL-6 production important for Th17 development, as well as their ability to secrete IGFγ and IL-2 (from Th1 cells) and IL-17 from Th17 cells [296–298]. The suppression of IL-12 also increases the development of Th2 cells and their production of IL-4, IL-5, and IL-13, which serve to further suppress Th1 while promoting Th2 cell number and function. $1,25(OH)_2D$

reduces IL-9 production by Th9 cells [299], which, like the products from Th1 and Th17 cells, plays a role in inflammatory responses. Treg cells, on the other hand, are induced by $1,25(OH)_2D$ [300]. Treg cells produce the regulatory cytokine IL-10 that suppresses the development of Th1 and Th17 leading to immune tolerance [301]. The regulation of a number of cytokines involved in the inflammatory process can be both direct and indirect. Inhibition of IL-2 expression involves blocking NFAT binding to the IL-2 promoter and sequestration of runx1 by VDR [297, 302]. Suppression of IFNα expression involves a negative VDRE in the promoter [303]. Suppression of IL-17 expression involves blocking NFAT binding to the IL-17 promoter and induction of Foxp3 [297]. $1,25(OH)_2D$ blocks NFκB by inhibiting its nuclear translocation, its binding to the consensus sequences in the genes it regulates such as IL-8 and IL-12, and by degradation of IFκB (inhibitor of NFκB) [304]. $1,25(OH)_2D_3$ has also been shown to bring an inhibitor complex containing histone deacetylase 3 (HDAC3) to the promoter of rel B, one of the members of the NFκB family, thus suppressing gene expression. The actions of $1,25(OH)_2D_3$ on B cells have received less attention, but recent studies have demonstrated a reduction in proliferation, maturation to plasma cells, and immunoglobulin production [293].

Although overall myelopoiesis and composition of lymphoid tissue are normal in VDRKO mice, abnormalities in immune responses to stimuli have been observed. Moreover, a number of experimental models of autoimmune diseases including rheumatoid arthritis, psoriasis, type 1 diabetes mellitus (NOD mouse), systemic lupus erythematosus (SLE), experimental allergic encephalitis (EAE, model for multiple sclerosis), and inflammatory bowel disease (IBD) have been prevented/ameliorated with the use of $1,25(OH)_2D$ or one of its analogs [305]. The severity in IBD is increased when IL-10 knockout mice are bred with VDRKO mice [306]. Rejection of transplanted tissues is reduced when the animals are treated with $1,25(OH)_2D$ or one of its analogs [307]. On the other hand, the promotion of Th2 numbers and function may have adverse effects on allergic diseases such as asthma and atopic dermatitis. Calcipotriol, an analog of $1,25(OH)_2D$, stimulated thymic stromal lymphopoietin (TSLP) in keratinocytes leading to an increased expression of Th2 cytokines and increased inflammatory responses to allergen-induced atopic dermatitis and asthma [308]. However, $1,25(OH)_2D$ was shown to be protective against experimentally induced asthma including a reduction in IL-4 production and eosinophilic infiltration in studies in normal mice [309] perhaps due to its suppression of IL-9, a potent part of the inflammatory response in the lungs [299]. Other studies have shown that mice lacking the VDR (VDRKO) are also protected from experimentally induced asthma [310]. The effects of $1,25(OH)_2D$ on infections are also mixed. $1,25(OH)_2D$ inhibition of IGFγ stimulation of reactive oxygen species and nitric oxide production [311] or suppression of IL-17 limiting its induction of AMPs and neutrophil recruitment [312] have been shown to reduce resistance to infectious organisms such as *Leishmania* [311], *Toxoplasma* [313], and *Citrobacter* [314].

Innate immunity. Stimulation of TLR 2/1 in macrophages [315] or TLR2 and its coreceptor CD14 in keratinocytes [195] leads to an increase in CYP27B1 and VDR expression enabling these cells to produce and respond their own $1,25(OH)_2D$.

$1,25(OH)_2D$ then induces antimicrobial peptides such as cathelicidin and defensins that kill intracellular organisms such as *Mycobacterium tuberculosis*. Cathelicidin also promotes the chemotaxis of neutrophils, monocytes, macrophages, and T cells into the skin thus linking the adaptive and immune responses in the skin and other tissues [316]. In this way, the innate immune function of these cells acts essentially as the first responder to invading organisms prior to the adaptive immune response. The murine cathelicidin gene lacks a VDRE and so is not responsive to $1,25(OH)_2D$. However, $1,25(OH)_2D$ stimulates the inducible NOS pathway by which it induces *M. tuberculosis* killing in these macrophages [317]. Unfortunately, supplementation with vitamin D of patients with *M. tuberculosis* has not been universally successful [318–321]. In diseases such as atopic dermatitis, the production of cathelicidin and other antimicrobial peptides is reduced, predisposing these patients to microbial superinfections [322]. Th2 cytokines such as IL-4 and IL-13 suppress the induction of AMPs [323]. Since $1,25(OH)_2D_3$ stimulates the differentiation of Th2 cells, its administration to patients with atopic dermatitis may not be useful in spite of its induction of cathelicidin in contrast to its proven value in patients with psoriasis in which suppression of Th1 and Th17 and their cytokines appears central to its therapeutic effect.

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Chapter 2
Vitamin D and Autoimmune Diseases

Shir Azrielant and Yehuda Shoenfeld

Abbreviations

AIH	Autoimmune hepatitis
AITD	Autoimmune thyroid diseases
BMI	Body mass index
CD	Crohn's disease
dcSSc	Diffuse cutaneous systemic sclerosis
IBD	Inflammatory bowel diseases
JoSLE	Juvenile-onset systemic lupus erythematosus
KO	Knockout
lcSSc	Limited cutaneous systemic sclerosis
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
PBC	Primary biliary cholangitis
RA	Rheumatoid arthritis
RR	Relative risk
SLE	Systemic lupus erythematosus

S. Azrielant, MD
Sheba Medical Center, The Zabludowicz Center for Autoimmune Diseases, Ramat-Gan, Israel

Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

Y. Shoenfeld, MD, FRCP, MaACR (✉)
Sheba Medical Center, The Zabludowicz Center for Autoimmune Diseases, Ramat-Gan, Israel

Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

Incumbent of the Laura Schwarz-Kip Chair for Research of Autoimmune Diseases, Tel-Aviv University, Tel-Aviv, Israel
e-mail: shoenfel@post.tau.ac.il; yehuda.shoenfeld@sheba.health.gov.il

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SS	Systemic sclerosis
TGF	Transforming growth factor
Th	T helper cells
Treg	T regulatory cells
UC	Ulcerative colitis
UV	Ultraviolet
UVB	Ultraviolet B
VDR	Vitamin D receptor

Introduction

Autoimmune diseases are characterized by an abnormal response of the immune system toward antigens of the body's own cells due to lack of self-tolerance. The various diseases differ from one another in the specific immune cells involved in the autoimmune process, the self-antigen targeted by the affected immune system, and, consequently, their signs and symptoms.

There are more than 80 known autoimmune diseases; their prevalence in the general population is estimated to be 5–20%. Genetic, immunological, hormonal, and environmental factors which may contribute to or increase the probability of autoimmune disease have been researched in recent years. One of the environmental factors studied has been vitamin D [1, 2].

In recent years, evidence has accumulated regarding vitamin D's immunomodulatory features and its significant role in various processes of the immune system [1, 3]. Vitamin D receptors (VDR) were found in immune cells, including macrophages, dendritic cells, B cells, and T cells [1, 4, 5]; in vitro studies demonstrated the vitamin's direct effect on their activity [6].

The vitamin D receptor is a nuclear hormone receptor. The gene for this receptor incorporates many polymorphisms, four of which have been extensively studied: *TaqI*, *BsmI*, *ApaI*, and *FokI*. These polymorphisms may affect the receptor's function and thereby affect the serum levels of active vitamin D [7]. Several of these polymorphisms were previously linked to autoimmune diseases [8, 9].

Modern living has shifted human activity away from the sunlight, and consequently exposed us to a variety of autoimmune diseases whose prevalence is constantly on the rise. Among the diseases found to be related to vitamin D deficiency are multiple sclerosis, type I diabetes, inflammatory bowel diseases, and rheumatoid arthritis [1, 10, 11].

Vitamin D Deficiency

The definition of vitamin D deficiency is controversial in the medical literature; an accepted definition is a serum level of 25-hydroxy-vitamin D (25(OH)D—the measured form of vitamin D in the blood) under 30–40 ng/mL, as lower levels lead to activation of the parathyroid glands, which results in bone absorption and damage [12] as well as increased risk for malignancies, cardiovascular diseases, and autoimmune

diseases [13]. Some researchers further divide deficiencies into insufficiency (21–29 ng/mL) and deficiency (under 20 ng/mL) [7, 14–16]; in some publications, the definition of vitamin D deficiency is even lower [12, 17]. An optimal level for the functioning of the immune system, within the normal range of serum vitamin D concentration, is yet to be determined [7].

Vitamin D deficiency is more common in patients with autoimmune diseases than in healthy subjects [18–21]. One risk factor for vitamin D deficiency is certain medications used for treatment of autoimmune diseases, such as glucocorticoids; kidney damage is another risk factor, one that can also be the result of autoimmune diseases [12, 22].

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease. Its manifestations involve almost all systems of the body, including the musculoskeletal, cardiovascular, and central nervous systems, as well as the kidneys and the skin. Signs and symptoms of the disease vary between patients, as does the severity. SLE is more common in women than in men, in a ratio of about 7:1 [23].

For SLE patients, sunlight is a double-edged sword, while sunlight produces vitamin D, which has a beneficial immunomodulatory effect, ultraviolet (UV) exposure in lupus patients worsens the disease's manifestations, primarily the cutaneous symptoms; therefore sun avoidance is advised [24].

Vitamin D deficiency is more common in lupus patients than in healthy subjects [20, 21]. Similar findings were demonstrated in teenagers and young adults diagnosed with juvenile-onset systemic lupus erythematosus (JoSLE) [25, 26]. There are several possible explanations for the difference between healthy subjects and patients with lupus. As mentioned previously, SLE patients are instructed to avoid exposure to sunlight due to photosensitivity, contributing to their vitamin D deficiency. In addition, the disease causes renal dysfunction, which interferes with the activation of the vitamin. Lastly, medical treatments for lupus, such as steroids, can harm the metabolism of the vitamin [4, 16, 27, 28].

In various molecular studies, vitamin D was found to have an attenuating effect on various immune cells that participate in the pathogenesis of the disease, including neutrophils [29], dendritic cells [30], and T regulatory cells (Treg) [31–33].

A study by Schoindre et al. examined the association between 25(OH)D levels and disease activity and flares. The investigators found an association between low levels of the vitamin and a higher SLE activity; however, they did not find a similar association with risk of flares during the 6 months following the measurement [17]. A study by Lertratanakul et al. presented the complementing finding that higher vitamin levels are associated with a lower disease activity [34]. Similar findings were also reported in studies performed on adults in Australia [35], India [36], Egypt [37], and Jamaica [15], as well in a study on adolescents [25]. These findings were also supported in recent literature reviews [4, 16, 28, 38]. Vitamin D levels were also associated with other manifestations of the disease, such as cognitive impairment [39].

However, in other studies of more limited scale, the investigators were unable to show a correlation between vitamin D deficiency and disease activity measured in different disease activity scales [14, 26, 27, 40, 41]. No link was found between vitamin D levels and SLE flares, even when flares were self-reported [20].

In a literature review published in 2014, intervening variables for the association between vitamin D levels and SLE manifestations were analyzed. The most significant variables that were found include medications (hydroxychloroquine, steroids, and vitamin D supplements), body mass index (BMI), renal function, and proteinuria [40].

The assumed link between vitamin D deficiency and lupus, which was supported by some studies, has led to attempts to better understand its nature and direction in interventional studies using vitamin D supplements. The conventional doses in the literature are 800, 2000, and 4000 IU per day, with some studies suggesting adjustment of the dose to patient baseline vitamin levels and individual risk factors (BMI, use of steroids, etc.) [4, 7].

In a large-scale cohort study, vitamin D supplementation was given to lupus patients with 25(OH)D levels lower than 40 ng/mL (50,000 IU of D2 per week and 200 calcium/D3 units each, two times a day). Improvement in proteinuria was shown in patients with higher 25(OH)D levels, and a correlation was observed between disease activity and the change in 25(OH)D values. Notably, this correlation was only seen in patients who were vitamin D deficient at the beginning of the study. No effect on disease activity was found after increasing the vitamin D levels above 40 ng/mL [42]. Similar findings were shown in adolescents and young adults; vitamin D supplements (50,000 IU per week, for 24 weeks) decreased disease activity and improved fatigue [43].

In contrast to the previously mentioned study, a recent review of the literature did not reach definite conclusions regarding the effectiveness of vitamin D supplements on patients [7]. A more recent interventional study, conducted in 2015, also failed to demonstrate vitamin D supplements effectiveness: although a high-dose regimen elevated serum levels of vitamin D, no significant difference was found between the treatment groups in disease activity and serological markers [44].

Currently, there is no across-the-board recommendation to give vitamin D supplements to lupus patients, apart from those patients that are treated with steroids, for whom a dose of 800–1000 units per day is recommended [45].

Type 1 Diabetes Mellitus

Type 1 diabetes mellitus, previously known as juvenile diabetes or insulin-dependent diabetes, is an autoimmune disease that is usually diagnosed during childhood or adolescence, typically before the age of 30. It is caused by an autoimmune response against beta cells in the pancreas, leading to their gradual destruction and to insulin deficiency [46].

The association between diabetes type 1 and vitamin D deficiency was investigated in an epidemiological study. In this study, researchers investigated the

relationship between ultraviolet B (UVB) irradiance in different locations worldwide and the incidence of type 1 diabetes in children. Researchers assumed that UVB irradiation, as the primary source of vitamin D in the human body, could be used as a rough estimation of the vitamin's circulating levels. They demonstrated a correlation between the disease's rates and the patients' distance from the equator: higher rates of the disease were found at higher geographic latitudes, in which UVB is scarce ($R^2 = 0.25$, $p < 0.001$) [47].

In a cohort study from Finland, 10,366 children were followed for 31 years, from 1966 to 1997. The researchers wished to investigate whether vitamin D supplementation in early life influenced the risk for development of type 1 diabetes later in life. They found that regular administration of vitamin D supplementation (2000 IU per day) in the first year of life was associated with reduced risk for the disease)0.12, 95% CI 0.03–0.51 relative risk (RR((. They also found that children suspected of suffering from rickets, which is caused by vitamin D deficiency, during their first year of life were more susceptible to develop type 1 diabetes later on) RR 3.0, CI 1.0–9.0 ([48].

Similarly, a large-scale population-based study from multiple centers in Europe demonstrated a reduction in risk for type 1 diabetes in countries where vitamin D supplementation during infancy is prevalent [49]; a meta-analysis done on this subject reached similar conclusions [50]. A different study from Norway concluded that children whose mothers who suffered from vitamin D deficiency during their pregnancy were more than twice as likely to develop type 1 diabetes [51]. These findings suggest the great importance of sufficient levels of vitamin D during infancy, neonatal, and even prenatal periods.

Vitamin D supplementation improved glycemic control of type 1 diabetes, as measured by Hemoglobin A1C levels, in pediatric patients who suffered from vitamin D deficiency [52]. Normal vitamin D levels in diabetes patients were correlated with lower prevalence of macroalbuminuria, a marker for diabetic nephropathy, but not with other complications of the disease, such as retinopathy and cardiovascular diseases [53].

Animal models studies also support this association, and further demonstrate a direct effect of vitamin D on the pancreas and its function. Zeitz generated mice with inactive mutant VDR. Compared to wild type mice, the mutant mice had elevated glucose levels and reduced insulin secretion in response to glucose administration [54].

Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory disease that causes demyelination of the central nervous system. The disease usually has a relapsing and remitting course; its clinical manifestations include sensory disturbances, motor weakness, diplopia, gait and balance disturbance, vertigo, and bladder dysfunction [55]. These symptoms are believed to be caused by inflammation, demyelination, and axonal damage [56].

An extensive review, done in 2008, suggests that distance from the equator is the strongest risk factor for MS, seemingly due to UV radiation exposure. Also, emigration from cold countries, with relatively low UV irradiation (e.g., United Kingdom), to sunny ones (e.g., South Africa) decreased the risk for MS [57]. A seasonal pattern was described for the clinical course of MS, which, at least in part, can be explained by vitamin D levels [58].

Munger et al. analyzed data from two very large cohort studies of women: "Nurses' Health Study" (92,253 participants) and "Nurses' Health Study II" (95,310 participants). Dietary and supplementary vitamin D intake was assessed at baseline and every 4 years. The study found that the risk for developing MS was lower in women with high vitamin D intake, compared to women with low intake (RR 0.67, 95% CI 0.40–1.12; $p = 0.03$), and vitamin D supplementation was inversely associated with MS risk. Notably, high dietary intake of vitamin D alone did not generate similar association [59].

MHC (major histocompatibility complex) class II are a family of molecules that is found in specific cells of the immune system known as antigen-presenting cells. Genetic variations in these molecules greatly influence the function of the immune system and were previously associated with autoimmunity [60]. MHC class II allele HLA-DRB1*15, which was previously linked to increased risk for MS, is relatively common among individuals of Northern European descent [61]. Ramagopalan et al. wished to demonstrate a mechanism by which genetic and environmental factors of MS interact. In their molecular study, they showed a direct impact of vitamin D on HLA-DRB1*1501, as the vitamin influences its expression in lymphocytes [62].

Vitamin D was reported to be an early predictor of MS activity and progression: slower progression and low disease activity were seen in patients with high serum vitamin D levels at the time of MS diagnosis. An increase of 50 nmol/L in average serum vitamin D levels in the first year from diagnosis was associated with less active lesions in magnetic resonance imaging (MRI) scans and lower relapse rate [63–65].

In an interventional study, increased levels of transforming growth factor-β1(TGF-β1) were measured in response to vitamin D supplementation in MS patients after 6 months' treatment [66].

Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are characterized by chronic inflammation of the gastrointestinal tract. The underlying mechanism for these diseases remains unknown, although the mechanism seems to involve overactivation of the immune system in response to certain antigens in individuals with genetic predisposition. Clinical manifestations of IBD vary and can include abdominal pain, diarrhea, weight loss, and anemia. There are two main diseases in this category: Crohn's disease (CD) and ulcerative colitis (UC); the two differ in their clinical, histological, and epidemiological characters [67, 68].

Vitamin D deficiency was found in 49.8% of IBD patients in a retrospective cohort study and severe deficiency in 10.9% of them. Moreover, low levels of vitamin D were associated with higher disease activity and lower quality of life in patients with CD (though not patients with UC) [69]. One factor that may mediate this association is malabsorption, a common condition in IBD patients, which can influence the absorption of vitamin D from food. However, the main source for vitamin D in the human body is its synthesis in the skin; therefore the influence of malabsorption as a mediator is limited. Another possible mediator for this association is the regulatory effect of vitamin D on the innate immune system's response to intestinal microbiota; the intestinal microbiota is one of the environmental factors that has been associated with IBD in recent year [70].

To examine the relationship between vitamin D and IBD, a team created an experimental animal model of IBD by creating interleukin 10 (IL-10) knockout (KO) mice, who developed the inflammatory bowel disease spontaneously. They created three groups: a control group, an experimental group that was vitamin D deficient, and an experimental group that was deficient early in life, but later supplemented with the vitamin. Both experimental groups showed severe clinical manifestations of the disease, however, vitamin D supplementation ameliorated symptoms of the disease. Mice in the control group did not develop the disease [71]. Other supporting evidence for this association can be found in another animal model experiment, in which IL-10 KO mice, as well as VDR KO mice, developed severe IBD with high mortality rates [72].

Inflammatory bowel diseases have extra intestinal manifestations and a deteriorating effect on bone health; IBD patients are more prone to suffer from osteoporosis and fractures. One suggested explanations for these issues is malabsorption of calcium from digested food. It has also been suggested that inflammatory mediators impact the activity of osteoblasts or bone-forming cells [73]. Hence, in IBD patients, vitamin D supplementation importance is doubly important, both for modulation of the immune system and for preserving bone health.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease that is usually characterized by symmetric polyarticular inflammation. Non-articular involvement of the disease can be seen in almost all systems of the body, including the skin, eye, lung, heart, kidney, and blood vessels.

Vitamin D deficiency is common among male RA patients, and even more so in patients who developed anti-cyclic citrullinated peptide antibodies—a specific, yet nonsensitive, marker of the disease [74].

When vitamin D levels and RA symptoms were compared between populations of Northern and Southern Europe, an inverse association was found between vitamin D levels and RA disease activity. Notably, this association was found only during the summer months [75].

A cohort study, comprised of 29,368 women over the course of 11 years, showed that intake of vitamin D supplementations was inversely associated with the risk of RA ($p = 0.03$), while dietary intake of vitamin D showed a similar trend but did not reach significance ($p = 0.16$) [76]. In a different study that analyzed data from the "Nurses' Health Study," no association was found between dietary intake of vitamin D and RA risk [77].

All three studies imply a fundamental difference between dietary and supplementary intake of vitamin D. This difference could be explained by the retrospective design of the studies and the methodological difficulty in assessing dietary intake of vitamin D, in contrast to intake of supplements.

Psoriasis

Psoriasis is a chronic inflammatory skin disorder. One of the disease's manifestations is hyperproliferation of the skin, which results in erythematous plaques with or without silver scale over them.

Lower levels of vitamin D deficiency were observed in psoriasis patients compared to healthy control patients in a case-control study by Orgaz-Molina et al. [78]. Also, VDR polymorphism in *ApaI* was associated with psoriasis, as well as with early onset of the disease [79].

In addition to the immunomodulatory effect of vitamin D, another important feature of the vitamin was discovered—as an antiproliferative agent, specifically in keratinocytes. Previous studies demonstrated that keratinocytes have VDR and that activated vitamin D inhibited their proliferation and stimulated their maturation in vitro [80], making activated vitamin D and its analogues good therapeutic agents for psoriasis [81]. Therefore, topical use of vitamin D analogs (calcipotriol, calcitriol, and tacalcitol) is an essential part of psoriasis treatment [82].

Other Autoimmune Diseases

Vitamin D deficiency was previously associated with several other autoimmune diseases.

Primary biliary cholangitis (primary biliary cirrhosis—PBC) and autoimmune hepatitis (AIH) are chronic autoimmune inflammatory diseases of the liver. Different genetic polymorphisms of VDR were associated with both diseases, *BsmI* polymorphisms in PBC patients ($\chi^2 = 9.49$, $p = .009$) and *FokI* in AIH patients ($\chi^2 = 9.71$, $p = .008$) [8].

VDR polymorphism (*BsmI* and *TaqI*) was also associated with autoimmune thyroid diseases (AITD) [83]. Moreover, vitamin D deficiency was found to be more common among patients with autoimmune thyroid diseases compared to patients with non-autoimmune thyroid diseases and to healthy controls; vitamin D deficiency also correlated with presence of antithyroid antibodies [84].

Low levels of vitamin D were inversely associated with disease's severity, both in Behçet's disease, a type of immune-mediated small-vessel systemic vasculitis [85], and in systemic sclerosis (SS), an autoimmune connective tissue disease [86]. Vitamin D levels were found to be lower in diffuse cutaneous type of SS (dcSSc) compared to limited cutaneous type (lcSSc) and negatively associated with patients' skin thickness [87].

Possible Underlying Mechanism

In the past decades, since the discovery of VDR on activated T cells [5], there has been growing interest in the various effects of vitamin D on T cells.

Type 1 T helper cells (Th1) and their subset of cytokine, including IL-2, IL-12, interferon gamma (IFN-γ), and tumor necrosis factor (TNF), have been previously associated with autoimmune processes. Specifically, Th1 are involved in organ-specific autoimmune diseases [88, 89] like many of the diseases mentioned above. Another subset of T helper cells, Th17, which was discovered more recently, also has a crucial role in various autoimmune conditions [90].

Vitamin D was shown to have an inhibitory effect on Th1 cells [91], leading to decrease in Th1 cytokine production [18, 91, 92]. Some studies also suggest vitamin D promotes an immunologic shift toward Th2, by increasing Th2 cytokines [91, 93]; however, there are conflicting reports on this matter, as some studies suggest vitamin D has an inhibiting effect on Th2 cytokines as well [94]. Th17 cells are also influenced by vitamin D; the activated form of vitamin D was shown to modulate Th17 activity and ameliorate symptoms of related autoimmunity [95].

T regulatory cells (Treg) are a subset of CD4+ T cells that also express CD25. They have an important role in modulation of the immune system, specifically in autoimmune diseases that are mediated by autoreactive T cells [96]. Previous studies have found that vitamin D increased the population of Treg cells, both in animal models [97] and in humans [92, 98].

Evidence suggests B cells are also affected by vitamin D. In an in vitro study, vitamin D inhibited autoantibody production and secretion [99].

Dendritic cells are antigen-presenting cells that activate naïve T cells and stimulate B cells for growth and differentiation [100, 101]. Differentiation and maturation of dendritic cells is modulated and inhibited by vitamin D, as well as their activity, which results in increased tolerance in autoimmune conditions [102–104].

Conclusion

Maintaining normal levels of vitamin D is important as vitamin D has a crucial role in regulation of the immune system.

Vitamin D deficiency is common in patients with autoimmune diseases. Epidemiologically, this deficiency was found to be a risk factor for various diseases, including MS, type 1 diabetes, IBD, and RA.

Many molecular studies concluded that through different mechanisms, vitamin D has an immunomodulatory effect on various cells of the immune system; however, the interaction between those mechanisms is yet to be determined. Although great progress has been made on this subject in recent years, there are still some missing parts of the puzzle.

Clinically, vitamin D deficiency affects the activity of numerous autoimmune diseases outcomes: end organ damage in SLE patients, glycemic control in type 1 diabetes patients, MRI-detected brain lesions and relapse frequency in MS patients, disease activity and quality of life in IBD patients, disease activity and serologic markers in RA patients, and others.

However, interventional studies done mostly on SLE patients failed to reach definite conclusions regarding treatment with vitamin D supplements for the disease, except for those performed on patients treated with steroids. Determining the safety, efficacy, and establishment of the exact doses for vitamin D treatment in patients with autoimmune diseases requires additional extensive interventional studies. Future studies should address possible intervening variables such as initial and final levels of the vitamin, VDR genetics, and sun exposure. However, since vitamin D has little, if any, known side effects in autoimmune patients, and its cost is rather low, vitamin D treatment should be considered based on existing evidence.

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Chapter 3
Vitamin D and Infectious Diseases

Christian Wejse and Cecilie Blenstrup Patsche

Why Would Vitamin D Have an Impact on Infectious Diseases?

As described in Chap. 1, Vitamin D (VD) is much more than a calcium metabolism-regulating hormone, and since lymphoid cells were discovered to richly express the Vitamin D Receptor (VDR) [1], there has been growing understanding of the overall importance of VD in various aspects of the immune system [2]. $1,25(OH)_2D_3$ has repeatedly been shown to enhance macrophage phagocytosis and upregulate numerous other pathways in the host immune response [3]. Hence, VD has been suggested as a possible adjuvant therapy for infections [4] or for prevention of infections [5]. In the following chapter, we will review the available literature on VD and a number of infectious diseases which have been associated with low VD.

Historical Aspects

Prior to modern knowledge of microbiology and causative mechanisms behind infections, VD was used to treat conditions which today are considered infections.

C. Wejse, MD, PhD • C.B. Patsche, BSc
Department of Infectious Diseases, Aarhus University Hospital, Aarhus N, Denmark

Center for Global Health, Dept of Public Health, Aarhus University, Aarhus N, Denmark
e-mail: wejse@dadlnet.dk

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Cod Liver Oil

The VD-rich cod liver oil was first recommended for consumption in 1766 by Darbey [6], and throughout the eighteenth century, cod liver oil was widely used to treat a number of conditions, such as tuberculosis (TB) [7]. Bennett initiated cod liver oil treatment at his TB sanatorium in Edinburgh with good results:

> "Cod liver oil has like no other remedy rapidly restored the exhaustive powers of the patient, improved the nutritive functions generally, stopped or diminished emaciation, checked the perspiration, quieted the cough and expectoration, and produced the most favourable influence on the local disease." [8]

From the Hospital for Consumption and Diseases of the Chest in London, it was reported from one of the first clinical trials that 1–2 tablespoons of cod liver oil two to four times daily given to 542 inpatients with TB arrested disease in 18% and improved disease in 63% [9]. This was remarkable because, among the 535 inpatients not given cod liver oil, only 5% were noted to have arrested TB disease, although the number of patients with improved disease was similar (61%) [10].

The widespread use of cod liver oil was even attributed to a 19% reduction in deaths due to consumption in Philadelphia between 1847 and 1852 [11]. It gradually became clear, however, that although perhaps a useful adjunctive therapy at a time when few other remedies were available, it was not a cure-all for TB, which was also noted by those advocating cod liver oil [11].

Light on Mycobacteria

In 1903, Niels Finsen was awarded the Nobel Prize for his discovery of the usefulness of light therapy for lupus vulgaris (skin TB) [12]. The theoretical background was believed to be a specific ultraviolet wave (UV) which induced bacteria killing [13, 14]. Yet, in 1958 VD was also considered to be the mechanism behind the effect of light therapy on skin TB; it was shown that the UV radiation in Finsen lamps could yield 100 IU VD per square cm—enough to equalize the obtained skin concentration after an oral dose of 500,000 IU [15]. Recently, production of singlet oxygen through radiation of porphyrins has been suggested as a plausible explanation of why Finsen's therapy worked [16]. The authors also suggested that possible effects of UV radiation on the immune reactions in the skin and granuloma (i.e., a VD-mediated reaction) could be responsible.

Vitamin D Used in Treatment

Cod liver oil remained an important part of TB treatment as late as in 1960 [7]. Using this knowledge, attempts were made to use calciferol in TB treatment. This was first described in 1943 by Charpy and later by Dowling [17, 18]. There are

numerous reports of a very convincing effect in treatment of lupus vulgaris with calciferol or its precursor, ergosterol [19–24]. The largest population treated was 1230 patients with various forms of skin TB: 748 patients had lupus vulgaris and of these 38.4% were completely cured [25]. A Dutch group found that the well-documented effect of Finsen's UV radiation on lupus vulgaris could be attributed to the skin formation of VD and even noted more rapid improvements when Finsen's lamps and ergosterol were used together in the treatment [15].

Various forms of VD were also used successfully in scrofula [20], in abdominal TB [26, 27], and, with varying results, in pulmonary TB [28–31]. In the literature, the use of VD for pulmonary TB was heavily debated [32–34], as well as the appropriate dosage. Charpy initially used 1,200,000 IU per week but later reduced to 600,000 IU per 60 kg every fifth day for lupus vulgaris [6, 17]. Most authors followed this regimen, and Marcussen even noted more relapses when using less than 100,000 IU per day [35]. One group found that 30,000 IU/day was sufficient for treating pulmonary TB [36], and current recommendations are not to exceed 50,000 IU/day to avoid toxicity [37]. Some data show that 100,000 IU/day may also be acceptable for a limited period of time [38], although only trials using ≤10,000 IU/day for longer periods have not reported toxicity [39]. Interestingly, the dosage of cod liver oil previously mentioned would be equivalent to 6000–24,000 IU per day [37].

Safety in terms of affecting calcium concentration, blood pressure, and electrocardiogram reading was found to be satisfactory [32, 40], but there were frequent cases of intoxication [27, 41, 42] and concerns of provoking exacerbation [23, 43, 44]. Even after streptomycin and isoniazid were widely used, some still recommended 600,000 IU calciferol twice a week as supplementary treatment [45], in particular in cases with streptomycin resistance [43].

Marcussen from the Finsen Institute reported that 83.5% of 280 lupus vulgaris patients treated with vitamin D_2 alone were clinically and histologically symptom-free during the course of treatment, but there were frequent relapses, and only 33% remained symptom-free after 5 years of follow-up [35]. This observation led them to conclude that VD acted on the host rather than on the tubercle bacillus.

Infections Where VD Has Been Hypothesized to Play a Role

A number of infectious diseases have been associated with Vitamin D deficiency (VDD), or the immune response toward these infections has been shown to be modulated by VD.

Tuberculosis

The infectious disease most frequently associated with VD is TB. This is partly because of the historical connection, as previously described, but also because of a rather strong documentation for specific associations with TB-relevant host immune

responses, a repeated epidemiological association, and several large treatment trials, although these have been with variable effect.

TB Immunopathology in Relation to Vitamin D-Mediated Immunology

The specific role of VD in immune reactions toward infection with *Mycobacterium tuberculosis* (MTB) was best described in a series of elegant experiments published in *Science* in 2006 [3]. Liu et al. showed that Toll-like receptor (TLR) 1 and 2 stimulation of human macrophages results in (1) reduced viability of intracellular MTB in human monocytes and macrophages but not in dendritic cells, (2) upregulation of the VDR gene in monocytes, and (3) hydroxylation of $25(OH)D_3$ into $1,25(OH)_2D_3$. They further showed that adding $1,25(OH)_2D_3$ to human monocytes led to upregulation of cathelicidin, an antimicrobial peptide, and a reduction of viable MTB in infected macrophages; in fact, simultaneous addition of $25(OH)D_3$ and TLR 2/1 ligand also upregulated cathelicidin. Additionally, Coussens et al. have shown that VD accelerates resolution of inflammatory responses during TB treatment [47].

Epidemiological Aspects: Vitamin D Status Among Patients with Active TB Infection

A number of studies have associated VDD with active TB. A report from Wilkinson on Gujarati Asians in London found significantly lower $25(OH)D_3$ among 91 patients with TB than in 116 healthy contacts, with an odds ratio (OR) of 2.9 for VDD being associated with TB [48]. The same group also reported a very high frequency (76%) of VDD among 210 consecutive patients with TB, mainly foreign-born [49]. The largest observational study on the subject matter was conducted in a high-burden setting in Africa, where we found an association between TB and Vitamin D insufficiency, but intriguingly no association with severe Vitamin D insufficiency [50] was found. A meta-analysis of case-control studies on VDD and TB risk concluded that serum VD level < 10 ng/mL (=severe VDD, sVDD) was significantly associated with an increased risk of TB; the range of 10–20 ng/mL (=VDD) was associated with a potential higher risk of TB, while the range of 20–30 ng/mL (=VD insufficiency) was not associated with an increased risk of TB [51].

The observation that African-Americans are more susceptible to infection with MTB [52], and also more frequently suffer from VDD [53], has been interpreted as a VDD-induced susceptibility toward TB. The abovementioned observations are frequently cited, and taken together, they point toward patients with TB having increased frequency of VDD and may suggest that this VDD was also implicated when the patient acquired MTB or developed active disease. But these are observational surveys with important risks of bias and confounding, and there are no prospective studies available in which patients with VDD are followed for risk of latent or active TB.

Genetic Aspects: Vitamin D Receptor and Susceptibility Toward TB

Certain VDR polymorphisms have been associated with higher risk of TB [54]. A study including TB cases and controls from Guinea-Bissau showed no association with single-nucleotide polymorphisms, but did show an association with TB in a family-based analysis at the haplotype level [55], and this was confirmed in a case-control study [56]. A group from Peru was even able to show a difference in treatment response toward TB, depending on genotype of the patient [57]. However, a large study from a highly TB-endemic region showed no association with *FokI*, *TaqI*, and *ApaI* loci [58]. A meta-analysis of 23 studies found major heterogeneity with a tendency toward a positive association with the *FokI* gene among Asians [59], and it therefore does not seem likely that VDR polymorphisms play a major role in TB susceptibility.

Intervention Studies Supplementing Patients with TB with Vitamin D

Several randomized clinical trials have been testing the effect of adding VD to TB treatment. The largest study conducted in a high-burden area in West Africa reported no serious adverse effects using an oral regimen of 100,000 IU three times during TB treatment [60]. The trial found no effect on morbidity or mortality, but reported a tendency for more rapid sputum conversion and reduced mortality among human immunodeficiency virus (HIV)-uninfected patients with low VD levels. A meta-analysis of six randomized controlled trials (RCT) with a total of 1005 patients with TB found a nonsignificant effect on sputum conversion in the VD supplementation group (OR = 0.86; 95% CI 0.62–1.19) and concluded that no beneficial effect of VD treatment for TB could be found [61].

Two small trials have assessed the effect of VD for prevention of active TB. A feasibility trial from a high TB incidence area in Mongolia enrolled 120 VDD children, supplying a daily dose of 800 IU [62]. They found that tuberculin skin test (TST) conversion occurred in 5/45 (11%) in the placebo group compared with 11/41 (27%) in the VD supplementation group. The other trial, from the UK, investigated the effect of a single dose of 100,000 IU on adult persons, who had contact to TB index cases [46]. A protective effect on the risk of acquiring latent TB infection, using interferon-gamma release assays, was not found.

There has been some hesitation to supplement patients with TB with VD for fear of hypercalcemia. Hypercalcemia in TB has been described mostly in case reports in which other conditions may also have played a role [63], but hypercalcemia has also been described in larger series, e.g., among 25% of patients with TB in Greece [64]. However, hypercalcemia was reported to be very rare among patients with TB in Hong Kong, Malaysia, and Turkey [65–67]. In one of the studies in which VD was given to patients with TB, no cases of hypercalcemia were found in neither those supplemented with VD nor those not supplemented [68]. Also noteworthy are older reports of high-dose therapy, in which calcium was monitored carefully and hypercalcemia not seen. An Indian study from 1957 used 600,000 IU/week in 8

pulmonary and 19 extrapulmonary TB cases resulting in improvement in clinical status, and they reported that hypercalcemia was absent in all [30].

Other Respiratory Tract Infections

The initial observation that there could be an association between VD status and respiratory tract infections was made through observational studies indicating an association between nutritional rickets and pneumonia. In an Iranian study on 200 children admitted to a children's hospital with radiologically proven rickets, Iran, 43% had bronchopneumonia [69], and in a study from Kuwait on 250 children with VDD rickets, 44% had pneumonia [70]. A case-control study from Egypt found acute respiratory infections in 81% of children with rickets, but only in 58% of controls [71]. Another case-control study from Ethiopia found that rickets was seen in 210 cases hospitalized with a diagnosis of pneumonia, but only in 20 of 500 sampled control children in the area (OR = 22.1) [72]. A systematic review including 12 studies with 2279 children found that children with lower respiratory tract infections (LRTI) had significantly lower mean VD levels as compared with controls; 29.7% of children with LRTI have VDD with an OR of 3.3 compared with controls [73]. This systematic review also found a correlation between VD levels and LRTI incidence and severity.

Among adults, a major observational study from a US National Health Interview Survey, involving 18,883 participants [74], reported recent upper respiratory tract infection (RTI) among 24% of those with VDD compared with 17% among participants with normal VD status (adjusted OR = 1.36), with stronger associations for individuals with asthma and chronic obstructive pulmonary disease. Another major observational study from the British Birth cohort study, involving 6789 participants [75], found a prevalence of respiratory infections with a strong seasonal pattern in the opposite direction to the pattern for 25(OH)D concentrations. Each 4 ng/mL increase in 25(OH)D was associated with a 7% lower risk of infection (95% CI 3–11%) after adjustment for adiposity, lifestyle, and socioeconomic factors. Another important cohort study on this topic, a Finnish study on 800 army recruits, showed that absence from duty due to respiratory tract infections was more frequent among recruits with 25(OH)D concentrations <16 ng/mL than in recruits with higher 25(OH)D levels [76].

The adult observational studies have not been reviewed separately, but a review of all the cross-sectional and cohort studies on VD and RTI by Jolliffe et al. [77] found consistent associations between low serum 25(OH)D concentrations and increased risk of RTI in the cross-sectional studies, in 5 out of 8 case-control studies and 7 out of 13 cohort studies. Yet, as noted by Bryson et al. in another review [78], the populations had symptoms of the infection at the time of the 25(OH)D measurement, and it is therefore difficult to distinguish if low VD status is a contributing factor or a consequence of the infection. None of the studies have examined if the infections actually had a direct effect on catabolism of 25(OH)D and $1,25(OH)_2D$, which could lead to an apparent VDD.

Intervention Studies on Respiratory Tract Infections

There have been a number of randomized clinical trials assessing the effect of VD administration on the risk of RTI; the largest was a trial enrolling 3046 children in Afghanistan [79], which showed low baseline 25(OH)D in the intervention as well as placebo arm, and upon 100,000 IU VD orally or placebo every 3 months for 18 months, it was found that 25(OH)D level in the VD group was consistently higher (32 ng/mL) than in the placebo group (10 ng/mL). Yet, no difference in incidence of pneumonia was seen between VD and placebo groups [79]. Several meta-analyses of the available trials have been conducted [78]. The most recent is by Zittermann et al. [61], which included 16 trials and found that the randomized controlled trials indicate a significant risk reduction by VD supplements (OR = 0.65; 95% CI 0.50–0.85). The trials further showed that daily administration was more effective than high-dose bolus administration (OR = 0.48; 95% CI 0.30–0.77 vs. OR = 0.87; 95% CI 0.67–1.14) and that individuals with deficient (10–20 ng/mL) circulating 25-hydroxyvitamin D levels benefit most.

Methicillin-Resistant *Staphylococcus aureus* (MRSA) Carriage in Airways

The association of methicillin-resistant *Staphylococcus aureus* (MRSA) and VD is similar to the association of other bacterial infections with VD. Patients with MRSA often have VDD [80, 81], and VDD has been found to be an individual risk factor for MRSA infection [81, 82]. Nasal carriage of MRSA increases the risk of MRSA infection, and VDD is hypothesized to inhibit the ability to clear nasal colonization of MRSA [82].

Sepsis

VDD is highly prevalent in sepsis patient populations [83], and sepsis patients admitted to intensive care units (ICUs) with low 25(OH)D have also been shown to have lower mean levels of cathelicidin and Vitamin D-binding protein, which may lead to reduced phagocytosis [84].

Observational Studies on Vitamin D and Sepsis

Several observational studies have described an increased mortality in sepsis patients with VDD—the largest is a retrospective cohort study from the USA involving 23,603 hospitalized patients with a 25(OH)D measured before admission [85]. In this cohort, where most of the subjects were not sepsis patients, the adjusted OR of 30-day mortality in patients with VDD was 1.45 (95% CI 1.21–1.74) and 1.30 (95% CI 1.10–1.54) for Vitamin D insufficiency (<30 ng/mL), both compared with

patients with normal prehospital 25(OH)D. A subgroup of 5628 patients had blood cultures drawn, and in this group VDD was associated with increased odds of community-acquired bloodstream infection (adjusted OR = 1.29; 95% CI 1.06–1.57) relative to patients with normal 25(OH)D. The observational studies have been well summarized in two recent systematic reviews by de Haan et al. [86] and Upala et al. [87]. The De Haans meta-analysis from 2014 included only critically ill patients from 14 observational studies involving 9715 patients and found that VDD was associated with increased rates of infection (risk ratio (RR) = 1.49; 95% CI 1.12–1.99), sepsis (RR = 1.46; 95% CI 1.27–1.68), 30-day mortality (RR = 1.42; 95% CI 1.00–2.02), and in-hospital mortality (RR = 1.79; 95% CI 1.49–2.16). The Upalas meta-analysis included a large retrospective cohort study by Lange including hospitalized patients without a sepsis diagnosis and reported on total ten observational studies involving 33,810 patients. The findings were similar with OR of sepsis in participants with VDD at 1.78 (95% CI 1.55–2.03) compared with controls.

Vitamin D Supplementation Trials

Only a few small trials have been conducted with the aim of testing VD supplementation for sepsis patients. In a trial from the USA, 67 critically ill patients with severe sepsis or septic shock [88] were administered 2 mcg of calcitriol ($1,25(OH)_2D_3$) as a single intravenous dose to determine the outcomes on plasma cathelicidin antimicrobial peptide (hCAP-18) protein levels and other pro-inflammatory markers. The median 25(OH)D levels in the placebo and intervention group in this cohort were both in the deficient range (17.6 and 16.5 ng/mL, respectively). The trial showed increased plasma $1,25(OH)_2D$ levels in the treatment group (median 75.7 pg/mL in the treatment group and 16.9 pg/mL in the placebo group, $p < 0.001$), but no significant change in plasma hCAP-18 or cytokine levels was found. The sample size was too small to detect clinical outcomes.

In another trial from Spain, Mata-Granados et al. studied calcifediol ($25(OH)D_3$) and calcitriol supplementation in 33 critically ill ICU patients with sepsis and 92 healthy controls [89]. The prevalence of VDD in the critically ill patients was 96.7 and 62% in the healthy controls. The critical care group was divided into 3 arms: (1) no VD given ($n = 12$), (2) treatment with 1.5 mg of oral calcifediol ($n = 11$), and (3) treatment with 2.0 mcg of intravenous calcitriol ($n = 10$). The study showed that calcifediol supplementation increased serum $25(OH)D_3$ and serum 1,25(OH) levels from baseline, but calcitriol supplementation did not show a significant improvement in serum $25(OH)D_3$ or $1,25(OH)_2D_3$ from baseline. No clinical outcomes were measured, so the benefit of supplementation is not clear.

The largest VD supplementation trial in critical illness was the VITdAL@ICU trial from Austria, which included 492 critically ill patients, who all had VDD [90]. Patients in the intervention arm received a single loading dose of 540,000 IU VD and a monthly maintenance dose of 90,000 IU VD from month 1 to 5. The trial showed no statistically significant difference in hospital length of stay or

ICU stay between the intervention group and the placebo group. There was no significant difference between the two groups for incidence of mortality, causes of death, severity of disease, or mechanical ventilation. The intervention group did show lower markers of inflammation by day 28 (procalcitonin and C-reactive protein), and higher albumin levels, compared with the placebo group. In a subgroup analysis comparing severe VDD with VDD, hospital mortality was significantly lower in the VD intervention group compared with the placebo group (hazard ratio (HR) = 0.56; 95% CI 0.35–0.90), which remained significant when adjusted for confounders. At the 6-month follow-up, mortality rates were lower in the VD treatment group (34.7%) compared with the placebo group (50%); however, this was not statistically significant.

Human Immunodeficiency Virus

HIV results in CD4+ T cell depletion and immune dysfunction. The modulatory effects of VD in HIV infection remain uncertain, but it has been hypothesized that $1,25(OH)_2D$ can reduce CD4+ surface expression on monocytes [91, 92], thus controlling viral entry and halting disease progression [93]. VDD impairs the ability to reduce pro-inflammatory cytokines and may lead to an increased proliferation of T helper 1 cells particularly susceptible to HIV infection [94]. Bergman et al. found that the antimicrobial peptide cathelicidin inhibits replication of HIV-1 [95]; however, VDD impairs the expression of antimicrobial peptides.

Several studies have demonstrated that VDD is common in patients with HIV [96–98], and in a recent review, the prevalence of VDD was estimated to be 70–84% [99]. Low baseline 25(OH)D levels have been associated with HIV disease progression [100–104], mortality [100, 102, 104], and virologic failure [100].

Association of Anti-retroviral Treatment (ART) and Vitamin D

Anti-retroviral agents are believed to affect the hydroxylation steps in Vitamin D metabolism, and VDD in HIV-infected individuals is therefore proposed to be a side effect of anti-retroviral agents. Treatment with the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz has been associated with decreased 25(OH)D levels [105–108], and Fox et al. demonstrated an increase of 25(OH)D in participants converting from an efavirenz-based treatment regimen to a darunavir-based treatment regimen [109]. Treatment with the NNRTI nevirapine has likewise been associated with decreased 25(OH)D levels [110]. In vitro studies have shown that protease inhibitors, especially ritonavir, decrease production of $1,25(OH)_2D$ [111]; however, Conesa-Botella et al. [110] and Cervero et al. [112] did not find decreased 25(OH)D levels among patients treated with protease inhibitors. Data on nucleoside reverse transcriptase inhibitors is scarce, but zidovudine has been reported to reduce serum VD concentrations [109]. The complex association of anti-retroviral drugs,

VDD, and HIV disease progression requires careful monitoring of VD status among HIV-infected individuals.

Vitamin D Supplementation to HIV Infected

Treatment of human monocytes and macrophages with $1,25(OH)_2D$ in laboratory settings have yielded conflicting results [91, 93, 113], and only a few placebo-controlled randomized trials have been performed. Kakalia et al. [114] randomly assigned HIV-infected children to receive (1) no supplementation, (2) 5600 IU VD weekly, or (3) 11,200 IU VD weekly for 6 months. Serum 25(OH)D levels increased significantly in both supplementation groups, but had no effect on CD4+ T cells. Similar results were found by Arpadi et al. [115] (100,000 IU bimonthly or placebo in children aged 6–16 years) and Giacomet et al. [116] (100,000 IU quarterly or placebo in VDD patients aged 8–26 years). According to Havens et al. [117], efavirenz-based treatment did not diminish the response to VD supplementation of 50,000 IU monthly for 3 months.

Hepatitis C Virus

The regulatory effects of VD on immunomodulation, inflammation, and fibrogenesis are believed to be involved in the pathogenesis of viral hepatitis, with VDD inducing fibrogenesis and impairing the ability to reduce pro-inflammatory cytokines. It has been suggested that VDD results in diminished T cell function and thus a reduced capacity to inactivate hepatitis C virus (HCV) [118]. Though the exact relationship between HCV and VD is uncertain, it is believed that HCV contributes to an altered lipid metabolism decreasing 25(OH)D levels [119], as is also seen in patients with advanced liver disease [120, 121].

Association of Vitamin D Deficiency with Disease Severity and Sustained Viral Response

Patients with HCV often have VDD, and multiple studies have focused on the impact of VDD on disease severity and sustained viral response (SVR) after treatment. Some studies have showed an association of VDD and the severity of liver fibrosis or SVR, while others have not. A meta-analysis by Garciá-Álvarez et al. [122] showed an overall significant association between VDD and advanced liver fibrosis, which was partially supported by Luo et al. [123]. Garciá-Álvarez et al. also analyzed the relationship between baseline VD status and SVR and found a significant association between low VD status and poor SVR, regardless of HCV genotype and HIV coinfection. The results were supported by a meta-analysis by Villar et al. [124], but not by Kitson et al. [125], who failed to find a significant

association between baseline 25(OH)D and SVR in neither of the HCV genotypes. Villar et al. included studies on HIV-HCV coinfected patients, whereas Kitson et al. excluded studies on HIV-HCV coinfected patients. The conflicting results may be a result of the great heterogeneity of existing studies, and a major source of heterogeneity is believed to be due to ethnicity [122]. Studies in patients with chronic hepatitis B and alcoholic hepatitis have yielded similar conflicting results.

Vitamin D Supplementation and Hepatitis

In 2011, Bitetto et al. [126] published results from a retrospective study considering the effect of VD supplementation on SVR among patients with recurrent hepatitis C being treated with pegylated interferon (PegIFN) and ribavirin (RBV). Patients received VD supplements on the basis of known osteopenia or osteoporosis and on average had begun VD supplement treatment more than 1 year prior to treatment for recurrent HCV. The authors showed that patients who were supplemented with VD more frequently achieved a SVR, instigating the idea that VD supplements might improve standard HCV treatment outcome. Since then, a few studies have examined the effect of VD supplement on treatment outcome, with special attention given to the difficult-to-treat HCV genotypes 1 and 4. Yokoyama et al. [127] evaluated the effect of VD supplementation (1000 IU daily) beginning 8 weeks in to PegIFN/RBV treatment in HCV genotype 1 patients, who did not achieve a rapid viral response after 4 weeks of treatment. A nonsignificant tendency toward an improved SVR was observed in favor of patients receiving VD supplementation. Esmat et al. [128] examined the effect of VD supplementation (15,000 IU weekly) on SVR in HCV genotype 4, but found no correlation between 25(OH)D levels and fibrosis stage, and no positive impact of VD on SVR. Three other trials examined the effect of a 4- to 12-week lead-in phase of VD supplementation combined with VD supplementation during the treatment period. Abu-Mouch et al. [129] evaluated the effect of 2000 IU daily in HCV genotype 1 treatment-naïve patients and found a significant improvement in SVR in the VD-supplemented group. Terrier et al. [130] examined the effect of 100,000 IU (weekly during lead-in, monthly during treatment) with PegIFN/RBV in null-responder patients with HCV genotype 1 or 4. No grade 3 or 4 adverse events were attributed to VD supplementation, and VD supplementation effectively increased 25(OH)D; however, early viral response did not improve, and the study was discontinued. Atsukawa et al. [131] examined the effect of 2000 IU daily in HCV genotype 1b (non-TT) treated with the new direct acting antiviral agent simeprevir in combination with PegIFN/RBV. VD supplementation was found to improve SVR rates among treatment-naïve patients and null responders. To date, only one study has examined the effect of VD supplements in patients with HCV genotypes 2–3: Nimer et al. [132] assessed the effect of 2000 IU daily (including a 12-week lead-in phase) and found that VD significantly improved the rate of SVR. Results from these studies propose a beneficial effect of a daily dose of 2000 IU VD on SVR after treatment, especially if combined with a lead-in phase to optimize 25(OH)D levels, whereas this effect is not seen for higher doses. Further

studies are needed to confirm this tendency. Current suggestions for VD supplement dosage is 4000 IU daily for patients with baseline 25(OH)D levels <10 ng/mL and 2000 IU daily for patients with baseline levels of 10–20 ng/mL [121].

In recent years, the association of interleukin and VDR polymorphisms with antiviral response has been given increased attention and may prospectively help predict treatment response.

Hepatitis C and HIV Coinfection

HIV-infected individuals are more susceptible to HCV and have a more rapid disease progression [133] with an accelerated rate of liver fibrosis and cirrhosis [134]. Furthermore, HIV-infected individuals with HCV have a reduced response rate to HCV treatment [133, 135]. It is uncertain if ART has a beneficial effect on HCV disease progression, and a recent meta-analysis concluded that ART was not able to fully correct the adverse effects of HIV infection on HCV disease progression [133]. Given the hypotheses that both HCV and anti-retroviral agents may affect Vitamin D metabolism, special attention to VD status and treatment of VDD must be given to HIV-HCV coinfected patients.

Vitamin D as Risk Marker for Infection and Role in Antimicrobial Treatment and Prevention

As illustrated above, there is a rich literature on VD and the association with several infectious diseases. The evidence points toward VDD being a risk marker for many infections, although the observational studies do not always agree on this. The meta-analyses overall agree that infections are more frequent in VDD, though the cause-effect relationship is more difficult to establish. Some have argued that VDD is a marker for ill health, which is then the true risk factor for infection [136], but the evidence for VD playing an independent role in the immune defense is abundant [137], so it is fair to conclude that, although VDD may to some extent be a confounder, it may very well also be a contributor to infections.

The hopes for VD supplementation have been high [4, 5, 137], but so far, the evidence from trials have been disappointing, and we have yet to see solid evidence supporting a role for VD as supportive treatment for infectious diseases. The role in prevention is more uncertain, but this is definitely a plausible hypothesis since VDD must be considered a form of immune deficiency. In particular when it comes to TB, there are data to support a role for VD supplementation in prevention [138], but definitive evidence will require enrollment of large populations with documented VDD, and groups without VD supplementation should be observed during a long period of follow-up. Such studies are very expensive and perhaps unethical since there is consensus that individuals with VDD should receive supplementation.

We may therefore conclude that VDD is a risk factor for infections, as a marker as well as a contributor, and that VD supplementation for deficient individuals may prevent some episodes of infection, though it is not a magic bullet in treatment of infections.

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Chapter 4
Vitamin D Hormone Action in the Endocrine Tissue: Implications for Prostate and Breast Carcinoma

Caleb Killer, Jungmi Ahn, Sulgi Park, and Bandana Chatterjee

Introduction

Calcitriol, the hormonally active metabolite of cholecalciferol (vitamin D_3), regulates diverse physiologies in addition to its classic endocrine role in the regulation of calcium and phosphate homeostasis, and thus bone mineralization. Calcitriol action in target cells is mediated by the cognate vitamin D receptor (VDR), which is a ligand-inducible transcription factor and a nuclear receptor. In extraskeletal tissues, calcitriol-activated VDR signaling inhibits cell proliferation and angiogenesis while promoting differentiation and apoptosis. The repertoire

C. Killer, BS
Research, South Texas Veterans Health Care System (STVHCS), San Antonio, TX, USA

Foundation for Advancing Veterans' Health Research, Audie Murphy VA Hospital, San Antonio, TX, USA

J. Ahn, PhD
Department of Molecular Medicine/Institute of Biotechnology, School of Medicine, The University of Texas Health San Antonio, San Antonio, TX, USA

S. Park, MS
Research, South Texas Veterans Health Care System (STVHCS), San Antonio, TX, USA

Department of Molecular Medicine/Institute of Biotechnology, School of Medicine, The University of Texas Health San Antonio, San Antonio, TX, USA
e-mail: ParkSG@UTHSCSA.EDU

B. Chatterjee, PhD (✉)
Research, South Texas Veterans Health Care System (STVHCS), San Antonio, TX, USA

Foundation for Advancing Veterans' Health Research, Audie Murphy VA Hospital, San Antonio, TX, USA

Department of Molecular Medicine/Institute of Biotechnology, School of Medicine, The University of Texas Health San Antonio, San Antonio, TX, USA
e-mail: chatterjee@uthscsa.edu

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of calcitriol action also includes anti-inflammatory response and induction of the drug metabolism machinery [1–3].

The normal and malignant tissues of prostate, breast, and other endocrine organs have an active vitamin D/VDR regulatory axis. Inhibition of prostate cancer and breast cancer in response to an activated VDR pathway has been extensively documented in cell culture, in xenograft settings, and in animal models of cancers arising from genetic changes or carcinogen-induced mutagenesis. Mechanisms attributed to the anticancer effects of vitamin D signaling include cell cycle inhibition at the G1/S checkpoint, reduced DNA damage, altered microRNA expression, pro-differentiation response, and apoptosis induction (reviewed in [4]). Relevance of prostate health to VDR/vitamin D signaling is highlighted by the significant association of low serum vitamin D status with aggressive prostate cancer [5–7]. Evaluation of prostate biopsies from prospectively enrolled men with abnormality in serum PSA levels and/or digital rectal examination revealed that African Americans with low (<20 ng/mL) serum 25-hydroxyvitamin D_3 have increased odds of prostate cancer diagnosis. This reinforces earlier results that prevalence of vitamin D deficiency among African Americans may partly account for higher incidences of aggressive prostate cancer and prostate cancer-related mortality in this race group. The same study on prostate biopsy outcomes further revealed that an increased race-independent probability for aggressive prostate cancer diagnosis, reflected in higher Gleason grades and advanced tumor stage, associated with severely low (<12 ng/mL) serum 25(OH) D_3 [5]. An intermediate range of 25(OH) D_3 (30–80 ng/mL) is considered a normal vitamin D status and may be optimal [5], since high serum vitamin D is thought to increase risks for advanced prostate cancer [8]. Inverse association has also been observed between serum vitamin D levels and breast cancer risks in postmenopausal women as well as breast cancer-related morbidity and mortality in premenopausal women [9, 10].

Prostate cancer is a common cancer in males worldwide and a leading cause of cancer deaths in US men, behind lung and colorectal cancer. Androgens, the male-prevalent sex steroids, and androgen receptor (AR), the transcription factor and nuclear receptor that mediates androgen action, regulate prostate cancer development, recurrence, and progression. Second-line inhibition of advanced prostate cancer by new-generation anti-AR/antiandrogen drugs and other intervention modalities is initially effective but non-durable [11]. A negative interplay of AR with VDR in prostate cancer cells, identified through our study and discussed in this review, leads to reduction of CYP24A1 (a cytochrome-P450 with 24-hydroxylase activity) and AR expression. Thus, a vitamin D/androgen combination may enhance inhibitory response of clinical prostate cancer to calcitriol, especially in the case of low-grade prostate cancer for which disease progression may be retarded by chemoprevention with vitamin D_3 supplementation.

In this article, we discuss (1) the enzyme machinery that drives calcitriol biosynthesis and metabolism to an inactive metabolite in the kidneys and local synthesis of calcitriol in prostate; (2) ligands, DNA response element, inter-domain allostery, and coregulators as determinants of VDR's transcriptional activity; and (3) inhibitory interaction of AR with VDR in prostate cancer cells. Finally, we describe

findings of preclinical studies and clinical trial outcomes for prostate cancer inhibition by calcitriol and analogs and comment on the prospects of uncovering novel interventions against prostate cancer by targeting downstream effectors of the VDR pathway in prostate. While this review primarily focuses on the current state of our understanding of the inhibitory vitamin D action on prostate cancer, a section briefly describing negative impacts of vitamin D signaling on breast cancer is also included.

Vitamin D Metabolism

Synthesis, Degradation, and Roles and Regulation of Key Enzymes

Calcitriol and its precursors are secosteroids with steroid-like structures. Figure 4.1 presents a schema of the biochemical pathway that leads to the cutaneous synthesis of cholecalciferol and its enzyme-catalyzed two-step hydroxylation, which ultimately produces 1,25-dihydroxy D_3 (calcitriol), the active metabolite [12]. Briefly, pro-D_2 (ergosterol) produced at the cell membranes of fungi and protozoa and pro-D_3 (7-dehydrocholesterol) produced in animal skin are secosteroid precursors of calcitriol, pro-D_2 being a weaker prohormone than pro-D_3. Upon exposure to sunlight-derived UVB radiation, the prohormones are converted first to pre-D_2 and pre-D_3, and then to D_2 (ergocalciferol) and D_3 (cholecalciferol), respectively—the latter conversion step requiring reversible heat-dependent isomerization. CYP2R1 is a 25-hydroxylase and the principal cytochrome P450 that converts D_3 or D_2 to the 25-hydroxy metabolite in the liver [13]. Additional 25-hydroxylase(s) may exist, since CYP 2R1 knockout caused 50% reduction of serum 25(OH)D_3 levels in mice [14]. A role for CYP 27A1 in the hydroxylation of vitamin D_3 at the carbon-25 position is ruled out, since CYP 2R1/CYP 27A1 double knockout mice did not show any further reduction of serum 25(OH)D_3 compared to CYP 2R1 single knockout mice. 25(OH) D_3 is the major circulating form of vitamin D in humans. In the event that endogenous synthesis of 25(OH) D_3 is not optimal, intake of cholecalciferol (vitamin D_3) from dietary and supplemental sources can maintain normal vitamin D status. Exposure to endocrine-disrupting chemicals (EDCs) such as bisphenol A (BPA) and phthalates may reduce blood levels of 25(OH) D_3, since urinary levels of these EDCs correlated inversely with serum vitamin D [15]. Intake of estrogen-containing oral contraceptives, in contrast, elevated serum 25(OH) D_3 [16]. It is of interest to know whether the effects of EDCs and estrogen are consequential to altered expression of 25α-hydroxylase or to altered calcium or phosphate homeostasis.

Bioactivation of circulating 25(OH) D_3 to the active hormone 1α,25(OH)$_2$ D_3 (calcitriol) occurs primarily in the kidneys and is mediated by 1α-hydroxylase (CYP27B1), which catalyzes additional hydroxylation at the carbon-1 position. Extrarenal tissues including prostate are also the sites of 25(OH)D_3 bioactivation to calcitriol [1, 12]. Homeostasis in cellular signaling by vitamin D is ensured by cal-

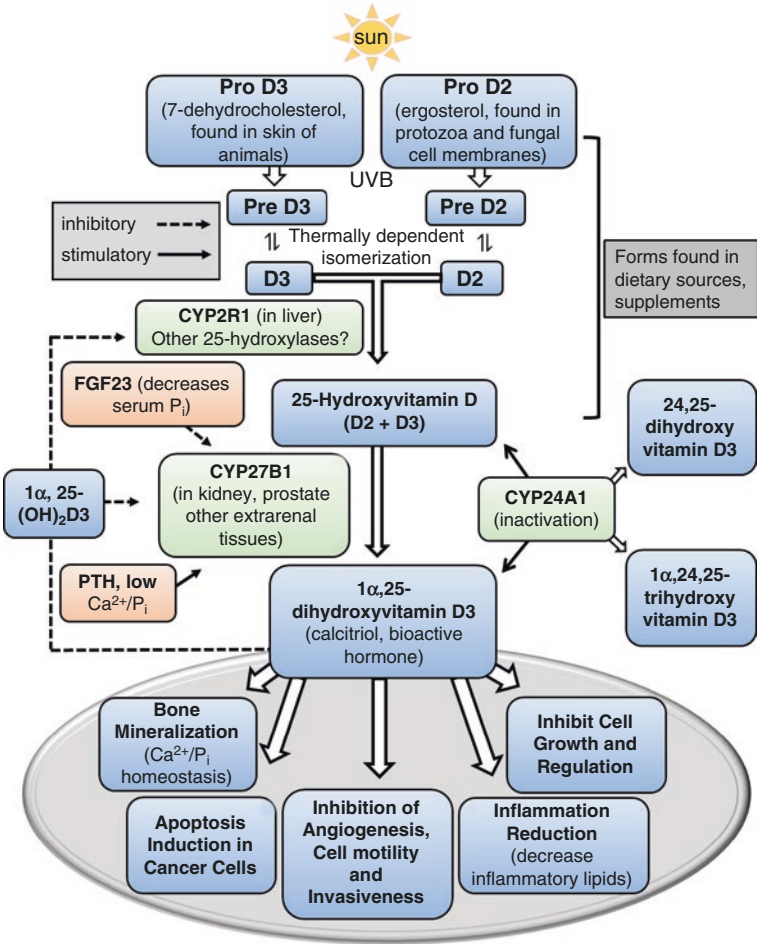

Fig. 4.1 The pathway to calcitriol (1α, 25$(OH)_2$ D_3) biosynthesis and metabolic inactivation. *UVB* ultraviolet B radiation, *CYP2R1* cytochrome P450 2R1, *FGF23* fibroblast growth factor 23, *PTH* parathyroid hormone, *CYP27B1* cytochrome P450 27B1, *CYP24A1* cytochrome P450 24A1, P_i, phosphate. Dotted arrows, inhibition; solid arrows, stimulation

citriol inactivation to 1α,24,25 trihydroxy D_3 (calcitroic acid) through a five-step oxidation process catalyzed by CYP24A1, which is a cytochrome P450 with 24-hydroxylase activity. CYP24A1 also converts 25(OH) D_3 to the inactive 24,25-dihydroxy D_3 (Fig. 4.1).

Calcitriol production is autoregulated as a result of the suppression of *CYP27B1* and *CYP2R1* gene transcription by calcitriol-activated VDR. Further, CYP24A1, the catabolic enzyme, is induced by a high serum concentration of calcitriol. Calcitriol homeostasis, which is vital for bone mineralization and bone health, is also regulated by calcium and phosphate levels. When phosphate levels are elevated, the bone-derived circulating peptide FGF23 (fibroblast growth factor-23), which

regulates secretion of the parathyroid hormone (PTH), interacts with a FGF receptor-Klotho complex, to generate intracellular signaling that leads to inhibition of *CYP27B1* gene transcription. This in turn prevents calcitriol production and phosphate reabsorption in the kidneys [17, 18]. Conversely, at low phosphate and calcium levels, *CYP27B1* gene is transcriptionally stimulated by PTH-induced intracellular signaling, leading to increased renal production of calcitriol and readsorption of Ca^{2+} and phosphate by the kidneys.

Clinical manifestations of vitamin D deficiency are linked to mutations of the enzymes that drive synthesis and degradation of calcitriol, as observed for the loss-of-function germ line mutation of the *CYP2R1* [13, 19]. *CYP27B1* mutations, which lead to vitamin D-dependent rickets type 1, are associated with hypocalcemia and hypophosphatemia and the resultant development of fracture-prone soft, weak bone and bowed legs [20]. The biochemical hallmark of CYP24A1 inactivation is the persistent escalation of calcitriol levels. *CYP24A1* mutations cause a range of clinical abnormalities due to hypercalcemia and hypercalciuria. These abnormalities include disrupted digestive homeostasis, aberrant renal functions, kidney stone, bone and muscle weakness, and abnormal brain functions. *CYP24A1* mutations leading to increased vitamin D sensitivity in patients with idiopathic infantile hypercalcemia have been reported [21].

Vitamin D Metabolism in the Prostate

Calcitriol is synthesized in the prostate from the precursor 25(OH) D_3 in the bloodstream. In a randomized clinical trial, a dose-dependent increase in calcitriol levels was detected in prostatectomy specimens from prostate cancer patients who received oral administration of cholecalciferol, and proliferation marker Ki67 levels correlated inversely with prostate calcitriol levels [22]. Ki-67 protein expression is strictly linked to cell proliferation. Calcitriol production in extrarenal tissues is essential for its intracrine, autocrine, or paracrine action, which is consistent with a ubiquitous expression pattern of CYP27B1 and vitamin D receptor [23]. Exogenously added 25(OH) D_3 converted to calcitriol in PC-3 and DU-145 (androgen receptor negative) prostate cancer cells and in cells isolated from normal and BPH prostate samples, and clotrimazole, a nonselective cytochrome P450 inhibitor, prevented this metabolic conversion [24]. 25-hydroxy D_3 did not metabolize to calcitriol in LNCaP prostate cancer cells, which lack CYP27B1 expression. It was reported that dietary supplementation of cholecalciferol and intraperitoneal injection of calcitriol reduced tumor burden from PC-3 prostate cancer xenografts at similar efficiency [25]. CYP27B1 expression was elevated in the xenograft tumor tissue in parallel to increased circulating calcitriol when cholecalciferol was added to mouse diet. Since the CYP27B1 level did not change in the kidneys under this condition, it was concluded that the elevated calcitriol level in circulation was due to conversion of diet-administered vitamin D_3 to calcitriol in the tumor tissue [25].

Transcription Regulation by VDR: Domain Structure and Roles for Ligands, DNA Response Elements, and Coregulators

Functional Domains of VDR and Inter-domain Allostery

VDR is a ligand-inducible transcription factor and part of the 48-member human nuclear receptor (NR) family. Similar to other NRs, VDR has a functionally segmented structural organization, having a 24-amino-acid-long unstructured N-terminal domain followed by a DNA-binding domain (DBD) with two zinc finger motifs, an unstructured hinge domain, and a ligand-binding domain (LBD). Of the two zinc fingers within DBD, one associates with the retinoid X receptor (RXR), while the other aids in the formation of VDR homodimer. The unstructured hinge region grants flexibility to VDR's LBD, permitting its association with the RXR LBD. Upon DNA binding, interaction at the interface of VDR and RXR DBDs as well as interaction of the two LBDs stabilize the dimer. This stabilization circumvents the otherwise low DNA-binding affinity of a VDR monomer [26–28]. Figure 4.2 presents the basic aspects of the modular structure of VDR and other NRs. A ligand-binding pocket (LBP) is located in the interior of LBD, and a variable region (known as the F domain) follows LBD for some NR members. A coactivator- or corepressor-binding surface is generated at the agonist-/antagonist-bound LBP as a consequence of conformational realignment of 12 alpha-helices present within LBD. Conformational details for each functional domain and for the ligand-bound, DNA-associated full-length receptor came into light from structural studies of VDR and other NRs utilizing X-ray crystallography and cryo-electron microscopy and from NR's solution structures, which were analyzed by small-angle X-ray scattering (SAXS), small-angle neutrino scattering (SANS), and hydrogen-deuterium exchange [29–32].

Allosteric interactions among various VDR domains are evident from structural and biochemical analyses [30–33]. Hydrogen-deuterium exchange profiling of the VDR/RXR heterodimer detected increased solvent exchange at the DBD

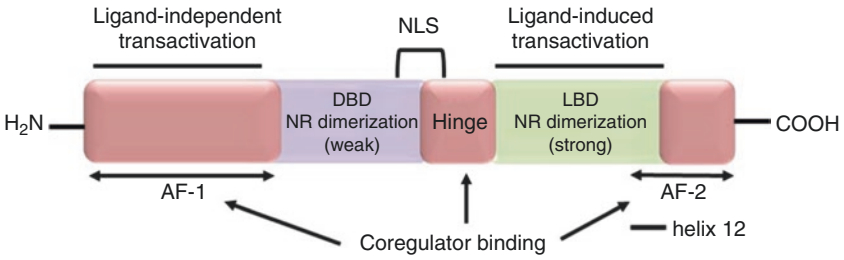

Fig. 4.2 Functional domains of a nuclear receptor (NR): a general scheme. *DBD* DNA-binding domain, *LBD* ligand-binding domain, *AF* activation function, *NLS* nuclear localization signal, NH_2 amino end, *COOH* carboxyl end, *Helix12* the alpha helix-12

upon ligand binding to VDR, indicating that the conformational change of LBD due to ligand occupancy impacted DBD conformation and, thus, the affinity of DBD for the cognate DNA element. This conformational change would potentially alter gene expression. Signal relay from DBD to LBD was evidenced by the result that interaction of the SRC1 coactivator with VDR and the VDR/RXR complex was altered when VDR is bound to a DR3 DNA element. NTD-DBD interplay is revealed from our result that a VDR polymorphic site (*FokI-FF*), which deletes three amino acids at the VDR NTD, abolished *CYP24A1* repression by unliganded VDR [34].

Naturally Occurring VDR Ligands

Calcitriol, Calcidiol, and Secondary Bile Acids

Calcitriol is the primary stimulus for VDR action, although 25(OH)D_3 (calcidiol) at high concentrations can bind and activate VDR and induce VDR-mediated gene transcription in vitro and in vivo [35, 36]. For example, calcidiol at high nanomolar range (400–1000 nM) induced Cyp24a1 mRNAs in primary mouse cells isolated from the prostate and kidneys of *Cyp27b1* −/− mice, and in the case of LNCaP human prostate cancer cells, treatment with calcidiol at 500 nM inhibited cell proliferation with or without the presence of the 1α-hydroxylase inhibitor SDZ, indicating that the inhibition was independent of calcitriol [36]. A synergistic effect of calcidiol and calcitriol in Cyp27b1-null cells was also observed.

Target genes for VDR include enzymes of the drug- and steroid-metabolizing machinery. VDR-mediated transactivation of genes encoding phase I oxidative enzyme, phase II conjugative enzyme, and phase III transporters has been widely reported [3, 37, 38]. Variable serum calcidiol and calcitriol levels arising from season-dependent differential sunlight exposures correlated with seasonal differences in the intestinal expression of *CYP3A4*, which is a VDR-regulated phase I gene [39].

The secondary bile acids LCA (lithocholic acid) and 3-keto LCA bind VDR with micromolar affinity as a result of relatively large ligand-binding pocket (LBP) of VDR. LCA-activated VDR facilitates metabolism of the toxic secondary bile acids by transactivating phase I and phase II genes in hepatic and enteric cells, and clearance of the LCA metabolites by inducing phase III transporter genes. Crystal structures of the VDR's LBD bound to LCA and 3keto-LCA have been solved [40]. For LCA to function as a VDR agonist, two LCA molecules must bind to the LBD [41]. The crystal structure of zebrafish LBD bound to LCA showed binding of one LCA molecule to the ligand-binding pocket (LBP) and the second LCA molecule residing on the LBD surface outside of LBP. The low-affinity second site stabilizes the active receptor conformation. The second binding site outside of LBP is a promising candidate target site for drug design and other computational studies.

Agonist Actions of Calcitriol and Calcidiol on VDR: Analysis by Molecular Docking

The X-ray crystallographic structure for calcitriol has been solved [42]. Molecular docking, which is a computational analysis of receptor interactions with synthetic ligands and chemically modified derivatives of natural ligands, can complement crystallography results. This approach is routinely utilized for rational drug design and development of more potent and less toxic analogs. On the basis of published information on a crystal structure of VDR in complex with calcitriol (*PDB ID: 1DB1*), we modeled interactions of the LBP of VDR with calcitriol (Fig. 4.3A1, A2) and with calcidiol (Fig. 4.3B1, B2) using the program AutoDock Vina™ [43]. The goal for this modeling exercise was to: (a) explore if calcitriol is able to bind to VDR more strongly at a site outside of LBP than a site inside of LBP—the former site potentially would lead to an allosteric influence on VDR activity; and (b) compare calcitriol vs. calcidiol binding to VDR's LBD.

We used AutoDock Vina™ in conjunction with PyRX© (version 0.8, [44]) interface to dock calcitriol and calcidiol to the LBD of VDR. The top-scoring binding pose (orientation) for calcitriol shared a pose that closely resembled the published crystal structure. Out of ten binding poses determined from our analysis, none detected ligand residency outside of LBP. Post-docking analysis using geometry optimization in Discovery Studio Visualizer© [45] revealed two polar hydrogen bonds between the hydroxyl groups (oxygen shown in red) on the carbon-1 and carbon-3 of calcitriol and polar amino acid residues SER-237 and CYS-288, respectively, of VDR (Fig. 4.3A1). An additional hydrogen bond appeared from interactions between carbon-25 of calcitriol and HIS-397 of LBP (Fig. 4.3A1). Upon calcidiol docking to VDR's LBD using similar conditions (as for calcitriol), the top-scoring binding pose of calcidiol with LBP is similar to what was reported in a molecular dynamics study [46]. The hydroxyl group at carbon-3 of calcidiol forms a hydrogen bond with CYS288 (Fig. 4.3B1) similar to the carbon-3 hydroxyl group of calcitriol. The hydroxyl group at carbon-25 of calcidiol forms hydrogen bond with HIS-305, not HIS-397 as seen for calcitriol (Fig. 4.3B1). Molecular surface rendition shows hydrophobicity of different regions of LBP residues (Fig. 4.3A2 and B2). Molecular surfaces show that hydrophobic interactions are formed mainly between the hydrophobic portions of calcitriol and calcidiol and hydrophobic residues of LBP, whereas hydrogen bonding occurs at the less hydrophobic regions of LBP. The scale for hydrophobicity goes from brown (more hydrophobic) to blue (more hydrophilic) (Fig. 4.3A2, B2).

From the above results, we infer that the additional hydrogen bond in the case of calcitriol is likely to account for the drastically increased potency of calcitriol over calcidiol observed from in vitro and in vivo experiments. Nonetheless, the favorable binding of calcidiol within the LBP, as reported earlier [46] and revealed here from our docking analysis (Fig. 4.3), supports the experimental findings that calcidiol can serve as a VDR agonist in vitro and in vivo, albeit at two orders of magnitude higher concentrations than calcitriol [35, 36]. Our modeling study is the further evidence that computational programs are useful tools in describing the agonist action of a molecule of interest. A docking approach can be insightful in the development of synthetic agonists and antagonists of VDR and other nuclear receptors.

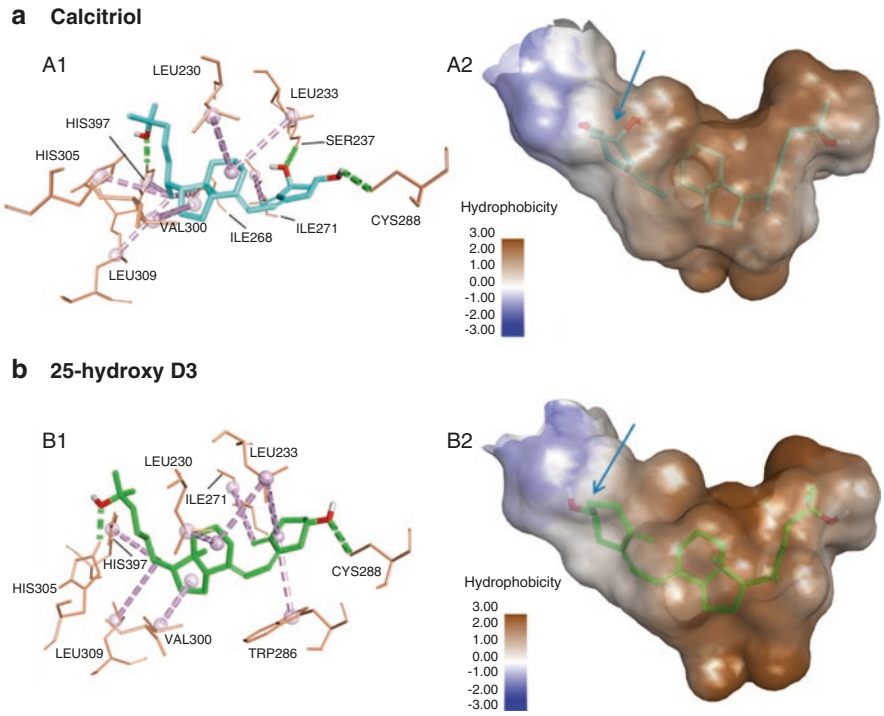

Fig. 4.3 Docking of calcitriol (**a**) and 25-hydroxyvitamin D_3 (**b**) to the ligand-binding domain (LBD) of VDR and post-docking analysis. Docking was done via AutoDock Vina™. A1: *Calcitriol (blue) docked to LBD*. The top-scoring binding pose of calcitriol at LBD occurred within the ligand-binding pocket (LBP). This pose (orientation) was optimized using a Dreiding-like force field in Discovery Studio Visualizer© to maximize strengthening interactions and minimize repulsive interactions. Purple and pink dotted lines indicate hydrophobic interactions (eight total); green dotted lines indicate hydrogen bonds (three total). A2: *Molecular surface rendition of hydrophobicity of calcitriol-bound LBP*. The hydrophobicity scale: brown, most hydrophobic; blue, most hydrophilic. The blue section at left depicts the region (blue arrow) where polar residues CYS288 and SER237 form hydrogen bonds with carbon-3 and carbon-1 hydroxyl groups (red atoms) of calcitriol, respectively. B1: *25(OH)D_3 (calcidiol, shown in green) docked to LBD*. Top-scoring binding pose at LBD occurred within LBP. This pose was optimized as in A1 to maximize strengthening interactions and minimize repulsive interactions. Purple and pink dotted lines signify hydrophobic interactions (eight total); green dotted lines signify hydrogen bonds (two total). B2: *Molecular surface rendition of hydrophobicity of calcidiol-bound LBP*. Hydrophobicity scale: brown, most hydrophobic; blue, hydrophilic. A small blue section at left depicts the region (blue arrow) where the polar CYS288 forms a hydrogen bond with the carbon-3 hydroxyl group (red atom) of calcidiol

Gene Regulation by VDR: Roles for Coregulators and DNA Response Elements

VDR regulates transcription of more than 1000 genes in skeletal and extraskeletal tissues and thus can impact diverse physiologies. In prostate cancer cells, by

comparing transcriptome profiling of calcitriol-treated vs. untreated prostate cancer cells, we found key genes in the androgen biosynthesis and metabolism pathways are regulated by VDR. Calcitriol robustly induced CYP24A1 mRNAs in prostate cancer cells (Fig. 4.6) and in primary prostate cells [36]. Regulatory elements driving CYP24A1 induction by ligand-activated VDR have been characterized [47]. VDR-targeted genes regulating calcium and phosphate homeostasis (such as osteocalcin, osteopontin, D9K, D28K, FGF2, PTH, SLC34A2) are most abundantly expressed in the intestine, kidneys, and bone where absorption and storage of calcium and phosphate is crucial. RANKL, another VDR target, regulates osteoclast survival and differentiation. A general scheme for VDR-mediated target gene induction by VDR and roles of several genetic and epigenetic factors in this process are schematically shown (Fig. 4.4).

Ligand-free nuclear VDR remains attached to the chromatin in a repressed state through association of its LBD with corepressors such as NCoR1 (nuclear corepressor1), SMRT/NCoR2, which in turn recruit a histone deacetylase (HDAC) complex, leading to a VDR-containing compact chromatin region and gene repression similar to what is observed for the thyroid hormone receptor and retinoic acid receptor [48]. Chromatin-bound non-liganded VDR stays associated with RXR, its heterodimer partner (the α isoform RXR-α in majority of cases). This overall picture is consistent with our result, which showed that VDR silencing elevated the basal expression of

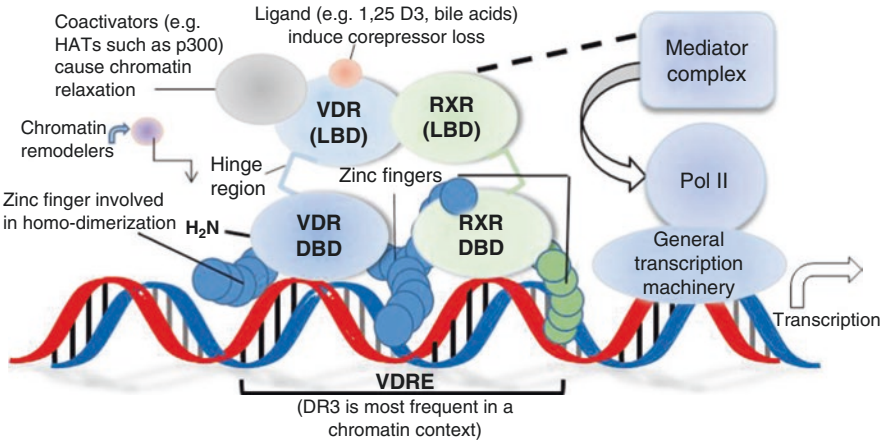

Fig. 4.4 Gene transcription by VDR: roles of upstream regulators. VDR/RXR heterodimer binds a VDRE (frequently DR3 type, especially in vivo). Zinc fingers maintain VDR binding to VDRE and its association with RXR. Agonist activation of VDR leads to release of VDR-associated corepressors including HDACs, which would otherwise maintain chromatin in a condensed form due to histone deacetylation. Corepressor to coactivator exchange at VDRE causes chromatin relaxation (from acetylation), recruitment of chromatin remodelers, as well as a mediator complex—the latter relaying signal (open arrow) to general transcription machinery. Dotted line reflects functional interaction. *VDR* vitamin D receptor, *RXR* retinoid X receptor, *LBD* ligand-binding domain, *DBD* DNA-binding domain, *H_2N* amino terminus, *HATs* histone acetyltransferases, *Pol II* RNA polymerase II, *VDRE* vitamin D response element, *DR3* direct repeat with a 3-nucleotide spacer

CYP24A1 in breast cancer cells [34]. As a nonpermissive heterodimer, the VDR/RXR complex is activated by calcitriol but not by the RXR ligand 9-cis retinoic acid [49]. Upon agonist binding, a conformational change involving the 12 helices within the VDR's LBD leads to repositioning of the helix-12 to create an interaction surface that favors recruitment of the p160 coactivators (SRC-1, SRC-2, SRC-3) and departure of the NCoR/SMRT corepressors. A pioneer factor is involved in the initial relaxation of the chromatin [47, 50]. The p160 coactivator makes physical contact with VDR through an LXXLL (leucine xx leucine leucine) motif and recruits histone acetyltransferases (HATs) such as CBP/p300 and p/CAF. The HATs mediate acetylation of lysine and arginine residues of histones H3 and H4 locally and also at other regions where promoters are present, thus initiating transcription throughout the gene locus [51]. Chromatin relaxation by acetylation leads to the assembly of additional coregulatory complexes including histone modifiers (methyltransferase/demethylase, ubiquitin ligase/deubiquitinase, kinase/phosphatase) and chromatin remodelers (such as SWI/SNF-containing WINAC complex) [48]. A 26-subunit mediator complex integrates the final transcriptional regulation by serving as the conduit for relaying the regulatory signal from DNA-associated VDR to the basal transcription machinery and RNA polymerase II at the transcription start site [52] (Fig. 4.4).

In the chromatin, the preferred genome-wide vitamin D-responsive binding element (VDRE) for the VDR/RXR heterodimer is a direct repeat of the consensus half-site sequence AG(G/T)TCA with three intervening nucleotides (DR3) [53]. Genes with clusters of DR3 type VDREs preferably respond to the transcriptional regulation by ligand-activated VDR. VDR contacts the 6-base 5′ half-site within the major groove of DNA via the first zinc finger module of DBD. The second zinc finger of VDR associates with the RXR DBD. A DR3 element mediates robust VDR-mediated transactivation of *CYP24A1* by 1,25-D_3 [29]. Other DR configurations (most frequently DR4, DR5) are also involved in vitamin D-mediated induction of target genes. We reported the involvement of a DR7-type VDRE in the VDR-mediated induction of *SULT2B1* sulfotransferase in transfected prostate cancer cells. SULT2B1b (in short SULT2B) is a cholesterol and DHEA sulfotransferase that is present at relative abundance in the normal human prostate. SULT2B levels are reduced in primary prostate cancer [54], and its expression is non-detectable in >90% cases of distant metastasis (B. Chatterjee, unpublished).

Gene repression by VDR can occur through a direct mechanism, known as transrepression [48]. Reduced *CYP27B1* gene transcription by calcitriol in the kidneys entails association of the ligand-bound VDR with several negative vitamin D response elements (nVDRE) [55]. The nVDREs in the *CYP27B1* upstream promoter are of two types—one having sequence organization resembling a positive VDRE with specific affinity for the VDR/RXR dimer in a ligand-dependent manner and the other with binding specificity for a specific transcription factor such as the VDR interacting repressor (VDIR), which is inhibited due to interference from the tethered VDR or by VDR bound to a nVDRE that bears no similarity to a classical VDRE. A DR4-type nVDRE mediates transrepression of the *CCNC* gene, which codes for cyclin C [29].

Interplay of Androgen and Vitamin D in Prostate: Impacts on Cell Proliferation, Vitamin D Metabolism, and the AR Pathway

Effects on Cell Cycle Regulation and E2F1, a Key Cell Cycle Regulator

Calcitriol-mediated inhibition of normal and malignant prostate cell proliferation has been widely documented in various experimental models such as primary cells isolated from normal and malignant prostate tissue, immortalized normal epithelial cell lines, tumor-derived prostate cancer cell lines, and xenograft tumors of prostate cancer cells. The inhibition involves diverse mechanisms including the G1/S arrest of cell cycle, apoptosis, pro-differentiation changes, microRNA regulation, and diminished angiogenesis (reviewed in [4] and references therein). E2F1 transcription factor activity drives expression of DNA synthesis genes, and loss of E2F1 function, upon its sequestration by accumulated hypo-phosphorylated retinoblastoma protein (Rb), leads to cell cycle block at the G1 → S checkpoint. Hypo-phosphorylated Rb accumulates in calcitriol-treated cells due to CDK2 inactivation by elevated p21/Waf1 (a CDK2 inhibitor). Also, calcitriol triggered CDK2 exit from the nucleus, thereby preventing CDK2-cyclin interaction and, hence, CDK2 activation. Calcitriol also caused nuclear accumulation of the CDK2 inhibitor p27/Kip1 [56]. CDK2 (cyclin-dependent kinase 2) is a serine/threonine kinase with a key role in cell cycle progression, especially during the G1 to S phase transition. Partner proteins such as cyclin E or cyclin A and the cell cycle inhibitor p21/Cip1 (CDKN1A) or p27/Kip1 (CDKN1B) regulate CDK2 activity.

The mitogenic impact of androgen on prostate cancer cells can be blocked by calcitriol, since increase in the proliferation of androgen-stimulated LNCaP prostate cancer cells was prevented by concurrent treatment of the cells with calcitriol. E2F1 induction by androgen was also blocked by calcitriol (Fig. 4.5a), and a parallel reduction of the androgen-stimulated E2F1 promoter activity in the presence of calcitriol could be demonstrated in transfected LNCaP cells in promoter-reporter assay. Chromatin immunoprecipitation (ChIP) demonstrated engagement of the androgen receptor (AR), the coactivator p300 (a histone acetyltransferase) and the transcriptionally competent RNA polymerase II (pol II, the serine5-phosphorylated form) at an androgen-responsive E2F1 upstream promoter region in androgen-treated LNCaP cells (Fig. 4.5b). VDR occupied the same region in the E2F1 promoter independent of calcitriol treatment, which is expected since nuclear VDR remains chromatin-bound in the ligand-free state as well. AR occupancy persisted at the androgen-responsive region during E2F1 de-induction by calcitriol, indicating that AR and VDR are part of the same corepressor complex (Fig. 4.5b). Characterization of individual components of the corepressor complex may identify druggable upstream regulators involved in *E2F1* gene repression by calcitriol in androgen-stimulated cells.

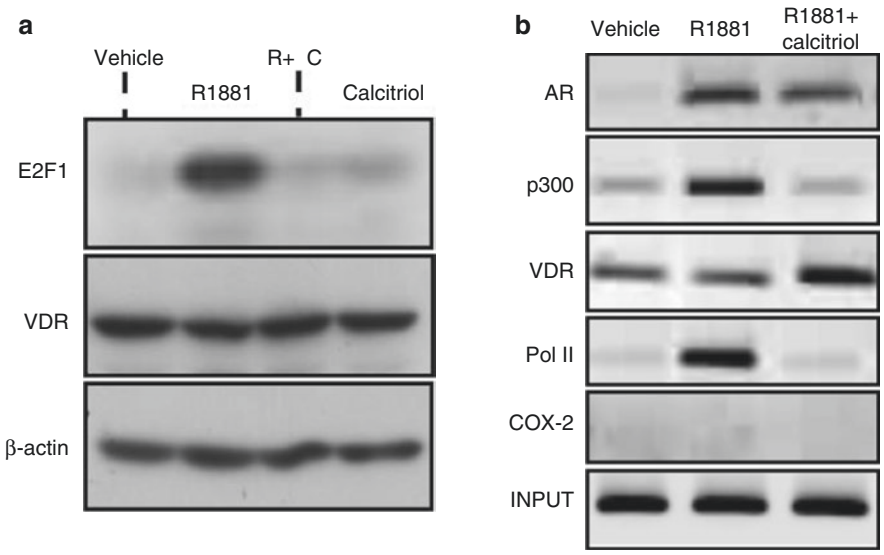

Fig. 4.5 Loss of androgen-mediated induction of E2F1 in prostate cancer cells in the presence of calcitriol. (**a**) Western blot assay of E2F1 levels in LNCaP cell lysates after various treatments. R1881: synthetic androgen, non-metabolizable; C: calcitriol. (**b**) Chromatin immunoprecipitation (ChIP) from LNCaP cells for analysis of the recruitment of AR, the coactivator p300 (a histone acetyltransferase), VDR, RNA polymerase II (Pol II) to the androgen-responsive region in the E2F1 promoter after cells were treated with vehicle, R1881 and R1881 plus calcitriol. The androgen-responsive sequence in the E2F1 promoter is located at around −400 upstream region. ChIP with anti-COX2 antibody was a negative control (COX-2, cyclooxygenase-2). An equal protein amount of the supernatant of sonicated cells from each treatment was used for ChIP of individual proteins, shown by similar input signals

Effects on Vitamin D Metabolism and on the Intratumoral AR Axis

In certain contexts, androgen facilitates the antiproliferative activity of calcitriol. For example, calcitriol reduced the prostate size of testis intact but not castrated Sprague-Dawley rats [57], and androgen-dependent, AR-positive LNCaP prostate cancer cells were inhibited by calcitriol more robustly than androgen-independent, highly aggressive PC-3 and moderately aggressive DU-145 prostate cancer cells [58]. PC-3 and DU-145 cells are mostly considered AR-negative, although very weak AR expression in these cells were noted in some reports. Androgen nearly completely blocked marked upregulation of CYP24A1 mRNAs by calcitriol in AR-expressing C4-2B castration-resistant prostate cancer cells, while androgen alone had no detectable effect on CYP24A1 (Fig. 4.6a). A similar result on the androgen-regulated inhibition of CYP24A1 induction by calcitriol in LNCaP cells was previously reported [59]. Since CYP24A1 converts

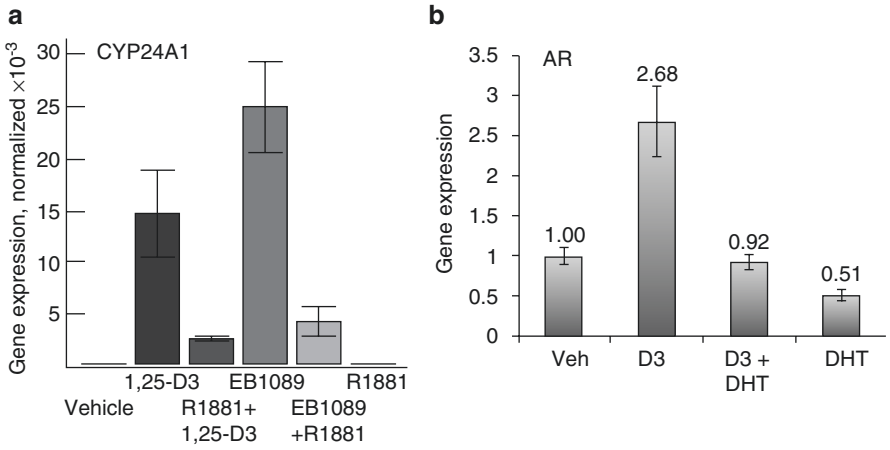

Fig. 4.6 Androgen-regulated loss of induction of CYP24A1 and androgen receptor (AR) mRNA in calcitriol-treated cells. Castration-resistant C4-2B prostate cancer cells were analyzed for the mRNAs using quantitative RT-PCR. The mRNA levels (indicated as gene expression) were normalized to β-actin mRNAs. *EB1089* a calcitriol analog, *D3* abbreviation for $1,25(OH)_2D_3$, *DHT* 5α-dihydrotestosterone

calcitriol to an inactive metabolite, its induction is normally beneficial for ensuring vitamin D homeostasis. By contrast, in the context of the vitamin D's role as an antiprostate cancer hormone, CYP24A1 induction assumes an adversarial role due to degradation of bioactive hormonal vitamin D. Indeed, pharmacologic inhibition of CYP24A1 by RC2204, a specific CYP24A1 inhibitor, or by ketoconazole, a general inhibitor of the cytochrome P450 family of enzymes, augmented calcitriol's anticancer effects on PC-3 prostate cancer cells, evidenced by heightened suppression of the clonogenic survival of the cells in culture, greater reduction of xenograft tumor burden, and more robust activation of caspase-independent apoptosis [60]. In the same study, pharmacokinetics showed that serum calcitriol levels were higher when normal mice (nontumor bearing) received calcitriol plus a CYP24A1 inhibitor compared to mice receiving calcitriol alone.

Androgen prevented calcitriol-mediated induction of AR (Fig. 4.6b). Thus inhibitory interplay between androgen and vitamin D signaling can interfere with an AR-driven growth pathway. We also observed that androgen prevented calcitriol-mediated induction of 3β-hydroxysteroid dehydrogenase-1 (HSD-3β1), which is a major driver of testosterone and DHT biosynthesis. HSD-3β1 catalyzes conversion of dehydroepiandrosterone (DHEA) to androstenedione. Association of advanced prostate cancer with HSD-3β1 mutations, which enhanced stability and activity of this enzyme, has been reported [61]. Results in Fig. 4.6 suggest that a vitamin D/androgen combination therapy may be beneficial due to higher calcitriol availability and reduced androgen-AR signaling.

Prostate Tumor Suppression by Calcitriol and Analogs: Outcomes of Preclinical Studies and Clinical Trials

Preclinical Studies

Calcitriol is consistently effective in reducing tumor burden and metastases for castration-sensitive and castration-resistant prostate cancer in xenograft and allograft tumors and in prostate tumors developed in genetically altered mice. Calcitriol can also act synergistically to enhance the efficacy of chemotherapy drugs against prostate cancer [1, 62]. Dietary supplementation of vitamin D_3 and intraperitoneally injected calcitriol reduced tumor growth of PC-3 prostate cancer cell xenografts with similar efficacy. These treatments caused intratumoral induction of mRNAs for CYP24A1, 15-PGDH (15-prostaglandin dehydrogenase), IGFBP-3 (insulin-like growth factor-binding protein-3), p21/Cip1 (inhibitor of cyclin-dependent kinases), and repression of cycloxygenase-2 and EP4 prostaglandin receptor mRNAs [25]. Consistent with these changes, a combination of calcitriol and a nonsteroidal anti-inflammatory drug (NSAID) caused synergistic inhibition of prostate cancer xenografts [25].

A chemopreventive role of calcitriol was revealed from its ability to prevent progression of PIN (prostate intraepithelial neoplasia) lesions in *Nkx3.1*; *Pten* mutant mice to dysplastic and neoplastic lesions [63]. Early intervention of TRAMP mice with calcitriol raised levels of E-cadherin (a pro-differentiation marker) in the tumor tissue and reduced early growth of primary prostate tumor under androgen stimulation [64]. TRAMP mice develop prostate cancer as a consequence of SV40 (simian virus 40) T antigen expression in the prostate. Continued calcitriol administration, however, enhanced distant organ metastases in these mice, indicating that long-term calcitriol therapy is potentially harmful. This result is somewhat reminiscent of the clinical finding that high serum levels of 25(OH) D_3 elevate risks for advanced prostate cancer [8].

A number of calcitriol analogs have been synthesized by introducing chemical modification either at the A ring, at the central CD ring, or at the side chain of the natural hormone in order to develop more potent and less calcemic vitamin D analogs [65, 66]. Many synthetic calcitriol analogs showed higher anticancer potency than the natural hormone [67, 68]. For instance, inecalcitol (19-nor-14-epi-23-yne-1,25$(OH)_2D_3$), a highly potent calcitriol analog, is several hundredfolds less hypercalcemic than calcitriol. In one study, the maximum tolerated dose of inecalcitol injected intraperitoneally three times per week was found to be 30 μg per injection per mouse, whereas the tolerated dose for calcitriol under the same condition was 0.0625 μg per injection per mouse [67]. Distinct conformations of the inecalcitol-bound versus calcitriol-bound VDR/RXR complex account for differential affinities of these complexes for VDR response elements, which results in an agonist-selective unique transcriptome profile. The markedly reduced hypercalcemia in inecalcitol-administered mice is thought to be the result of a unique profile of gene expression driven by inecalcitol-bound VDR [69].

Clinical Trial Outcomes

Several large-scale trials led to the conclusion that as monotherapy, calcitriol or its synthetic analogs did not provide any clinical benefit against prostate cancer. The hypercalcemic effect of hormonal vitamin D at clinical doses elevated risks for cardiovascular disease and lethal prostate cancer [1, 67, 70]. In randomized controlled trials, patients with metastatic prostate cancer receiving a combination of α-calcidol and docetaxel had no benefit for overall survival or PSA response [71]. An ongoing randomized Vitamin D and Omega-3 Trial (VITAL) on ~26,000 US participants with no prior history of major illnesses is assessing whether daily supplements of cholecalciferol (vitamin D_3) at the physiological dose of 2000 international unit with or without daily omega-3 fatty acid (fish oil, 1 g) supplements will reduce risks for various cancers including prostate cancer and lower risks for stroke and cardiovascular diseases [72]. Inecalcitol combined with docetaxel is being investigated for safety and pharmacodynamics in a phase I study on metastatic castration-resistant prostate cancer patients [62].

A more promising clinical benefit may arise from the chemoprevention activity of vitamin D, especially for low-risk prostate cancer. A pilot study evaluated the effect of enhancing serum vitamin D status by vitamin D_3 supplements (4000 IU per day) on prostate cancer progression for 44 patients who are on active surveillance due to diagnosis of low-risk prostate cancer. When these patients took a daily physiological dose of vitamin D_3 supplement for 1 year, positive cores decreased in number at repeat biopsy, and Gleason scores dropped for more than 50% of the subjects. Serum PSA levels were unchanged [73]. The yearlong vitamin D_3 intake had no adverse health impact. In a second pilot study with 37 patients awaiting elective prostatectomy, inflammatory lesions decreased dramatically in resected prostate cancer specimens from men who took a daily physiological dose (4000 IU) of vitamin D_3 for 2 months prior to prostatectomy [74]. For 60% of the patients in the vitamin D_3 group, tumors in resected tissues either shrank or disappeared, and inflammation was drastically reduced, revealed from lower levels of inflammation-related lipids and proteins. The growth factor differentiation factor-15 (GDF-15, also known as macrophage-inhibiting anti-inflammatory cytokine-1, MIC1), which is an anti-inflammatory cytokine, was elevated in the vitamin D_3 arm, whereas specimens from the placebo arm had biochemical evidence for severe inflammation and showed marked reduction of GDF-15. These results are consistent with an anti-inflammatory activity of the hormonal vitamin D_3 [2] and add credence to the concept that inflammation is closely linked to the etiology and progression of various cancers including prostate cancer [75]. Preclinical studies showed that 1,25 D_3 reduced the prostaglandin level in prostate tumors by causing diminished expression of COX-2 and the prostaglandin receptor and elevated expression of 15-prostaglandin dehydrogenase. Therefore, as proposed earlier [76], impacts of combined use of vitamin D_3 supplements with a nonsteroidal anti-inflammatory drug (NSAID) on prostate cancer should be thoroughly assessed.

Insensitivity to VDR/vitamin D signaling can contribute to a lack of response to vitamin D-based therapy. Altered VDR expression and transcriptional activity due to single nucleotide polymorphism of the *VDR* gene can occur. For VDR *FokI* polymorphism, the longer, less active VDR variant arises from the *f* allele, and a more active shorter VDR is produced by the *F* allele due to mRNA translation from a downstream ATG initiation codon. An *f* allele *VDR* combined with a low serum vitamin D status associated with ~twofold greater prostate cancer risk compared to an *FF* or *Ff* genotype and high serum 25(OH)D_3 [77]. The VDR *TaqI* polymorphism giving rise to synonymous mutation at exon-9 reduces mRNA stability for VDR, thereby lowering VDR abundance and its transcriptional activity. Vitamin D homeostasis is expected to be impacted by reduced VDR activity since genes involved in vitamin D biosynthesis and metabolism (e.g., *CYP2R1*, *CYP27B1*, *CYP24A1*) are transcriptionally regulated by VDR. In meta-analysis, VDR *TaqI* polymorphism significantly associated with prostate cancer risk in Asian and African-American men [78].

Epigenetic mechanisms may also contribute to vitamin D insensitivity. Critical genes influencing vitamin D signaling and vitamin D homeostasis, such as *VDR*, *CYP2R1*, *CYP27B1*, and *CYP24A1*, are potential targets for epigenetic silencing from DNA hypermethylation since long CpG islands localize in the promoter regions of these genes. Furthermore, certain histone demethylases such as the lysine-specific demethylases and Jumonji C domain-containing histone demethylases are direct targets of VDR-mediated transcription regulation [79]. Trichostatin A (TSA), which is a pan-HDAC (histone deacetylase) inhibitor, restored antiproliferative activity of calcitriol in PC-3 cell cells [80]. In another example, calcitriol did not inhibit proliferation of prostate cancer cells that overexpressed the corepressors NCoR1 and NCoR2/SMRT, whereas corepressor silencing rescued castration-resistant prostate cancer cells from the loss of response to calcitriol [80, 81]. Elevated expression of NCoR1 and SMRT was detected in prostate cancer cell lines and in the primary culture of malignant cells isolated from prostate tumor xenografts [81, 82]. NCoR1 and SMRT physically interact with VDR, and they are part of the repressive complex that associates with ligand-free, transcriptionally inactive VDR. They are also components of the corepressor complex that regulates VDR-mediated transrepression [83].

Breast Cancer Suppression by Calcitriol

An inverse association of serum vitamin D levels with breast cancer risks in women is indicated from epidemiology and meta-analyses, although confounders such as disease heterogeneity and variable estrogen status of the study cohort have precluded any definitive conclusions in this regard. Recent results from a prospective cohort study of 1666 breast cancer survivors showed that patients with advanced breast cancer had lower serum levels of 25-hydroxyvitamin D_3, with lowest levels

in premenopausal women who present with triple negative breast cancer [10]. The triple negative phenotype signifies a lack of the estrogen receptor (ER), progesterone receptor (PR), and growth factor receptor HER2/ErbB2, thus making triple negative breast cancer nonresponsive to available targeted therapies. Furthermore, serum vitamin D levels inversely correlated with hazards of breast cancer progression and death, and women at the highest tertile of serum vitamin D showed improved overall survival compared to women at the lowest tertile [10]. However, interpretation of these results did not take into account confounders such as smoking and alcohol consumption, which may reduce serum vitamin D status while increasing risks for cancer in general [84]. Clinical trials for assessing benefits of vitamin D supplementation for breast cancer chemoprevention are in progress [85].

In experimental models, calcitriol inhibited breast cancer, revealed from studies in cell culture, in mammospheres, and in animal models of mammary tumors [1, 86–88]. Calcitriol downregulated the expression of ER-α as well as aromatase (CYP19A1), the latter regulating estrogen biosynthesis by catalyzing aromatization of testosterone. The calcitriol effect on aromatase expression was tissue selective, since in MCF7 breast cancer xenografts, calcitriol reduced aromatase expression in both the tumor tissue and in the surrounding mammary adipose tissue while elevating aromatase in bone marrow cells and having no effect on aromatase levels in the ovary and uterus [87]. Blocking of the estrogen signal by an aromatase inhibitor (such as letrozole) is a standard-of-care for managing ER-positive mammary tumor—a phenotype carried by ~70% of breast cancer patients.

The vitamin D/VDR signaling axis suppressed breast cancer in a tumor autonomous manner. A recently reported study demonstrated that xenografts from VDR-ablated breast cancer cells grew more rapidly and showed enhanced metastasis to distant organs, whereas ectopic expression of VDR in VDR-silenced breast cancer cells reduced xenograft growth and metastasis [89]. Enhanced cancer cell metastasis in VDR-knocked down xenografts was in part due to derepression of the ID1 (inhibitor of DNA binding 1), which is known to aid in tumor progression. For breast cancer patients, serum 25-hydroxy vitamin D_3 levels at presurgery inversely correlated with the abundance of ID1 in the tumor tissues of resected specimens. A negative VDRE in the *ID1* gene mediates the repressive effect of ligand-activated VDR [89]. Other examples of VDR-suppressed genes that are involved in breast cancer invasion and metastasis include those encoding uPA (urokinase-type plasminogen activator), periostin (a secreted heparin-binding protein), HbEGF (a heparin-binding EGF-like growth factor), and hyaluronan synthase [90]. VDR-mediated repression of these genes was revealed from a comparative analysis of the gene expression profiles of breast tumor cells from carcinogen-exposed wild-type vs. Vdr-null mice [86]. Periostin promotes epithelial to mesenchymal transition (EMT), while uPA is functionally linked to stromal invasion and migration of cancer cells owing to its role in the cleavage of plasminogen that leads to the activation of metalloproteases. Periostin and uPA expressions are elevated in breast cancer. Binding of the cell surface receptor CD44 to hyaluronic acid (HA), the extracellular proteoglycan which is synthesized by the catalytic action of hyaluronan synthase, triggers a sequence of events that lead to the activation of intracellular signaling induced by specific

ligand-activated growth factor receptors, thereby promoting cancer cell survival [86]. CD44-mediated cell survival signaling in triple negative breast cancer cells was suppressed by calcitriol [91]. Furthermore, repression of the breast cancer stem cell-like population by calcitriol was also demonstrated [88]. Insights into selected downstream effectors of the VDR pathway may be leveraged in pursuit of novel interventions against breast cancer.

Conclusion

Most extraskeletal human tissues express vitamin D receptor (VDR) and thus are subject to transcription regulations by vitamin D/VDR signaling, which targets more than 1000 genes [92]. Insufficient serum 25-hydroxyvitamin D_3 and an errant VDR pathway elevate risks for a number of pathologies, most notably those arising from malignant cell growth, insulin resistance, atherosclerosis, and illnesses from immune system dysfunctions. Biosynthesis of calcitriol ($1\alpha,25(OH)_2\ D_3$), the natural ligand for VDR, occurs primarily in the kidneys. Local synthesis of calcitriol from circulating $25(OH)D_3$ also occurs in prostate, breast, and other VDR-responsive extraskeletal tissues. CYP27B1, the 1α-hydroxylase that converts 25(OH) D_3 (calcidiol) to calcitriol, is expressed in both normal and cancerous cells of endocrine tissues such as the prostate and breast.

In a randomized dosing study, orally administered vitamin D_3 (cholecalciferol) caused a dose-dependent increase of calcitriol in the surgical specimens of prostate cancer patients who were treated for 2 months prior to prostatectomy. Serum PSA and parathyroid hormone levels also decreased, albeit modestly [22]. Reduced expression of the proliferation marker Ki-67 in the resected samples correlated with intra-prostate elevation of calcitriol. This is consistent with growth inhibition of prostate cancer cells by calcitriol—a finding that has been validated extensively in cell culture and animal experiments. Calcitriol analogs with markedly lower calcemic toxicity and robust agonist action on VDR at sub-nanomolar concentrations have demonstrated antiprostate cancer activity. Inecalcitol, a potent VDR agonist, was tolerated in mice at a ~400-fold higher dose than calcitriol [67]. However, neither calcitriol nor analog monotherapies showed a clear benefit against clinical prostate cancer. A treatment protocol that combines inecalcitol with docetaxel (a standard chemotherapy drug) is currently under evaluation for the treatment of metastatic castration-resistant prostate cancer. The combination protocol was designed following the preclinical finding that VDR agonists can sensitize prostate cancer to chemotherapeutic interventions. In a small-scale study, vitamin D_3 supplementation at a physiological dose prevented disease progression for low-grade, organ-localized clinical prostate cancer. A final verdict on the chemoprevention activity of vitamin D_3 awaits outcomes of large-scale clinical trials.

We observed that androgen prevented vitamin D-mediated induction of androgen receptor and CYP24A1 in castration-sensitive and castration-resistant prostate cancer cells. This data raises the intriguing possibility that androgen in conjunction

with calcitriol (or analog) may stem prostate cancer progression in a therapeutic setting. In this regard, we note that a high testosterone dose promoted ablation of castration-resistant prostate cancer cells in preclinical studies [93]. Further, in a pilot clinical study, bipolar androgen therapy, which entails rapid cycling from supraphysiologic to near-castrate serum testosterone levels over a 4-week cycle, lowered serum PSA and reduced radiographic progression in asymptomatic patients with castration-resistant prostate cancer and limited metastatic burden [94]. Whether daily vitamin D_3 supplementation can further enhance antiprostate cancer response to bipolar androgen therapy merits clinical evaluation.

Novel therapeutics against prostate and breast cancer may be identified by leveraging insights from upstream regulators and downstream effectors of the vitamin D/VDR pathway. Distinct mechanisms such as G1/S cell cycle block, apoptosis, cellular differentiation and inhibition of angiogenesis account for cell growth arrest by vitamin D action. The anti-inflammatory response elicited by vitamin D signaling may also interfere with malignant cell growth, given the close link of inflammation to cancer initiation and progression. Indeed, when patients with low-grade prostate cancer received daily vitamin D_3 supplementation for 2 months prior to radical prostatectomy, induction of the anti-inflammatory cytokine MIC-1 (known also as GDF-15) and reduction of inflammation in resected specimens were observed [74].

Insights into the epigenetics that play a role in the regulation of genes encoding VDR, CYP2R1, CYP27B1, CYP24A1, as well as the epigenetic mechanisms that direct VDR-mediated transcription regulation of genes encoding certain histone demethylases (such as lysine-specific demethylases and Jumonji C domain-containing demethylases) may uncover a new path to the inhibition of prostate and breast cancer. VDR-mediated microRNA regulation may also be leveraged in search of a new intervention strategy. As an example, microRNA-98, which inhibited clonogenic growth of LNCaP prostate cancer cells, was induced in calcitriol-injected mice leading to its increased level in the blood. The potential of microRNA-98 as a candidate therapeutic as well as a biomarker for vitamin D action in vivo is being explored [95]. Above examples highlight the possibility that upstream or downstream effectors of the vitamin D/VDR pathway may provide opportunities for novel targeting of breast and prostate cancer.

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Chapter 5
Vitamin D and Colorectal Cancer

Albert Do and Petr Protiva

Introduction

Vitamin D metabolites and analogs are known to play an important role in the development and prevention of bone mineral diseases and bony fractures. However, vitamin D is involved in a variety of cellular processes including innate immunity, cell proliferation, differentiation, and apoptosis. There have been a variety of studies investigating the role of vitamin D in the risk of developing cancer, particularly colorectal cancer. In this chapter, we will briefly review vitamin D biochemistry and will summarize and discuss the current knowledge of colorectal cancer risk associated with vitamin D deficiency and supplementation.

Biology Review

Vitamin D is a fat-soluble vitamin, obtained in humans from dermal ultraviolet B (UVB) light exposure and dietary sources (food and/or supplementation) in two forms: D3 (cholecalciferol) and D2 (ergocalciferol). Both forms are subsequently converted to its active metabolites (primary circulating form 25-hydroxyvitamin D [25[OH]-D] by CYP2R1 produced mainly in the liver and the primary active form 1,25-hydroxyvitamin D [1,25[OH]-D] by CYP27B1 formed in the kidney).

A. Do, MD, MPH
Department of Medicine, Yale University School of Medicine, New Haven, CT, USA
e-mail: albert.do@yale.edu

P. Protiva, MD, MPH (✉)
Department of Medicine, Yale University School of Medicine, New Haven, CT, USA

Department of Medicine, VA Connecticut's Healthcare System, West Haven, CT, USA
e-mail: petr.protiva@yale.edu

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Renal production of 1,25[OH]-D is tightly regulated by serum parathyroid hormone, calcium, phosphorus, and fibroblast growth factor 23 (FGF23) levels [1]. Although 1,25[OH]-D is the most potent and biologically active form of vitamin D, serum 25[OH]-D levels are generally used as a marker of an individual's vitamin D status owing to abundance in circulation and longer half-life (2–3 weeks rather than 4–6 h of 1,25[OH]-D). Vitamin D metabolites act on the vitamin D receptor (VDR) to exert its biological effects. 1,25-Hydroxyvitamin D [1,25[OH]-D] is a potent inducer of CYP24A, an enzyme that hydroxylates 1,25[OH]-D into its inactive form 1,24,25[OH]3-D. With the discovery that most cells in the human body contain the vitamin D receptor, and that extra-skeletal cells throughout the human body respond to vitamin D metabolites in a variety of ways, research has been ongoing regarding the effect of vitamin D on health, infections and other immunological effects, cancer risk, as well as bone health [2, 3].

Original Findings Driving Further Investigations

In 1937, Peller and Stephenson proposed that sunlight exposure could result in decreased rates of various cancers [4]. Subsequently in 1941, it was observed that cancer risk was higher in individuals who resided at higher altitudes [5]. Decades later, the observation that vitamin D levels associated with sunlight exposure may play a protective role from colorectal cancer resulted in a new line of inquiry in assessing the effect of vitamin D status on colorectal cancer risk [6]. Further studies suggested that UVB radiation exposure and associated circulating vitamin D levels may prevent the development of colorectal cancer, as well as numerous other cancers [7–9].

Since then, in vitro animal and human studies have investigated sunlight, vitamin D, and colorectal cancer risk. Vitamin D and its metabolites have been found to play a role in inhibition of colorectal cancer progression in animal models, in both initial development and progression of colorectal cancer [10].

Molecular Mechanisms

The strong evidence of protective effect of vitamin D against colorectal cancer from epidemiological studies led to numerous studies attempting to address the underlying mechanism of this protective effect. The VDR is abundantly expressed in the intestinal cells, and it is involved in regulation of calcium absorption. All key enzymes involved in activation of circulating cholecalciferol or ergocalciferol and deactivation of 1,25 (OH)2-D also show low baseline expression level in the colon and can be transcriptionally regulated in autocrine or paracrine manner [11]. VDR expression is diminished in poorly differentiated colorectal carcinomas and virtually lost in metastatic lesions when compared to normal

mucosa, adenomas, or well-differentiated colorectal adenocarcinomas [12]. Furthermore, levels of CYP27B1 are decreased, while levels of CYP24A are elevated in poorly differentiated colorectal neoplasms [12]. The colorectal tumors were also shown to downregulate the CYP27B1 levels in apparently normal adjacent mucosa, further bolstering the role of paracrine regulation of vitamin D system in tumor microenvironment [13].

The majority of work on antitumor effects of 1,25 (OH)2-D was conducted in vitro and, to a lesser extent, in animal models. In vitro animal studies using animal models with deficient and absent vitamin D receptors (VDR) demonstrated higher risk of developing breast and intestinal cancer [14, 15]. This phenomenon is thought to occur through a variety of mechanisms, including modulation of the beta-catenin-TCF pathway, and through expression control of over 200 genes, including some responsible for regulation of cellular proliferation, differentiation, apoptosis, and angiogenesis [10, 16]. Such effects have been observed in brain, prostate, breast, and colonic tissues [17].

The in vitro studies have shown that vitamin D activates VDR nuclear translocation, and it acts as a potent transcriptional activation or repression of the target genes resulting in a wide range of molecular and cellular effects. Calcitriol induced **differentiation** in colon cancer cells, as evidenced by increased expression of alkaline phosphatase and CDH1 (E cadherin) and inhibition of WNT/beta-catenin pathway [18–21]. In addition, calcitriol exhibited **antiproliferative** and **pro-apoptotic effects** in colon cancer cells by regulating activity of cyclin-dependent kinase inhibitors and altering the levels of pro- and anti-apoptotic proteins [19, 20, 22, 23]. Furthermore, calcitriol was shown to be also involved in regulation of **antitumor micro-RNA**, **antiangiogenesis**, and **immunity**. There is a complex interplay between innate and adaptive immunity and calcitriol, and evidence suggests a strong immune-modulatory effect of 1,25OHD3 on both immune systems [24]. The VDR is also expressed in activated T cells and in antigen-presenting cells, and 25-alpha-hydroxylase, 1-alpha-hydroxylase, and 24, 25-hydroxylase enzymes involved in vitamin D metabolism are expressed by cells of the immune system [25]. Active vitamin D also exerts pronounced effect on dendritic cells [26]. The immune system of VDR-null or vitamin D-deficient mice shows increased sensitivity to autoimmune diseases such as inflammatory bowel disease or type 1 diabetes [27]. VDR-deficient mice do not have a spontaneous increase in cancer but are more prone to oncogene- or chemocarcinogen-induced tumors [27]. The effect on immunity in human studies is complex. For example, the low levels of vitamin D are associated with a poorer clinical outcome in some autoimmune disorders such as Crohn's disease [28]. However, randomized double-blind placebo-controlled study showed that high-dose cholecalciferol 50,000 IU weekly for 12 weeks followed by 50,000 IU every other week for 40 weeks did not significantly alter circulating inflammatory markers at the end of the study [29–33], a finding that was confirmed by three other studies [29–33]. Thus, the immune-modulating effects of the vitamin may only be clinically apparent in states of abnormal immune activation. The complex relationship between the calcitriol and immune system is reviewed in Chap. 2 [34].

The dietary animal models of colorectal cancer use a long-term Western-style diet that induces both benign and malignant intestinal lesions in both wild-type or genetically modified animals predisposed to intestinal tumorigenesis [35]. Supplementing the Western-style diet with calcium and vitamin D reduces the intestinal tumorigenesis in these models [35]. Calcitriol or its analogs were also shown to inhibit intestinal neoplasms in both genetic and carcinogen-induced models of intestinal neoplasms, and it also reduced the growth of cancer xenografts in murine models [36].

Effect in Human Colorectum

There are only few human intervention studies examining the effects of vitamin D and calcium supplementation directly in colorectal mucosa. One randomized, double-blind, controlled study of vitamin D and calcium supplementation utilizing a 2×2 design by Bostick [37] showed that vitamin D favorably altered several markers of colorectal cancer risk such as pro-apoptotic marker BAX, antiproliferative marker p21, and also CDH1, MSH2, and CYP27B1 [38–42]. More recently, two randomized, double-blind controlled crossover studies of vitamin D and calcium supplementation were reported by Protiva et al. [43]. These studies examined the molecular pathways altered by a Western-style diet supplemented with and without 2 g/day of calcium carbonate and a Western-style diet supplemented with calcitriol (0.5 μg/day) with or without 2 g/day of calcium carbonate. The Western-style diet induced modest upregulation of genes involved in inflammatory pathways, including interferon signaling, and calcium supplementation reversed these toward baseline. The supplementation of the Western-style diet with calcitriol induced striking upregulation of genes involved in inflammation, immune response, extracellular matrix, and cell adhesion. Calcium supplementation largely abrogated these changes suggesting a biological interaction with vitamin D at the level of colorectal mucosa. Figures 5.1 and 5.2 demonstrate the interplay between Western-style diet, calcitriol, calcium, and genome-wide gene expression pathways in the human colorectum. The few human translational studies are congruent with the preclinical evidence.

Evidence in Support

Most data supporting vitamin D's role in colorectal cancer prevention are derived from moderate- to long-term prospective observational and epidemiological studies which demonstrated an inverse relationship between vitamin D levels and incident colorectal cancer risk [44]. A nested case-control study of the Nurses' Health Study found a significant inverse linear association between serum 25[OH]-D levels and colorectal cancer risk (odds ratio [OR] = 0.53 in highest versus lowest quintile) [45]. A large nested case-control study found a strong inverse association between

pre-diagnostic 25[OH]-D levels and risk of colorectal cancer in Western European populations [46]. Another nested case-control study drawn from an ethnically diverse population found a significantly reduced relative risk of cancer (OR = 0.68) associated with doubling of serum 25[OH]-D, with risk reduction of 37–46% when levels were analyzed as quintiles [47]. More recently, an observational study of 1598 patients with stage I–III colorectal cancer who received treatment showed that low postoperative vitamin D levels were associated with lower all-cause as well as colorectal cancer-specific survival, with most pronounced findings in stage II disease. However, this study also identified statistical interaction between vitamin D receptor genotype and 25[OH]-D levels, thus suggesting the possibility of causation between vitamin D and survival in this population [48].

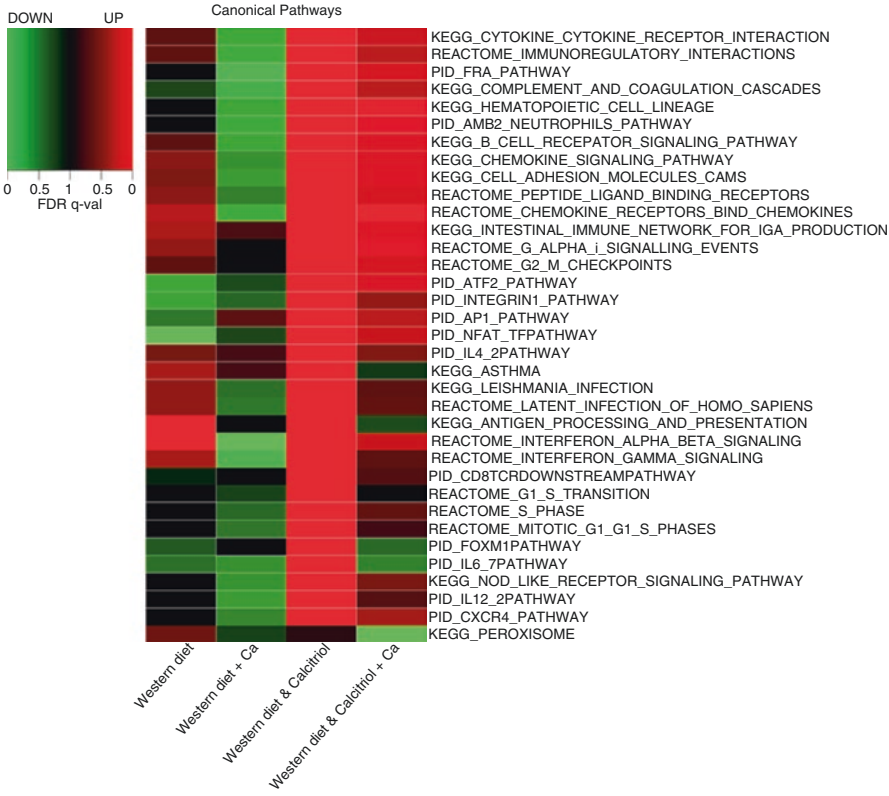

Fig. 5.1 Heatmap of gene set enrichment analysis of canonical pathways and gene ontology. The Western-style diet induced small but significant upregulation of genes involved in immunity-modulated pathways such as interferon signaling or antigen processing and presentation. Supplementation with calcium partially reversed these changes toward baseline. Supplementation with vitamin D induced striking upregulation of genes involved in immune response and inflammation, extracellular matrix, and cell adhesion and cell cycle. Addition of calcium largely reversed these changes. Only most significant pathways and categories are presented in the figure (FDR < 0.005), reproduced from Protiva et al. [43]

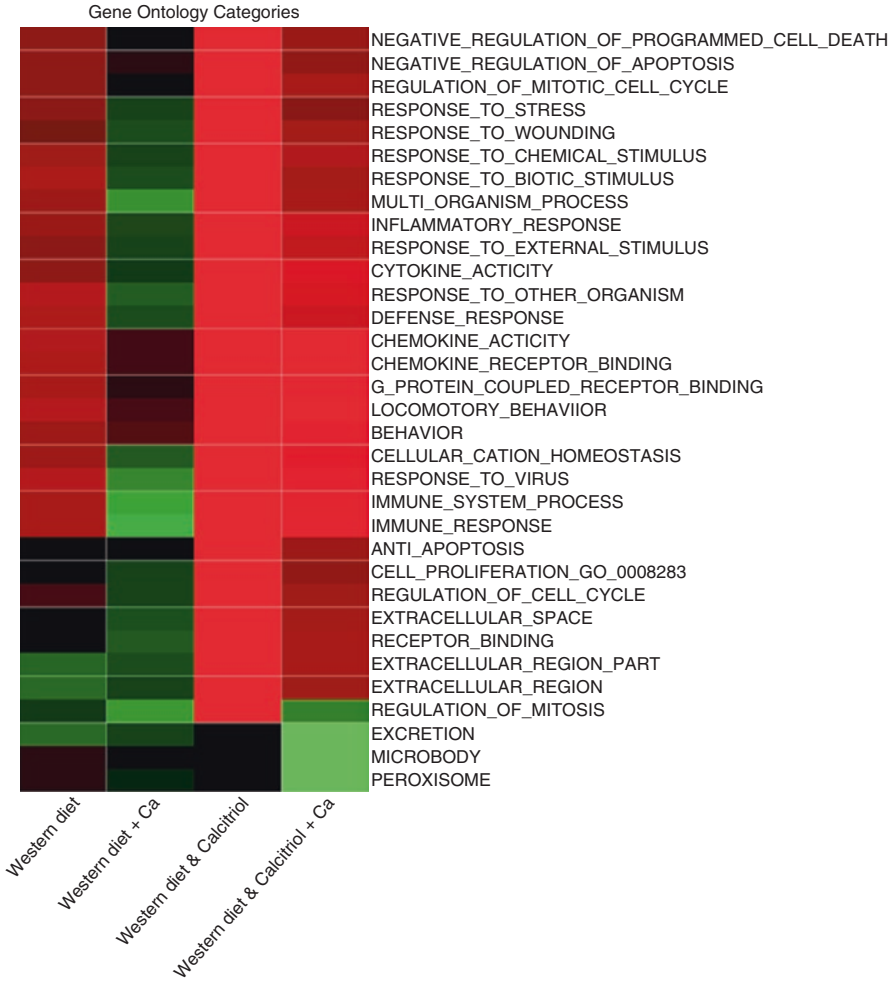

Fig. 5.1 (continued)

Consolidation of studies evaluating vitamin D and colorectal cancer in an updated meta-analysis including nine studies (three prospective clinical trials and six observation studies including studies reporting null findings as below) found that for each 10 nmol/L increase in serum 25[OH]-D, a 6% reduction in risk of colorectal cancer was observed, though a similar trend was not observed for breast and prostate cancer [49].

Vitamin D deficiency has been associated with other types of cancers including breast, ovarian, pancreatic, and prostate cancers [50, 51]. However, an analysis by the World Health Organization (WHO) determined that colorectal cancer is the type of cancer with the greatest risk associated with deficient vitamin D [52]. Additionally, vitamin D deficiency has also been associated with noncancer, extra-skeletal effects,

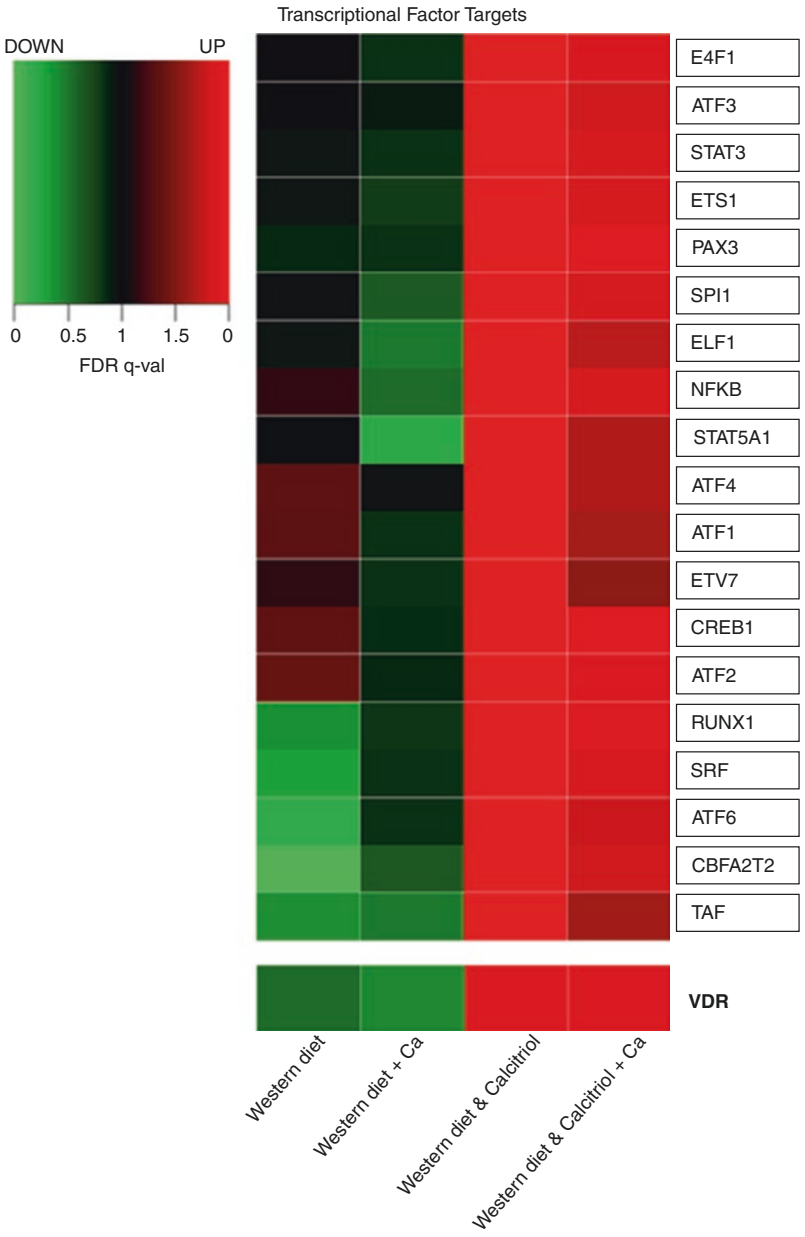

Fig. 5.2 Heatmap of gene set enrichment analysis of transcriptional factor (TF) targets. As expected the Western-style diet supplemented with vitamin D induced significant upregulation of VDR target genes (VDR, FDR = 0.09) and target genes of other transcription factors that have key roles in immune response, proliferation, and extracellular matrix function. Addition of calcium reversed these changes toward baseline. Note that only most significant pathways and categories are presented in the figure (FDR < 0.01), reproduced from Protiva et al. [43]

including cardiovascular disease, insulin resistance, and immunological effects [53]. Although some clinical trial data also suggest vitamin D supplementation resulted in reduced all-cancer risk in postmenopausal women, which included colorectal cancer, the study length was only 4 years, and low outcome frequency did not allow for colorectal cancer stratification [54].

Null Evidence

However, evidence has been conflicting, with other observational studies yielding no association between 25[OH]-D levels and colorectal cancer risk or possibly increased risk. The largest prospective clinical trial was conducted through the Women's Health Initiative—this double-blind, placebo-controlled trial included 36,282 postmenopausal women who were randomized to receive calcium and vitamin D or placebo and found no difference in incidence of colorectal cancer [55]. Some observational studies have found no difference in colorectal cancer risk based on 25[OH]-D levels, though one also reported higher odds of rectal cancer in the cohort with the lowest quartile of 25[OH]-D levels [56, 57]. Similarly, a matched, nested case-control study of Finnish patients with colorectal adenocarcinoma did not find a significant association between vitamin D levels and cancer risk but reported lower relative risk estimates with increasing 25[OH]-D levels [58]. After pooling the results from the HPFS and NHS, Wu et al. found higher plasma 25(OH)D concentrations were statistically significantly associated with decreased risks of both colorectal cancer (highest versus lowest quintile, OR = 0.66, 95% CI = 0.42–1.05; P(trend) = 0.01) and colon cancer (highest versus lowest quintile, OR = 0.54, 95% CI = 0.34–0.86; P(trend) = 0.002) [59].

Quality of Evidence

Some challenges with the current evidence are the utilization of case-control study design and relative paucity of prospective controlled trials, inability to adjust for family history of cancer, heterogeneity in study populations (Finnish, American, Asian, healthcare provider cohorts have been utilized), and heterogeneity of methodology reporting quality and statistical analysis.

Modulators of Vitamin D Effect

Genetic factors, age, sex, ethnicity, as well as dietary and lifestyle factors contribute to the risk of colon cancer and may modulate or confound the effect of vitamin D.

The genetic polymorphisms in components of VDR pathway and risk of colorectal cancer were examined in multiple studies. The majority of studies report little effect of VDR variants on CRC risk. The most common polymorphisms linked to increased risk are *Apa*I, *Bsm*I, and *Taq*I, but the risk varies with age, sex, race, and ethnicity [60]. For example, the meta-analysis demonstrated that VDR BsmI polymorphism increased risks in colorectal cancer in Caucasian population, but no significant associations were observed in other VDR polymorphisms in the overall analysis [61]. Detailed information on subject's characteristics and the next-generation sequencing should provide more comprehensive picture on this subject.

It is estimated that about one fifth of all cancers are caused by obesity. Obesity is consistently associated with low circulating vitamin D levels, and it is believed to be one of the underlying causes of the high BMI cancer link [62]. It is currently not clear if low vitamin D status actually promotes obesity. However, a recent meta-analysis and systematic review showed that the significance of the mediating role of vitamin D in the biological pathways linking obesity and cancer is low [63]. The relationship between low vitamin D, high BMI, and cancer risk is likely to be modulated by dietary and genetic factors as well as physical activity, even after adjusting for majority of additional factors such as sex, age, and ethnicity. Nevertheless, some intriguing preclinical evidence involving transgenic mice overexpressing VDR in adipose tissue suggests that VDR signaling in adipocytes promotes obesity and marked reduction of energy expenditure and is associated with paracrine/autocrine regulation of fatty acid beta-oxidation and lipolysis [64]. Despite this evidence, a causal link between low vitamin level and obesity is unconfirmed.

Current Clinical Recommendations and Further Research

A recent meta-analysis found that vitamin D and calcium supplementation can reduce fracture risk, but there is not sufficient evidence to draw definitive conclusions regarding benefits and harms for use of vitamin D with or without calcium for cancer prevention [49].

Currently, evidence is insufficient to recommend vitamin D supplementation for cancer prevention or treatment. Further studies are needed on determination of whether a protective effect is seen, in light of conflicting studies. Additionally, an effective dose should be established owing to evidence of a possible dose-response protective effect, coupled with other studies suggesting that higher doses of vitamin D could further reduce the risk of colorectal cancer with minimal risk of adverse side effects [65, 66]. Further clinical and translational research is needed on the effectiveness of vitamin D supplementation on colorectal cancer risk, and with more prospective clinical trials, efficacy will be better assessed.

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Chapter 6
Vitamin D and Leukaemia

Geoffrey Brown, Andrzej Kutner, and Ewa Marcinkowska

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G. Brown, BSc, PhD (✉)
Institute of Clinical Sciences, Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham Edgbaston, Birmingham, UK
e-mail: g.brown@bham.ac.uk

A. Kutner, BSc, PhD, DSc
Department of Pharmacology, Pharmaceutical Research Institute, Warsaw, Poland
e-mail: a.kutner@ifarm.eu

E. Marcinkowska, MD, PhD
Laboratory of Protein Biochemistry, Faculty of Biotechnology,
University of Wroclaw, Wroclaw, Poland
e-mail: ema@cs.uni.wroc.pl

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Introduction

The basic forms of vitamin D, derived from steroids (ergosterol and cholesterol), are ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). The key step of this transformation is a photolytic cleavage of the 9,10 single bond of 7-dehydro steroids. This is why vitamin D molecules are termed *seco*-steroids. The difference between vitamin D_2 and vitamin D_3 is solely in the aliphatic side chain: the same difference is seen in the above parent steroids. The side chain of vitamin D_2 contains an additional double bond between carbons C-22 and C-23 and a methyl at carbon C-24, of 24*R* absolute configuration.

The term vitamin ("vital amine") was conceived by Casimir Funk at the beginning of the twentieth century [1]. However, vitamin D is not a true vitamin because it does not contain an amine group and is biosynthesised by the body rather than ingested with food as an essential nutrient. Moreover, vitamin D is like other hormones in that it is produced in one organ of the body (skin via ultraviolet B (UVB) irradiation of 7-dehydrocholesterol), activated in different organs and then transported to other places in the body as to its function. Although some of the vitamin D is derived from the diet, from fish, milk and eggs, as vitamin D or in the form of provitamins (as ergocalciferol and cholecalciferol), most of the vitamin D comes from exposure of the body to the sunlight. The source of ergocalciferol (vitamin D_2) is plants as this is not synthesised by humans. Both vitamin D_2 and D_3 are biologically inactive and must be hydroxylated, in the liver by vitamin D 25-hydroxylase (CYP27A1), to give rise to 25-hydroxyvitamin D_2 (25D2) and 25-hydroxyvitamin D_3 (25D3, calcidiol), respectively. These major circulating metabolites of vitamins D are further hydroxylated, mainly in the kidney and primarily by 25(OH)D-1α-hydroxylase (CYP27B1), to 1α,25-dihydroxyvitamins D (1,25D): 1α,25-dihydroxyvitamin D_2 (1,25D2) and 1α,25-dihydroxyvitamin D_3 (1,25D3, calcitriol), respectively (Fig. 6.1). The activity of endogenous 1,25D3 has been widely believed to be comparable to that of 1,25D2.

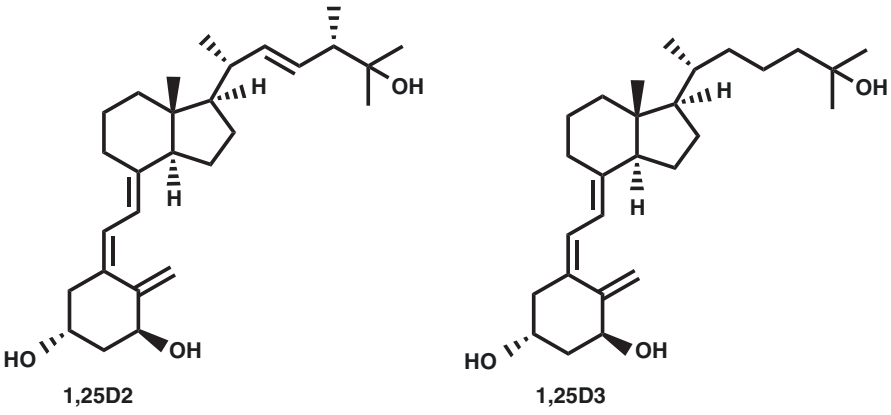

Fig. 6.1 Structures of 1α,25-dihydroxyvitamin D_2 (1,25D2) and 1α,25-dihydroxyvitamin D_3 (1,25D3)

However and very recently, Danilenko and Kutner [2] have shown by a "head-to-head" comparative study that 1,25D2 is less effective in driving differentiation of acute myeloid leukaemia cells (AML) than 1,25D3.

To the best of current knowledge, the most active vitamin D metabolite is 1,25D3, and 25D3 is a partially activated precursor [3]. The hydroxylation at C-1 is strictly controlled as CYP27B1 expression is regulated by feedback loops. Parathyroid hormone is produced in response to a low serum calcium level and upregulates the expression of CYP27B1, whereas fibroblast growth factor 23 is produced by bone in response to high 1,25D levels and downregulates the expression of CYP27B1[4–6]. Interest is focused on the use of 1,25D as an anticancer agent, and the vitamin D status of individuals is routinely assessed by measuring the blood level of 25D. The concentration of 25D is 100 times higher than that of 1,25D because it has an exceptionally long half-life.

An important role of 1,25D in the body is the maintenance of calcium homeostasis and regulation of calcium and phosphate in the bloodstream to promote the growth and remodelling of bones. This is controlled by 1,25D increasing calcium absorption by the kidneys and decreasing the extent to which calcium is excreted by the kidneys [3]. Additionally, 1,25D has other diverse actions throughout the body. An appropriate level of 1,25D is essential for a healthy innate and adaptive immune system. People who are vitamin D deficient, and who have inactivating mutations in the receptor for 1,25D (vitamin D receptor; VDR), are unable to mount adequate response to infections [7, 8]. 1,25D is also important to inhibiting inflammatory responses, and vitamin D supplementation has been shown to be of therapeutic benefit in mouse models of autoimmunity [9, 10]. The use of 1,25D3, 1,25D2 and analogues of these metabolites as anticancer agents is supported by the finding that they can drive cell cycle arrest and differentiation of a wide variety of cell types [11].

Epidemiological Studies Support the Use of Vitamins D as Anticancer Agents

A considerable number of studies have revealed a link between the level of 25D and both the incidences of various cancers and survival from these diseases. The findings are not conclusive as they do not prove that a deficiency is the cause of the risk or of a poor outcome for patients receiving treatment. A caveat is that a low level of 25D might also be considered a marker of a bad lifestyle and poor health. Large systematic reviews and follow-up meta-analyses have focused on 25D levels and the incidence and survival from carcinomas. In 1941, Apperly proposed, from studies of North Americans, that there is a link between the level of exposure to solar radiation and cancer mortality [12]. In 1990, Hanchette and Schwartz hypothesised that vitamin D deficiency is a risk factor for prostate cancer [13] and later provided evidence, from examination of the geographic patterns of prostate cancer mortality, that UVB radiation has a protective effect against prostate cancer [13]. That serum levels of 25D correlate with prostate cancer risk was observed in studies undertaken

in Scandinavia which showed that men living at high altitude have very low serum 25D and a higher risk of developing prostate cancer [14]. Of particular interest to risk is that normal prostate cells can convert 25D to 1,25D [15].

Many epidemiological studies have focused on the relationship between serum levels of 25D and colorectal and breast cancer. Case control studies together with meta-analyses have revealed an association between an increased risk of developing colorectal cancer and a low level of 25D in the blood [16–20]. Studies have also shown that patients with colorectal cancer that have low levels of 25D have a poorer outcome, and the lower the level, the worse the outcome as to mortality [18, 20]. As to breast cancer, systematic reviews and meta-analyses have shown associations between an increased risk of developing this disease and a poor outcome for patients and a low blood level of 25D [18, 21, 22]. A low serum level of 25D is also associated with an increased risk of developing melanoma and an unfavourable prognosis [23]. Whilst there is good evidence for the above associations, the relationships between a low level of 25D in the circulation and the risk of developing other cancers and the importance of 25D levels to disease outcome are less certain [24, 25].

Early Studies of the Activity of 1α,25-Dihydroxyvitamin D_3 Against Leukaemia Cell Lines

The findings from studies of leukaemia cell lines are very clear regarding the action of 1,25D and support the use of 1,25D as an anticancer agent. 1,25D drives the growth arrest, differentiation and apoptosis of a wide variety of cancer cell lines. In 1981, Suda and co-workers reported that 1,25D3 drives the differentiation of mouse myeloid leukaemia cells towards macrophage-like cells [26]. The human promyeloid leukaemia cell line HL60 can differentiate to neutrophils and monocytes, and a number of early studies showed that 1,25D3 induces monocyte differentiation of these cells [27–29]. 1,25D3 drives an initial burst of rapid proliferation, a transient increase in the proportion of cells in the G2/M phase of cell cycle followed by growth arrest in the G0/G1 phase and differentiation towards phagocytic and macrophage-like cells [30, 31]. Other myeloid leukaemia cell lines that differentiate towards macrophages in response to 1,25D include U937, THP-1, MV4 and MOLM-13 (Reviewed in [32]). Preclinical studies using mouse models of systemic acute myeloid leukaemia confirmed the antileukaemic action of 1,25D3 [33, 34]. As considered in the next section, 1,25D triggers a complicated set of events in leukaemia cells involving the coordinated actions of transcription factors and intracellular signalling molecules.

Modes of Action of 1α,25-Dihydroxyvitamin D

Despite extensive in vitro studies of the actions of 1,25D on cancer cells, the cellular events that lead to growth arrest and cell differentiation are still not entirely clear. A first consideration that is germane to this change in cell behaviour is how do 25D

and 1,25D get inside cells? For many years it was believed that these lipophilic compounds freely diffuse through the cell membrane. The precursor of 1,25D, namely, 25D, must be efficiently taken up by kidney cells, which are the major source of 1,25D in the body. The viewpoint as to diffusion, and the so-called free hormone hypothesis, was confounded by the discovery of megalin, a member of the low-density lipoprotein receptor-related proteins. Megalin is essential to uptake of 25D by kidney cells [35], and another endocytic receptor, cubilin, cooperates with megalin in the endocytosis of 25D [36]. Later studies have shown that these proteins are important to the transport of vitamin D metabolites into mammary [37], skeletal muscle cells [38] and bones [39].

Once inside cells, 1,25D needs to bind to the vitamin D receptor (VDR) to be active. This receptor belongs to the superfamily of steroid/thyroid nuclear receptors and acts as a ligand-activated transcription factor, as a heterodimer with retinoid X receptor, to regulate expression of the target genes [40]. VDR is encoded by a single gene located on chromosome 12. Translation of VDR protein starts from exon 2 and, in some people, due to a T to C polymorphism, starts from the second in-frame ATG codon. Thus, two variants of VDR may be produced, and the shorter (424aa) variant exerts higher transcriptional activity than the longer (427aa) one [41]. The 5′ region of the *VDR* gene is very complex, consisting of seven exons that are used variously in different tissues to transcribe mRNA for VDR [42, 43]. This complexity means that expression of the *VDR* can be differentially regulated by the same factor in different cells [43]. A few factors are known to regulate VDR expression and in some tissues 1,25D upregulates VDR [44–46], whilst in others it does not [47, 48]. Additional factors that regulate VDR expression are dexamethasone and retinoic acid [43, 49, 50].

VDR protein has a domain structure consisting of the N-terminal, the DNA-binding domain (DBD), the hinge region, the ligand-binding domain (LBD) and the transactivation domain [51]. The N-terminal is short, and its deletion does not affect the transcriptional activity of VDR. The DBD binds the vitamin D response elements (VDREs) in the promotor regions of target genes by using two zinc fingers. The hinge region between DBD and LBD is highly flexible and allows the receptor to bind many response elements. The LBD changes its shape substantially when the ligand is attached and helix H12, which acts as a lid, closes the ligand-binding cavity and creates the interface for the binding of transcriptional co-activators (CoAs) [52].

VDR protein is unstable when ligand is not bound and the mechanism of stabilisation is as yet unclear [47, 48]. It has been shown that ligand binding decreases the interaction of VDR with proteasome components, to extending the lifetime of the protein [53]. Another mechanism is based on ligand-induced nuclear translocation of VDR [54], since protein degradation in the nucleus is not as efficient as in the cytosol. Upon ligand binding, the nuclear localisation sequence in VDR becomes accessible to the transport proteins, and the receptor is rapidly translocated to the cell nucleus. Therefore, important factors that influence the actions of 1,25D are the level of VDR protein in the cells [55] and its efficient transport to the cell nuclei [56]. There are hundreds of genes that are regulated either directly or indirectly by VDR [57, 58], and many of them are responsible for calcium-phosphate homeostasis [59].

Some of the VDR-regulated genes are important to the control of cell cycle and the conduct of cell differentiation [60]. Target genes include ones that encode the features of macrophages, such as cluster of differentiation (CD) 14.

The extent to which 1,25D is catabolised within cells is important to its action against cancer cells. 1,25D is active at pico-molar concentrations, and its level in tissues is strictly regulated. The half-life of 1,25D is only 10–20 h [61], due to efficient catabolism to an inactive metabolite by 24-hydroxylase, which is located in the inner membrane of mitochondria. The gene encoding this enzyme, *CYP24A1*, is the most strongly regulated VDR-target gene, and its promoter contains multiple VDREs [62]. Expression of *CYP24A1* may be upregulated to the extent of thousands of times in response to 1,25D in both healthy and leukaemia cells [63], to provide a negative feedback loop as to the activity within cells of 1,25D. The level of expression of this enzyme is important to the responsiveness of cancer cells to 1,25D as overexpression and multiple copies of the gene have been detected in colon cancer. Accordingly, *CYP24A1* has been proposed to be a tumour suppressor [64, 65].

Many researchers hold the viewpoint that the biological actions of 1,25D are solely mediated by VDR via "a genomic effect". However, there are effects of 1,25D on cells that cannot be attributed to VDR-driven gene regulation. Gene transcription requires at least a few hours, and intracellular events have been observed to occur within seconds or minutes after adding 1,25D to cells. These "rapid responses", leading to activation of the RAS/RAF/ERK-MAP kinase signalling pathway, are required for 1,25D-driven differentiation of leukaemia cells [66–70]. The existence of a specific membrane receptor that is responsible for the rapid response of cells to 1,25D was proposed long time ago [71], but the nature of this receptor is still far from clear. It has been postulated that a small proportion of a cell's canonical VDR is localised at the cell membrane where it plays a role in rapid intracellular signalling, through binding of 1,25D to an alternative ligand-binding domain of VDR [72]. VDR has been detected in the caveolae of some cells. However, this can't be the sole reason for the rapid response of cells to 1,25D because such is retained by cells from VDR-knockout mice [73]. One of the most rapid cellular responses to 1,25D is calcium and phosphate uptake by intestinal cells [66]. This has been attributed to the membrane receptor protein disulphide isomerase family A member 3 (Pdia3) [74], which has been carefully studied as to a role in 1,25D-mediated signalling in the musculoskeletal system [75] and bones [76]. Whether this receptor plays a role in other tissues is unclear. As above, there is uncertainty about whether the action of 1,25D on leukaemia cells is mediated by a single receptor, namely, nuclear VDR, or VDR together with other putative receptors. However, it is clear that interplay between genomic events and rapid intracellular signalling is important to the 1,25D provoking a change to cell status (Fig. 6.2). Also, and as considered later, selective events, mediated by different receptors, may be the reason why some analogues of 1,25D are potent in driving differentiation of leukaemia cells and have little calcaemic action.

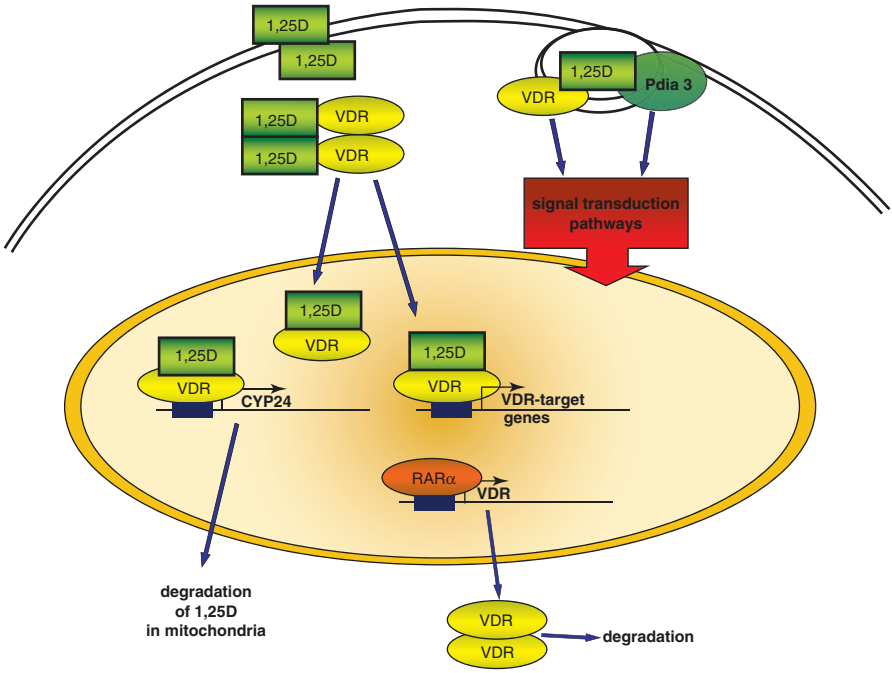

Fig. 6.2 Overview of the mechanisms of action of 1α,25-dihydroxyvitamin D (1,25D). 1,25D enters the cells either by free diffusion or by receptor-mediated endocytosis. VDR is unstable in the cytosol if not bound to 1,25D. Liganded VDR translocates to the cell nucleus, where it regulates transcription of VDR-regulated genes. The most strongly regulated VDR-target gene is *CYP24A1*, which encodes 24-hydroxylase of 1,25D. 24-Hydroxylase in mitochondria degrades 1,25D to inactive compounds. 1,25D also binds to not fully defined receptors localised in caveolae and rapidly activates multiple signal transduction pathways

Responsiveness of Primary AML Cells to 1α,25-Dihydroxyvitamin D

The results from in vitro and in vivo preclinical studies provided good support to the use of 1,25D to treat leukaemia [34, 77–79]. In contrast, the findings from early clinical trials conducted on small groups of patients with myelodysplastic syndrome (MDS) and AML were not conclusive ([80–83] and see later). Of importance to this outcome is the extent to which cells from patients with AML do or do not respond to 1,25D.

AML is not just one disease, but a very heterogeneous group of acute leukaemias. More than 200 different chromosome translocations and mutations have been detected in blast cells from patients diagnosed with AML [84]. AML is a rare disease in children and constitutes 80–85% of acute leukaemia in adults, and the incidence

of AML increases with age [85]. Initially AML was divided into eight subtypes (M0–M7), based on the phenotype of the cell from which the leukaemia developed and the level of cell maturation [86]. In 2001, the WHO introduced a new classification, which was revised in 2008 to divide AML into four main groups: AML with recurrent genetic abnormalities, AML with MDS-related changes, therapy-related myeloid neoplasms and AML not otherwise specified [87]. This classification took into consideration the existence of genetic abnormalities in AML but put all of them into one group. This does not really reflect differences in disease progression, prognosis and treatment, since some mutations confer a good and others a poor prognosis [88]. Targeted therapies exist for some subtypes of AML, and they are based on a mutation which occurs in the given subtype. The most successful one is differentiation therapy using all-*trans*-retinoic acid (ATRA) for acute promyelocytic leukaemia (APL), which has revolutionised treatment of this disease by converting it from a fatal to a curable one [89]. Pathogenesis of APL is associated with a translocation between chromosomes 15 and 17 which disrupts retinoic acid receptor α (RARα) and fuses it to promyelocytic leukaemia protein (PML) to cause the formation of the chimeric oncoprotein PML-RARα [90]. ATRA, at pharmacological doses, causes a conformational change to PML-RARα, leading to protein degradation, the chromatin structure becoming relaxed, relief of transcriptional repression and APL blasts undergoing terminal differentiation into granulocytes [91].

It was therefore tempting to speculate that 1,25D is only effective against certain subtypes of AML and that some mutations might confer resistance and others susceptibility to 1,25D-based differentiation therapy. Studies were conducted to examine the correlations between the extent to which 1,25D and analogues were able to drive differentiation of AML cells and the most common genetic abnormalities [92, 93]. These studies have shown that cells which carried activating mutations in *Flt3* gene are less susceptible than all other AML blasts to 1,25D-induced differentiation [92]. Cells from AML patients that had a complete or partial deletion of chromosome 7, or a mutated nucleophosmin (*NPM1*) gene, were found to be sensitive to 1,25D and its analogues [92, 93]. Unfortunately, the cell line models with mutations in the gene encoding Flt3 receptor are not resistant to 1,25D-induced differentiation, and, therefore, the molecular mechanism of the observed correlation could not be studied [94].

Studies of the KG1 cell line have shed some light on the mechanism of resistance to 1,25D-induced cell differentiation. This cell line is a unique model of the disease entity called 8p11 myeloproliferative syndrome, which transforms rapidly to AML [95]. In this leukaemia, the fibroblast growth factor receptor 1 (*FGFR1*) and its oncogene partner 2 (*FOP2*) fusion gene were identified, which results in the generation of a constitutively active fusion protein FOP2–FGFR1 [96]. The KG1 cell line has been defined as resistant to 1,25D-induced differentiation [97], and the resistance has been attributed to low level of expression of the VDR gene and protein [55]. Also, the FOP2–FGFR1 fusion protein is constitutively active in KG1 cells and causes permanent activation of the signal transducer and activator of transcription (STAT) pathway [96, 98]. The very low level of VDR protein in KG1 cells can be upregulated moderately by treatment with RARα agonists, and then these cells

start to differentiate in response to 1,25D [43]. Importantly, disruption of *FOP2–FGFR1* fusion gene, and resulting silencing of the STAT signalling, caused massive upregulation of *VDR* gene expression, a high level of VDR protein and very efficient differentiation in response to 1,25D [99]. These experiments show that an aberration in signal transduction, which often occurs in AML blasts, can interfere with differentiation therapy.

Clinical Trials of 1α,25-Dihydroxyvitamin D

Clinical trials using 1,25D with chemotherapy in leukaemia and breast, colon and prostate cancer have resulted in conflicting and often disappointing results. One reason is that it is difficult to achieve an effective therapeutic dose. The dose is severely limited by the calcaemic action of 1,25D: the risks from hypercalcaemia are coma and cardiac arrest. As to this barrier, seocalcitol, developed by Ernst Binderup at Leo Pharmaceuticals and coded as EB1089 (22,24-dihydro-24,26,27-trishomo-1α,25$(OH)_2D_3$), is more potent (50–100-fold) in its anticancer action than 1,25D and has a reduced calcaemic activity. However, all of the patients with advanced colorectal cancer and breast cancer receiving a prolonged high dose of EB1089, in a phase I study, experienced hypercalcaemia [100]. Clinical trials of EB1089 in cancer patients appear to have ceased despite low doses of EB1089 being well tolerated. Inecalcitol, developed by Pierre De Clercq at Thermex and coded TX-522 [19-*nor*-14-epi-23-yne-1,25-$(OH)_2D_3$], is also more potent than 1,25D3 and when tested in a tumour regression model did not have a major effect on the level of blood calcium. However, dose-limiting toxicity was seen in advanced prostate cancer patients receiving the highest dose in a phase I study. Though the benefit from adding inecalcitol to docetaxel treatment was unclear [101], this agent is being tested in current clinical trials (see later).

It has been known for over 30 years that 1,25D and analogues drive differentiation of AML-like cell lines and AML cells towards a monocyte-like phenotype. Harrison and Bershadskiy have reviewed 24 clinical reports of the use 1,25D/analogues to treat patients with AML and myelodysplasia (MDS) [102], and Kim and co-workers have examined 17 clinical trials of these agents in these diseases [103]. The overarching observations are that translating findings from in vitro studies into clinical benefit has been difficult and the effective and routine use of 1,25D/analogues, either alone or as an adjunct to chemotherapy, to treat leukaemia has still to be achieved.

In the late 1980s, there were reports of the use of 1,25D and analogues alone to treat single or small groups of patients with AML and various MDS subtypes. Minor or partial responses were observed, and Nakayama and co-workers reported a favourable response in a single case of AML [81, 82, 104]. Some patients developed hypercalcaemia. For example, hypercalcaemia occurred in 9 of the 18 MDS patients given 2 μg/day of 1,25D [104]. In the 1990s, cohorts of between 20 and 30 patients with MDS were treated with 1α-hydroxyvitamin D_3 or 1,25D [105–107].

Again, hypercalcaemia was the main toxicity. Some low-intermediate risk MDS patients responded, in terms of an increase in granulocytes, platelets or haemoglobin levels, without developing hypercalcaemia. However, the overall conclusion was that use of 1,25D as a single agent has very limited benefit.

A number of groups have examined the use of 1,25D/analogues in combination with other agents to treat AML and MDS. A variety of agents have been used in combination with 1,25D/analogues, in phase II and phase III trials, including 13-*cis*-retinoic acid with or without prednisone [108], 13-*cis*-retinoic acid with valproic acid [109], low-dose conventional antineoplastic chemotherapy such as cytarabine [80, 83, 110–113] and erythropoietin with 13-*cis*-retinoic acid [114]. Overall the response rates have been highly variable. Even so, a minority of patients experienced a transient or persistent benefit as to their blood profile and the few successes supported continued investigation of the potential use of new analogues of 1,25D to treat leukaemia. There are ongoing trials of inecalcitol in patients with AML and chronic myeloid leukaemia [115], and this agent has been given orphan designation by the European Medicines Agency and the US Food and Drug Administration for the treatment of AML and chronic lymphocytic leukaemia (CLL) [116, 117]. The results from a phase II trial in which 15 CLL patients received a huge dose of 2 mg of inecalcitol are encouraging. At 10 months, there was a 90% decline in the level of blood lymphocytes in one patient, and lymphocyte levels had failed to increase in eight more patients [115].

Low Calcaemic Analogues of 1α,25-Dihydroxyvitamin D That Retain Anticancer Action

In order to overcome the barriers to the clinical use of 1,25D, a substantial number of analogues have been synthesised with reduce calcaemic action and that retain potency against cancer cells. The intent is to generate analogue with no/negligible calcaemic action that are potent anticancer agents. Analogues that have been widely investigated for antiproliferative activity, including evaluation in clinical trials for some, include BXL0124 (1α,25-dihydroxy-20*R*-21(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol), calcipotriol (22-ene-26,27-dehydro-1α,24(OH)2D3, also coded as PRI-2201), EB1089, EMI [(5Z,7E)-(1S,3R)-1,3-dihydroxy-9,10-secochola-5,7,10(19)-trien-23-in-24-yl] and inecalcitol (reviewed in [118]). As mentioned above, there is still the need to provide a 1,25D that is entirely suitable for use to treat leukaemia and other cancers.

Figure 6.3 shows the structures of novel double-point modified analogues of 1,25D [119] that have been synthesised recently at the Pharmaceutical Research Institute (PRI) in Warsaw, Poland. The structure of these analogues resulted from consecutive modifications to the structural fragments of the vitamin D molecule

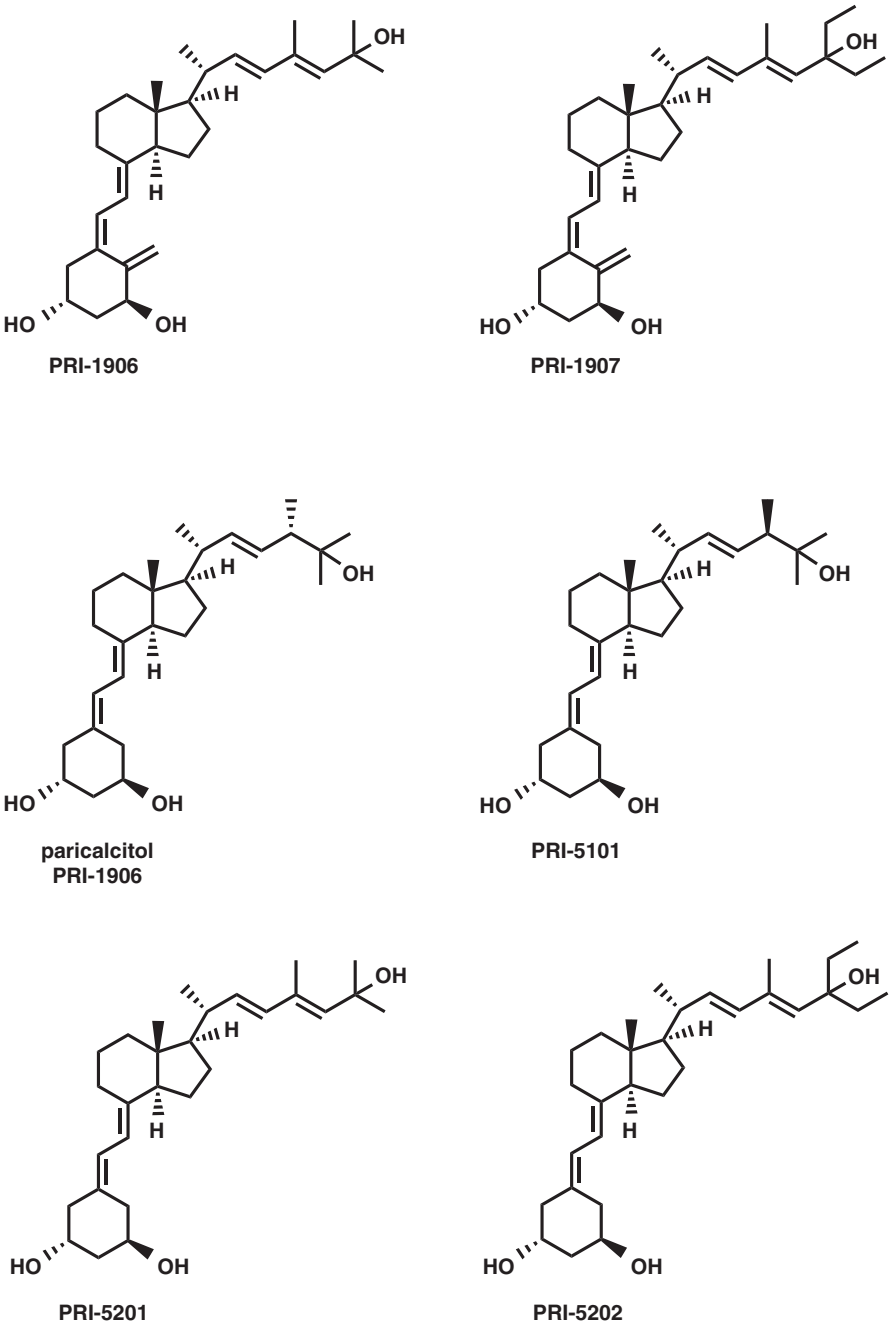

Fig. 6.3 Structures of the leading analogues of 1α,25-dihydroxyvitamin D_2 synthesised at the Pharmaceutical Research Institute

that contribute the most to the binding of the analogue to LBD of VDR. Synthesis and biological evaluation of several generations of side-chain modified 1,25D analogues have resulted in the most effective overall modification, including the extension of the side chain and additional unsaturation. Mono-homologated side chain containing a 22,24-conjugated diene system and D_2-like skeleton (24-methyl) combined with a 19-*nor* modification in the A-ring results in a synergy as to increasing the anticancer action of 1,25D2. The 1,25D2 analogues have been evaluated for activity against AML cells grown in vitro and as to their calcaemic action in mice [2, 120, 121]. The new analogues are more potent than 1,25D3 as inducers of monocyte differentiation of AML cells and in driving VDR-mediated transcription. Importantly, double-point modified analogues do not increase serum calcium levels in mice as much as 1,25D3, and overall these agents were less toxic. PRI-5201 and PRI-5202 [122] are the most active analogues of 1,25D that have been designed and synthesised at PRI (Table 6.1). Further refinement of these structures may provide analogues that are suitable for long-term therapy of leukaemia and other cancers.

Interestingly, the anticancer capacities of the low-calcaemic analogues of 1,25D2 do not correlate with the affinities of the analogues to VDR (Table 6.1). All of the actions of 1,25D on leukaemia cells cannot be attributed to VDR-driven transcription. As considered above, 1,25D induces rapid events within cells, and there might be alternative receptors. The rapid action of 1,25D on intestinal cells has been attributed to binding to Pdia3. Furthermore, megalin and cubilin transport vitamin D metabolites, coupled to serum vitamin D-binding protein (DBP), through the cell membrane. Receptor endocytosis, and recycling, can rapidly generate intracellular signals through the Rab5/PI3-kinase pathway. In essence, interplay between genomic and non-genomic events, different signal transduction pathways and the ability of a given analogue to stabilise VDR in the cell nucleus are all important to the biological activity of analogues. A resolve as to the extent analogues of 1,25D2 and 1,25D3 can elicit particular events within cells is important to the development of the very best, particularly non-calcaemic analogue, for use in antileukaemia therapy.

Table 6.1 Differentiation-inducing and calcaemic activities of 1,25D and PRI analogues and their binding to VDR Cell differentiation activity is given as EC50, and the VDR binding is expressed as IC50. Calcium levels are the means of values obtained for five mice treated with 0.3 μg/kg of 1,25D or analogue every other day for 3 weeks and measured on day 21. The serum calcium level in ethanol-treated mice was 62.3 ± 0.6 mg/dL. EMR: effective molar ratio (EC50 analogue/EC50 1,25D3). The potency of 1,25D as to binding to VDR is normalised to 100. RBA: relative binding affinity

	Differentiation EC_{50} M e^{-11} (EMR)	Calcaemic action Ca^{2+} (serum) mg/dL	VDR-binding IC_{50} M e^{-10} (RBA)
1,25D3	53.47 (1)	107.0 ± 4.5	23.20 (100)
PRI-1907	5.952 (0.11)	74.8 ± 2.2	61.72 (37)
PRI-5100	112.8 (2.12)	85.5 ± 3.7	5.599 (414)
PRI-5101	117.9 (2.22)	90.3 ± 3.2	4.921 (471)
PRI-5201	3.397 (0.063)	93.3 ± 2.3	11.193 (194)
PRI-5202	1.788 (0.033)	80.5 ± 1.9	35.98 (64)

Prospects for the Future

The prospect of using analogues of 1,25D to treat leukaemia, and other cancers, has by no means reached the end of a very long road. AML is a disease of the elderly, as are many cancers. The proportion of older people is increasing steadily. Previous estimates of the proportion of people facing cancer at some point during their life, most likely in old age, have been around one in three. The latest estimate of the chance of developing cancer is one in two for people born after 1960, because most people are living much longer. Correspondingly, cancer is more and more a prevalent disease of aged people. Such has given rise to the need for a different approach to treatment to meet the demands of very elderly patients with leukaemia and other cancers. Aggressive chemotherapy cannot be used in very old people. They often are unable to tolerate such due to comorbidities, and common and intractable fears from treatment toxicity result in no treatment in old age as the preferred/advised option. When chemotherapy is given to elderly patients, they often face poor health or disability after treatment, and the dosage has to be reduced for some patients, to avoid debilitating side effects. As to these sound reasons, there is the need for new and gentler drugs, for use alone or to reduce substantially the dosage of chemotherapy in a combined treatment. The development of new analogues of 1,25D for use alone or to reduce substantially the dosage of chemotherapy for leukaemia offers the prospect of a treatment that is likely to be well tolerated by elderly patients.

Differentiation therapy provides a radically different, though proven, approach to treating cancer. To achieve the aim of 1,25D-based differentiation therapy of leukaemia, there still needs to be progress in three areas in particular. The development of analogues of 1,25D that have no or negligible calcaemic action and that are potent antileukaemia agents is a prospect that is highly feasible. It is unlikely that such compounds will be used alone. As seen for the benefit of combination chemotherapy, the search for agents that act synergistically with 1,25D is important. Finally, cells obtained from a meaningful proportion of AML patients respond to 1,25D. To extend the potential use of 1,25D analogues in differentiation therapy of leukaemia, it is important to develop a better understanding of why particular patients' cells fail to respond and to find a means to circumvent this problem. In summary, there are still very good rationales to pursuing 1,25D analogue-based differentiation therapy for leukaemia.

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Chapter 7
Vitamin D and Diabetes

Emilia Pauline Liao

Introduction

Pre-vitamin D in the skin is converted to vitamin D by ultraviolet radiation from the sun. Vitamin D is converted to 25-hydroxyvitamin D (25-D) in the liver, which is the stored form of vitamin D and is still biologically inactive. The biologically active hormone, 1,25-dihydroxyvitamin D (calcitriol), is formed by 1α-hydroxylase in the kidney and binds to the vitamin D receptor (VDR) in target tissues. The presence of VDR and 1α-hydroxylase in extraskeletal tissues suggests functions of vitamin D beyond its well-known skeletal effects.

Beta cells in the pancreas express both VDR [1] and 1α-hydroxylase [2], which suggests that vitamin D can have a direct effect on beta cell function by binding to its VDR or an autocrine effect via 1α-hydroxylase within the beta cell. Vitamin D also has an indirect effect by regulating calcium concentrations. Calcitriol mediates calcium influx from intracellular stores and the extracellular space, influencing insulin release from beta cells. Beta cells also express calbindin, a calcium-binding protein in the cytosol, which is a regulator of intracellular calcium, and thereby can modulate depolarization-stimulated insulin release [3]. Since insulin release is dependent on calcium, vitamin D deficiency, which causes hypocalcemia, reduces insulin secretion. Vitamin D deficiency also reduces expression of proteins involved in the glucose response, including insulin, and this action is partially reversed with calcitriol [4, 5] (Fig. 7.1).

In vitro studies by Sergeev showed that calcitriol induces oscillations in calcium concentrations that parallel pulsatile insulin release from beta cells and the insulin release is independent of glucose concentrations. While this phenomenon has not

E.P. Liao, MD
Division of Endocrinology, Department of Medicine, Hofstra Northwell School of Medicine, Lenox Hill Hospital, Northwell Health, New York, NY, USA
e-mail: eliao@northwell.edu

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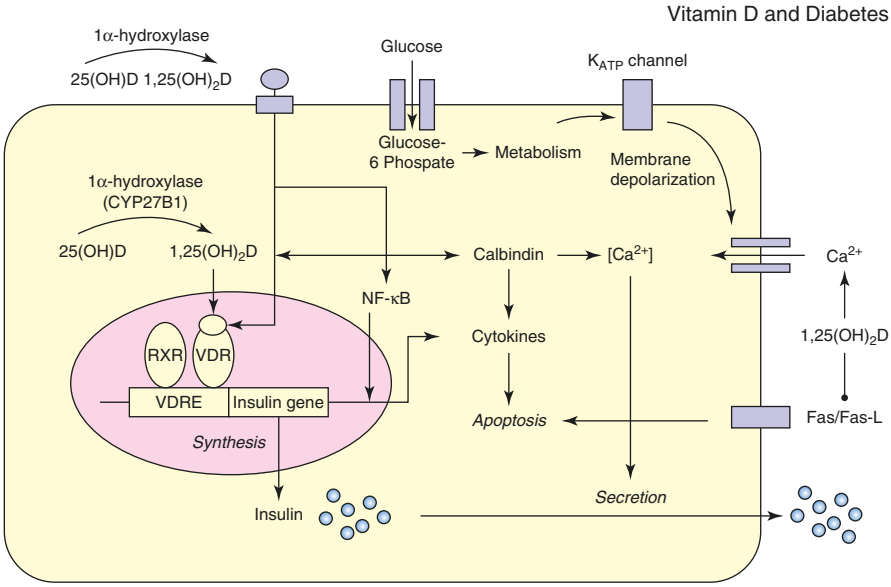

Fig. 7.1 Vitamin D and the beta cell. Vitamin D has a direct effect on the beta cell via the vitamin D receptor. Beta cells also possess 1α-hydroxylase, suggesting an autocrine effect. Vitamin D regulates calcium concentrations in the beta cell from both extracellular sources (through voltage-gated channels) and intracellular sources (such as calbindin). Expression of the insulin gene is promoted through the vitamin D response element. Vitamin D downregulates inflammatory cytokines, such as NFkB and the Fas pathway, to reduce beta cell apoptosis (From Eliades M, Pittas AG. Vitamin D and type 2 diabetes. In: Holick M, editor. Vitamin D: physiology, molecular biology and clinical applications. New York: Humana Press, 2010; with permission of Springer)

yet been demonstrated in animals and humans, the physiological role for this mechanism is hypothesized to be for maintaining basal insulin secretion [6].

Vitamin D and Type 1 Diabetes

Type 1 diabetes is characterized by autoimmune destruction of islet cells in genetically susceptible individuals mediated mainly by T cells, resulting in absolute insulin deficiency. Antibodies against islet autoantigens (GAD, islet cell, insulin) develop and usually predict future clinical disease. Pro-inflammatory mediators are implicated in the pathogenesis of type 1 diabetes, with a predominance of Th-1 and Th-17 cells over anti-inflammatory Th-2 and Treg cells. Shifting this balance presents a potential therapy for new-onset type 1 diabetes, to prevent progression of disease. Dendritic cells, which take up antigen (by endocytosis or phagocytosis) and transport them to T cells, also play a key role in the pathogenesis of type 1 diabetes. Dendritic cells can activate Th cells and killer T cells, which, in turn, produce additional immune responses. Activation of dendritic cells produces either an

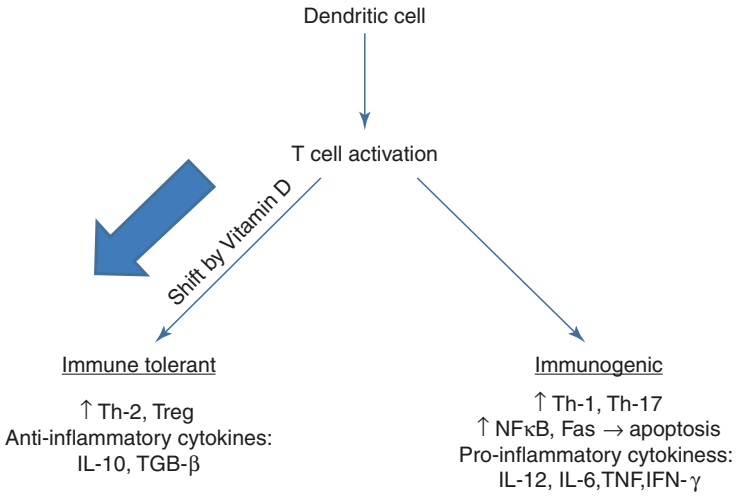

Fig. 7.2 Vitamin D and immune response in type 1 diabetes. Dendritic cells present antigen to T cells, activating them to produce an immune-tolerant response (with anti-inflammatory cytokines) or immunogenic response, resulting in increased pro-inflammatory cytokines. Calcitriol can arrest maturation of dendritic cells and alter their function to produce more anti-inflammatory cytokines

immunogenic response, with increased release of pro-inflammatory cytokines (IL-12, IL-6, TNF), or immune-tolerant response, which releases anti-inflammatory cytokines (IL-10, TGF-β) which, in turn, can suppress T cell responses. VDR is expressed in dendritic cells and T and B cells, and expression is increased in activated T cells [7]. Calcitriol can arrest maturation of dendritic cells and alter their function to produce less inflammatory cytokines and more anti-inflammatory cytokines. B and T lymphocytes are affected in a similar manner by calcitriol, which inhibits production of Th-1 and Th-17 and increases Th-2 and Treg [8]. Inflammatory cytokines induce Fas expression in islet cells, which normally do not express Fas. Fas is a transmembrane surface receptor that is a member of the TNF receptor family and is believed to be a mediator of beta cell apoptosis. Riachy showed that calcitriol was able to revert elevated nitrite (reflecting NO synthesis) and NFkB levels that are seen with Fas induction [9] (Fig. 7.2).

Animal Studies

Giving high-dose calcitriol to the nonobese diabetic (NOD) mouse, an animal model of type 1 diabetes, prevented insulitis and diabetes [10, 11]. This was accompanied by decreased islet chemokine production and Treg cell induction [12]. In addition, T lymphocytes from calcitriol-treated NOD mice did not transfer diabetes into young irradiated NOD mice, while untreated mice did develop diabetes [13].

NOD mice rendered deficient in vitamin D in utero or in early life showed earlier and more aggressive disease [14]. In streptozotocin-treated mice (another animal model of type 1 diabetes), calcitriol was also shown to arrest diabetes development [15]. Because the high doses of calcitriol used to achieve disease suppression also cause hypercalcemia, vitamin D analogs with calcitriol-like activity without hypercalcemia have been developed, and they have also been shown to reduce insulitis and type 1 diabetes in NOD mice [16]. In another study, vitamin D analog given in combination with cyclosporin A arrested diabetes development in NOD mice [17].

Studies with VDR knockout mice are conflicting, but most demonstrated that lacking VDR does not cause impaired glucose tolerance or diabetes, provided that calcium levels are normal [18–20].

Human Studies

Human observational studies show an association between vitamin D deficiency and prevalence of type 1 diabetes in multiple countries [21]. Diagnosis of type 1 diabetes follows a seasonal pattern, with higher incidence during winter months, when vitamin D levels are lower [22]. There is also higher incidence of type 1 diabetes at latitudes further from the equator and low UVB irradiance [23].

In a case control study, women in late pregnancy whose vitamin D concentrations were in the lowest quartile demonstrated over twofold higher risk of their child developing type 1 diabetes [24]. Supplementation of vitamin D in infancy and early childhood appears to reduce future risk of type 1 diabetes. In a Finnish birth cohort study following approximately 10,000 children over 30 years, children who took 2000 IU of vitamin D daily were at lower risk for type 1 diabetes than children who received less than the recommended amount [25]. Children with a history of rickets also were at increased risk for developing type 1 diabetes. In a Norwegian study, infants given cod liver oil, which is rich in vitamin D, in the first year of life also showed reduced risk of diabetes. Furthermore, timing of cod liver oil supplementation between 7 and 12 months of age conferred a lower risk of developing type 1 diabetes compared to supplementation between 0 and 6 months of age [26]. A meta-analysis including observational studies from many European countries, totaling 6455 patients, concluded a 29% risk reduction of developing type 1 diabetes with vitamin D supplementation [27]. The DAISY (Diabetes Autoimmunity Study in the Young) trial, which recruited children at high risk for type 1 diabetes based on HLA-DR phenotype or family history, initially reported that maternal vitamin D intake (from food, not supplements) in the third trimester was associated with a reduced risk of islet autoantibodies [28]; however, intake was self-reported (and may have been overestimated), and a 7-year follow-up did not show association between vitamin D intake or vitamin D level and islet autoantibody or diabetes progression in antibody-positive children [29].

Children with type 1 diabetes have lower vitamin D levels [30], and there may also be an association between levels of vitamin D and autoantibodies [31]. In an Italian study, vitamin D levels were inversely related to diabetic ketoacidosis (DKA)

severity and diabetes onset. And in patients with diabetes for more than 1 year, vitamin D levels were inversely related to hemoglobin A1c and insulin requirements. However, supplementing with cholecalciferol reduced hemoglobin A1c only in Italian patients and not in a migrant population [31].

There have been few intervention studies with calcitriol or vitamin D analog in type 1 diabetes. One trial using calcitriol (0.25 μg every other day) or nicotinamide in children with recent onset of type 1 diabetes (and being treated with intensive insulin therapy) showed reduced insulin doses in the calcitriol group at 3 and 6 months, but hemoglobin A1c and C-peptide after 1 year were not significantly different [32]. In another trial involving patients with new-onset type 1 diabetes, calcitriol showed no benefit in hemoglobin A1c or insulin requirement at 9 or 18 months [33]. A similar study with younger patients and known positive baseline C-peptide (>0.25 nmol/L) also showed no benefit in beta cell preservation after 2 years [34]. In one small pilot study enrolling patients with latent autoimmune diabetes of adults (LADA), there was protective effect on beta cells with calcitriol (assessed by C-peptide) compared to insulin use alone after 1 year [35]. Similarly, another small trial in patients with new-onset type 1 diabetes given 2000 IU/day of cholecalciferol showed higher stimulated C-peptide at 1 year and slower decline of C-peptide at 18 months compared to placebo. In addition, Treg cells were significantly increased at 12 months in the cholecalciferol group [36]. These latter two trials enrolled patients with higher C-peptide (>0.60 nmol/L) than the previously mentioned two studies, which may account for the difference in results.

Some, but not all, studies have reported associations with vitamin D polymorphisms in type 1 diabetes; however, a recent meta-analysis evaluating four most common polymorphisms (Bsml, TaqI, FokI, Apal) found no association with type 1 diabetes [37].

In summary, vitamin D may play a role in preventing type 1 diabetes by modulating the immune response in disease. Timing and dose of vitamin D appear to be important, as there is a positive correlation with vitamin D dose and disease risk reduction. However, intervention in established disease appears to have little, if any, benefit.

Vitamin D and Type 2 Diabetes

Vitamin D and Insulin Resistance

While type 1 diabetes is characterized by an absolute deficiency of insulin, type 2 diabetes results from relative insulin deficiency due to beta cell failure in the setting of insulin resistance, typically due to obesity and/or aging. As mentioned above, vitamin D is required for insulin release, and in addition to impairing insulin secretion, vitamin D deficiency can contribute to insulin resistance through a number of mechanisms (Table 7.1). Chronically elevated glucose and free fatty acid concentrations (glucotoxicity and lipotoxicity) result in beta cell dysfunction and eventually beta cell death.

Table 7.1 Vitamin D effects on insulin resistance

Increased insulin receptor number (via VDRE)—increases insulin sensitivity
PPAR activation—increases insulin sensitivity
Negative regulator of RAS system—increases insulin sensitivity
Deficiency causes secondary hyperparathyroidism—increases insulin resistance
Deficiency increases hepatic steatosis—increases insulin resistance

Vitamin D increases expression of insulin receptor number (but not affinity), via vitamin D response element (VDRE) in the human insulin receptor gene, thus increasing insulin sensitivity [38]. Vitamin D also activates peroxisome proliferator activator receptor (reported with PPARδ and PPARγ), which is involved in the regulation of fatty acid metabolism in skeletal muscle and adipose tissue, thereby increasing insulin sensitivity [39–41]. Deficiency of vitamin D causes hyperparathyroidism, which is thought to increase insulin resistance, as patients with primary hyperparathyroidism have higher rates of glucose intolerance and diabetes [42].

The renin-angiotensin system (RAS) is upregulated in hyperglycemia and contributes to glucose toxicity and islet apoptosis. Vitamin D is a negative regulator of RAS, and VDR knockout mice demonstrate overactive pancreatic RAS. Administration of calcitriol prevented and corrected RAS upregulation in hyperglycemic conditions in animal studies [43, 44].

The liver is a major target organ for insulin, as insulin is a regulator of gluconeogenesis. Hepatic lipid accumulation leads to inflammation and insulin resistance. Hypovitaminosis D is associated with nonalcoholic fatty liver disease (NAFLD) [45], and degree of vitamin D deficiency has been correlated with histologic NAFLD severity [46]. Vitamin D-deficient rats who were fed Western diet demonstrated increased hepatic steatosis and inflammatory markers compared to rats fed low-fat diet [47]. In a rat model of NAFLD, phototherapy increased 25-hydroxyvitamin D and calcitriol levels and reduced hepatic inflammation, fibrosis, and apoptosis [48].

Observational Studies

Observational studies consistently show an association between low vitamin D levels and incidence of type 2 diabetes. Low vitamin D concentrations predict increased future risk of type 2 diabetes [38]. Vitamin D deficiency is also associated with multiple risk factors for diabetes and its associated conditions, such as obesity [6], metabolic syndrome [49, 50], and nonalcoholic fatty liver [45].

In the Diabetes Prevention Program which enrolled patients with prediabetes, the highest risk of developing diabetes was seen in subjects in the lowest tertile of 25-D (mean concentration 12.8 ng/mL), compared to the highest tertile (25-D 30.1 ng/mL) [51]. Similarly, the Nurses' Health Study, which included over 83,000 healthy patients, showed an odds ratio of 0.52 for developing type 2 diabetes

between the highest quartile of 25-D and the lowest quartile (mean concentrations 33.4 versus 14.4 ng/mL). A combined intake >1200 mg of calcium with >800 IU vitamin D was associated with a 33% lower risk of type 2 diabetes [52].

Studies in human subjects regarding the relationship between vitamin D and insulin resistance are conflicting. In 126 healthy subjects, a positive correlation between insulin sensitivity index (ISI = average glucose infusion/average insulin concentration) was found. Increasing vitamin D concentration from 10 to 30 ng/mL improved insulin sensitivity by 60% [53]. A review by Alvarez showed that most, but not all, studies demonstrated an inverse association between vitamin D and HOMA-IR (which is derived by fasting and insulin concentrations) [54].

Intervention Studies

There have been numerous randomized controlled studies using vitamin D as intervention; however, they are difficult to compare because they vary in form of vitamin D supplementation (ergocalciferol, cholecalciferol, calcitriol), duration of treatment, and subject population (diabetes of varying length of duration and severity, prediabetes).

A meta-analysis including 35 randomized controlled trials and 43,000 patients found no benefit of vitamin D supplementation to prevent diabetes in healthy subjects or improve insulin resistance and glycemia in patients with prediabetes or type 2 diabetes [55]. Most of the trials were short in duration (<1 year), which is likely not long enough to detect benefit of vitamin D in a disease such as diabetes which develops over many years.

In large-scale, long-term trials (Women's Health Initiative, RECORD) designed for nondiabetes outcomes, post hoc analyses showed that vitamin D and calcium did not reduce future diabetes risk, but these trials used low doses of vitamin D, and optimal vitamin D concentrations (>30 ng/mL) were not attained [56, 57]. However, more recent trials using larger doses of vitamin D (>2000 IU/day) with confirmed increased concentration of 25-D also did not show significant benefit in the vitamin D groups [55].

It is quite plausible that any vitamin D effect would be significantly discernable only in a population at high risk for diabetes, such as prediabetes. In one study [58], vitamin D benefit was seen in subjects with impaired fasting glucose but not in those with normal glycemia at baseline. Patients who received 700 IU/day of vitamin D and 500 mg/day of calcium for 3 years had a reduction in fasting glucose and lower HOMA-IR. Jorde et al. followed 511 individuals with prediabetes and randomized them to receive 20,000 IU vitamin D weekly versus placebo for 5 years. The vitamin D group showed lower rate of progression to diabetes (40 versus 43%); the difference was not statistically significant, but the study had not been powered to detect such a small difference [59]. There is currently a trial in progress with 2400 patients with prediabetes, randomized to 4000 IU of vitamin D versus placebo, which will hopefully be able to clarify if vitamin D will delay progression from prediabetes to diabetes [60].

In summary, vitamin D deficiency is very prevalent in type 2 diabetes. Causality has not been determined, and the association may be due to shared risk factors and mechanisms, such as obesity and insulin resistance. Vitamin D supplementation has not shown benefit in improving glycemia or insulin resistance in patients with normoglycemia or patients with established diabetes. Vitamin D may delay progression to diabetes in patients with prediabetes, but larger and longer-term studies are needed, and optimal dosing regimen also needs to be determined.

Vitamin D and Diabetes Complications

Long-standing diabetes, especially if not well controlled, leads to macrovascular (cardiovascular disease, stroke) and microvascular (neuropathy, nephropathy, retinopathy) complications. In the FIELD (Fenofibrate Intervention in Event Lowering in Diabetes) trial, which enrolled over 9500 patients with type 2 diabetes followed for 5 years, patients with 25-hydroxyvitamin D concentration less than 20 ng/mL at baseline were more likely to have cardiovascular disease, hypertension, retinopathy, and nephropathy at baseline, independent of treatment of duration of diabetes. The authors also found higher baseline dyslipidemia, microalbuminuria, hemoglobin A1c (A1c), and BMI. Over 5 years of follow-up, low 25-D concentration was an independent predictor of macrovascular events, with patients in the lowest quartile (25-D < 14 n/gmL) demonstrating 21% higher risk compared to those in the highest quartile (25-D > 25 ng/mL). A higher rate of new microvascular complications was seen in patients with 25-D < 20 ng/mL over this period (18% higher risk) though after adjustments for A1c, physical activity, and seasonality, the increased risk was reduced to 11% and was not significant [61]. Zoppini et al. found an inverse relationship between 25-D and severity of retinopathy and nephropathy in a cross-sectional study of 715 patients with type 2 diabetes [62]. In the EURODIAB prospective complications trial which included 532 people with type 1 diabetes followed for 7–9 years, low 25-D2 and 25-D3 concentrations correlated with macroalbuminuria, but not microalbuminuria, retinopathy, or cardiovascular disease [63]. However, in 220 patients with type 1 diabetes followed for 20 years, severe vitamin D deficiency (25-D < 6 ng/mL) predicted higher mortality but did not predict development or progression of nephropathy or retinopathy [64].

By upregulating RAS, vitamin D deficiency contributes to hypertension, which exacerbates nephropathy and retinopathy. Chronic inflammation also contributes to diabetic complications, and the absence of the anti-inflammatory effect of vitamin D (i.e., vitamin D deficiency) may be permissive in the development of complications.

Vitamin D and Diabetic Neuropathy

Diabetic neuropathy is the most common diabetic complication and is often the earliest complication patients experience. From NHANES 2001 to 2004, which included 591 patients with diabetes, vitamin D deficiency was found to be

independently associated with self-reported symptoms of diabetic neuropathy, after adjusting for obesity, duration of diabetes, glucose control, and use of medications for neuropathy [65]. In a meta-analysis including six studies and 1484 patients with type 2 diabetes, vitamin D deficiency was associated with a higher risk of diabetic peripheral neuropathy (OR 2.88) [66].

Nerve growth factor is a neurotrophic growth factor (NGF) involved in growth, maintenance, proliferation, and survival of neurons. NGF is synthesized in a pro-form (pro-NGF) and cleaved to produce its active form. Reduced NGF levels have been reported in both animal models of diabetes and patients with diabetes [67]. In diabetic patients, maturation of pro-NGF is impaired, leading to reduced mature NGF and a higher ratio of pro-NGF/NGF. This imbalance is reported in early retinal inflammation in diabetic retinopathy, as NGF is important for differentiation and survival of neurons in the retina [68]. Vitamin D regulates expression of NGF, as well as other neurotrophic factors, such as neurotrophin-3, neurotrophin-4, and glial-derived neurotrophic factor [69]. In streptozotocin-induced diabetic rats, administration of vitamin D analog prevented NGF depletion in sciatic nerves [70]. In a study including 91 patients with type 1 diabetes, lower 25-D concentrations were seen in patients with neuropathy (along with lower GFR, increased age, and longer duration of diabetes), but no difference in NGF or oxidative stress markers was seen. A positive correlation between 25-D and NGF was reported, but the most important risk factor for neuropathy was determined to be duration of diabetes [71].

Clinical trials using vitamin D for diabetic neuropathy are few and often flawed, due to very small subject number or lack of blinding. In one study, 51 patients with type 2 diabetes and hypovitaminosis D (mean 25-D 18 ng/mL) received a mean dose of 2059 IU of vitamin D for 3 months. Significant improvements in pain scores (48.5% in visual analog scale and 39.4% in McGill pain score) were reported and thought to be higher than expected for maximal placebo effect (30%) [72]. In another study, 148 patients with diabetes (95% type 2, 5% type 1) received 1 dose of 600,000 IU of vitamin D3 intramuscularly. At 20 weeks, 25-D concentration improved from 31 to 46.2 ng/mL (though 58% did have 25-D concentrations below 20 ng/mL), A1c improved from 8.6 to 8.2%, and pain scores improved on three different pain questionnaires [73].

Foot ulcers, which are the precursor to amputation (the end-organ result of neuropathy), are associated with low vitamin D levels. It is not clear if low vitamin D is a secondary effect of having foot ulcers or is contributing to the development of foot ulcers. Tiwari et al. found that diabetic patients with foot ulcers had lower vitamin D concentrations than controls [74]. They also found increased levels of inflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ, and significant negative correlations between 25-D concentration and IL-1B and IL-6 were observed [75]. In one recent RCT, 60 patients were randomized to receive 50,000 IU of vitamin D bi-weekly for 12 weeks or placebo. The vitamin D group demonstrated reduced ulcer length, depth, and width, along with reductions in ESR and high-sensitivity CRP [76]. Presumably, vitamin D's beneficial effects on wound healing involve reduction in inflammation and possibly promoting immune system defenses against infection.

Vitamin D and Diabetic Nephropathy

Because the kidney is one of the main target organs for vitamin D, chronic kidney diseases such as diabetic nephropathy will result in vitamin D deficiency. Impaired hydroxylation of 1α-hydroxylase causes secondary hyperparathyroidism with markedly elevated parathyroid hormone levels and can result in tertiary hyperparathyroidism if left untreated. With nephrotic-range proteinuria, there will be urinary losses of vitamin D-binding protein, which will result in lower concentrations of 25-D. Treatment of diabetic kidney disease includes RAS blockade with either ACE inhibitor or angiotensin receptor blocker (ARB) in patients with albuminuria and vitamin D supplementation to maintain 25-D > 30 ng/nL and active vitamin D (calcitriol or vitamin D analog) to reduce secondary hyperparathyroidism, in stage 3–5 chronic kidney disease [77, 78].

Fernando-Juarez et al. reported that low 25-D levels (25-D < 15 ng/mL) were independently associated with renal disease progression (>50% increase in creatinine or development of end-stage renal disease) in patients optimally blocked with ACE inhibitor, ARB, or combination of both [79]. In patients with type 2 diabetes and albuminuria who were already taking ACE inhibitor or ARB, addition of 2 μg paricalcitol, a vitamin D analog, significantly reduced albuminuria by 18% [80].

In renal biopsy specimens, VDR protein was nearly absent in tubular epithelial cells in patients with type 2 diabetes, compared to normal controls [81]. VDR expression in peripheral blood mononuclear cells was also found to be downregulated in patients with type 2 diabetes, and expression was inversely associated with degree of albuminuria. Furthermore, expression of TNF-α was increased, with the highest levels seen in patients with macroalbuminuria (versus microalbuminuria or normal controls). These findings support the concept that inflammation contributes to diabetic nephropathy and that vitamin D attenuates the progression of kidney disease. Also, the renoprotective effects of vitamin D appear to be synergistic with RAS blockade.

Vitamin D and Diabetic Retinopathy

Diabetic retinopathy is characterized by a nonproliferative phase featuring abnormal vessel permeability and vascular occlusion with ischemia, followed by a proliferative phase featuring neovascularization, with vessels prone to hemorrhage. VDR are expressed in the retina, and calcitriol was shown to inhibit retinal neovascularization in a dose-dependent manner in an animal model of ischemic retinopathy [82].

Not all studies show an association between vitamin D deficiency and diabetic retinopathy [63, 64, 83], but a meta-analysis including 15 observational studies and over 17,000 patients showed that diabetic patients with vitamin D deficiency had significantly increased risk of retinopathy (OR 2.03). Patients with retinopathy also

had significantly lower 25-D concentration [84]. In one study involving patients from the NHANES survey from 1998 to 1994, the percentage of patients with vitamin D deficiency increased with severity of retinopathy (28% with vitamin deficiency in those with no retinopathy, 43.2% in those with moderate to severe retinopathy, and 64.6% in those with proliferative retinopathy), though no statistically significant correlation between the two factors was found [85]. There are no studies of vitamin D for treatment or prevention of diabetic retinopathy.

Conclusion

In conclusion, there is compelling evidence for the role of vitamin D in the development of both type 1 and type 2 diabetes, through different mechanisms. For both diseases, timing and dose appear to be important, suggesting a role in disease prevention, and vitamin D intervention in established disease does not show benefit in improving glycemia or reducing insulin requirements. Vitamin D deficiency is also associated with diabetes complications, with the evidence being strongest for a role of vitamin D in diabetic nephropathy.

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Chapter 8
Vitamin D and Cardiovascular Disease

Kaushal Sondarwa, Rupinder S. Buttar, Victoria Hensley, and Michal L. Melamed

Introduction

Vitamin D's role in bone health and mineral metabolism has been recognized for a long time, but discovery of vitamin D receptors in multiple human tissues, including the heart and blood vessels, generated a surge of research interest and consequently a surge of publications on the vitamin D's relationship with cardiovascular disease [1]. In the early 1980s, Dr. Scragg provided an alternative explanation for the high cardiovascular morbidity and mortality at high latitudes and in the winter months by correlating it with low ultraviolet radiation resulting in low levels of vitamin D [2]. This was followed by a surge of research interest and publications in the coming decades, which now stands at more than 5000 articles listed in PubMed with the search terms vitamin D and cardiovascular disease. Many animal models, observational studies, and clinical interventional randomized, controlled trials have tried to understand complex relationship between vitamin D and cardiovascular health. In this chapter will review animal models, observational studies, and randomized, controlled trials to summarize our current understanding and offer clinically relevant information about vitamin D and cardiovascular health.

K. Sondarwa • R.S. Buttar • V. Hensley
Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

M.L. Melamed, M.D., M.H.S. (✉)
Department of Medicine (Nephrology), Albert Einstein College of Medicine/Montefiore Medical Center, Bronx, NY, USA
e-mail: michal.melamed@einstein.yu.edu

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Mechanistic Studies

Several animal models suggest an association between vitamin D and cardiovascular health. Mice with knockout vitamin D receptor (VDR) or Cyp27b1 (1-alpha-hydroxylase) genes, mice who effectively have low-activated vitamin D levels, have myocardial hypertrophy, hypertension, increased thrombogenicity, and overexpression of renin-angiotensin-aldosterone system (RAAS) [3].

Myocardium

The VDR is present in cardiac myocyte and fibroblast, which in cardiomyocyte-specific VDR knockout mice results in myocardial hypertrophy [3]. In mice models, the VDR regulates the extracellular matrix by altering the expression of the matrix metalloproteinases [4]. Also, the VDR has an effect on cardiac contractility. Mice with 1-alpha-hydroxylase knockout have reduced systolic function, which is reversed by activated vitamin D treatment [5].

Vasculature

Experimental studies on macrophages from diabetic patients suggest a crucial role of the vitamin D receptor signal pathway in the inhibition of foam cell formation and increased cholesterol efflux in macrophages [6]. Vitamin D and its molecular effects on blood vessels have been extensively reviewed [7]. In vascular smooth muscle cells, 1,25-dihydroxyvitamin D induces production of vascular endothelial growth factor (VEGF) and promotes endothelial repair [7]. Other effects on endothelial cells and macrophages include downregulation of tissue factor (F3) and upregulation of thrombomodulin (THBD) and increased endothelial production of nitric oxide [7]. Endothelial cells in VDR knockout mice show significant alteration in vascular function, showing increased sensitivity to circulating angiotensin-2 when compared to controlled mice [8]. Vitamin D also plays a major role in controlling inflammation and the immune response within physiological limits. Major actions include downregulation of pro-inflammatory cytokines (such as IL-1, IL-2, IL-6, IL-23, interferon-γ**,** tumor necrosis factor-α) and upregulation of anti-inflammatory cytokines (IL-4 and IL-10) [6]. Vitamin D acts as an immune modulator by suppression of T_H1 and T_H17 cells and favoring T_{reg} and T_H2 cells [9]. These immune functions may play a role in atherosclerosis as administration of oral calcitriol reduced atherosclerosis by inducing tolerant dendritic cells and by inducing regulatory T cells [10].

Modulation of Vascular Calcification

Effects of vitamin D on vascular calcium balance are complex, as both excess and deficiency of vitamin D result in vascular calcification [7]. VDR activation increases phosphate levels, which in turn activates fibroblast growth factor-23 (FGF-23), a regulator of phosphate excretion by kidney. VDR activation also enhances expression of Klotho, which has a co-receptor function for FGF-23 and is protective against vascular calcification [7]. This suggests complex interplay between vitamin D, FGF23, and klotho-regulating vascular calcification.

Cardiovascular Risk Factors: Mechanistic Studies

Hypertension: Antihypertensive effects of vitamin D are mediated by RAAS. VDR activation blocks cAMP response in the renin gene promoter and blocks transcription of renin gene [11]. Other studies have suggested that, low calcium and high parathyroid hormone (PTH) levels aside, vitamin D deficiency can directly increase vascular resistance and vasoconstriction and contribute to arterial hypertension [12].

Diabetes mellitus: Antidiabetic effects of vitamin D include increased insulin secretion, insulin sensitivity, and reduced β-cell death and dysfunction induced by cytokines [13].

Lipid Metabolism: Effects of vitamin D on lipid metabolism remains to be determined [14]. High-calcium diet-fed VDR null mice when compared to wild-type mice showed less body fat mass, lower plasma cholesterol, and triglyceride levels even when placed on high-fat diet [15]. In another study done by Wang et al. showed VDR null mice on regular diet had increased cholesterol in both male and female mice [16], while HDL cholesterol and apolipoprotein A-I (APOA1) increased only in male VDR null mice [16]. Effects were reversed by changing to a special diet which normalized mineral metabolism suggesting minimal direct role of VDR in cholesterol regulation [16]. Cell culture studies showed that 1,25 dihydroxyvitamin D decreased both APOA1 and its mRNA in a dose-dependent manner, and effects were reversed by a vitamin D antagonist [17].

PTH regulation: VDR activation has been shown to have suppressive effect on PTH levels. High PTH levels have been linked to chronic kidney disease, obesity, and with increased risk for adverse cardiovascular events [18]. As vitamin D and PTH are intertwined, it is hard to separate out effects of high PTH versus low vitamin D.

Other: VDR activation has been implicated in regulation of inflammation by blocking nuclear factor κB (NFκV) activation. Vitamin D activation is also associated with prevention of kidney diseases through protecting podocytes and other proteinuric effects and associated with regulation of coagulation and oxidative stress [19].

Observational Studies

An association between low concentrations of vitamin D and increased cardiovascular risk has been consistently reported across various observational studies. These observational studies fail to provide a causal relationship due to study design.

A large meta-analysis with prospective studies from across Europe and the United States using individual participant data from the CHANCES Cohort (Consortium on Health and Aging: Network of Cohorts in Europe and the United States), as well as the NHANES III (National Health and Nutrition Examination Survey), found that the lowest 25-hydroxyvitamin D (25(OH)D) quintile was associated with increased all-cause mortality (relative risk (RR), 1.57; 95% confidence intervals (CI), 1.36–1.81) including cardiovascular mortality (RR, 1.41; 95% CI, 1.18–1.68) in subjects with no cardiovascular disease at baseline [20]. Researchers analyzed results for differences in population, sex, age, and seasons; the blood was drawn and found that the results were consistent across all parameters [20]. These results were similar to that reported by Wang et al. in a meta-analysis of 65,994 patients with a RR of 1.03 (95% CI, 1.00–1.06) per 25 nmol/L decrement in 25(OH)D levels for CVD [21]. The pooled RR was 1.52 (95% CI, 1.30–1.77) for total CVD and 1.42 (95% CI, 1.27–2.10) for CVD mortality when comparing lowest to the highest 25(OH)D levels [21]. This association remained significant even on exclusion of studies with baseline CVD and controlling for various confounding factors [21].

Another large meta-analysis of 73 studies involving 849,412 participants found inverse associations of circulating 25(OH)D with risks of deaths due to cardiovascular disease [22]. The data showed pooled relative risks of 1.35 (95% CI, 1.13–1.60) for deaths from cardiovascular disease and 1.35 (95% CI, 1.22–1.49) for all-cause mortality when comparing the bottom versus top thirds of baseline 25(OH)D levels [22]. These results have been consistently reproduced in similar systematic reviews of observational studies with an increased risk of cardiovascular mortality and cardiovascular events associated with 25(OH)D deficiency [20–30].

Although the association of low vitamin D levels and cardiovascular mortality is fairly consistent, less consistent data is available in regard to specific underlying diseases and mechanisms. Several authors have reported that low vitamin D levels are associated with a higher risk of coronary artery disease (CAD) and congestive heart failure [31, 32]. However, other studies failed to find a similar association [33, 34].

Despite some contrary studies, there is much evidence of an association between vitamin D deficiency and cardiovascular disease mortality and events. However, a cause and effect relationship cannot be determined without larger randomized clinical trials.

Cardiovascular Risk Factors: Observational Studies

Hypertension: The relationship between vitamin D and blood pressure was initially described in studies that reported increased risk of arterial hypertension with low UVB exposure and increased prevalence of hypertension at higher latitudes and

during the winter season [2, 35–37]. Kunutsor et al. in their meta-analysis of 283,537 individuals reported a 12% reduction in the risk of future hypertension for every 10 ng/mL increment in circulating 25(OH)D levels [35]. The relative risk was 0.70 (95% CI, 0.58–0.86) when comparing the incidence of hypertension in extreme tertiles of baseline 25(OH)D levels [35]. Another recent meta-analysis of 53,375 individuals from seven different studies found significant associations between vitamin D deficiency and incident hypertension (hazard ratio (HR), 1.24; 95% CI, 1.08–1.41) [38]. Although accumulating evidence suggests the association between low vitamin D levels and the risk of hypertension, these studies fail to show a causal relationship and are limited by confounding factors.

Diabetes mellitus: Various observational studies have reported vitamin D deficiency as a risk factor for developing type 1 and 2 diabetes mellitus [13, 39–42]. These include studies using data from NHANES and other large cohorts from the United States and across Europe. A recent meta-analysis by Ye et al. which combined data from 22 longitudinal cohorts reported an increased risk of type 2 diabetes mellitus (RR, 1.21; 95% CI, 1.16–1.27) for 25 nmol/L lower 25(OH)D concentration [43]. Similarly, Song et al. showed an inverse association between 25(OH)D levels and risk of type 2 diabetes mellitus with a 4% lower risk of diabetes (95% CI, 3–6) for each 10 nmol/L increase in 25(OH)D levels [42]. The association between vitamin D and diabetes mellitus is reviewed in Chap. 7 in this publication.

Lipids: The relationship between levels of vitamin D and lipid profile is not well defined. Various cross-sectional studies have linked low 25(OH)D concentrations to elevated LDL cholesterol and triglyceride levels along with low HDL cholesterol levels [14, 44–49]. Ponda et al. also reported an increase in mean total cholesterol (TC) and HDL cholesterol levels when 25(OH)D levels increased from <20 to ≥30 ng/mL over time [44]. Nevertheless, no significant change was noted in LDL cholesterol or triglyceride levels [44]. Similar findings were reported by Faridi et al. in a study of 13,039 Atherosclerosis Risk in Communities (ARIC) participants studied over a period of 5 years with cross-sectional and prospective associations between 25(OH)D deficiency and higher LDL cholesterol and non-HDL cholesterol and triglycerides [48]. There was no prospective association seen for LDL cholesterol [48]. This increased risk for incident dyslipidemia in demographic adjusted models (RR, 1.19; 95% CI, 1.02–1.39) was however attenuated on adjusting for other clinical variables [48].

Mendelian Randomization Studies

Several studies have examined the relationship between single nucleotide polymorphisms (SNPs) in the VDR and other vitamin D-related genes and cardiovascular health, but results have been mixed. In the context of heart failure, low levels of 25(OH)D were independently associated with incident heart failure in white but not in black participants in ARIC and in those genetically predisposed to have high vitamin D-binding protein gene [50]. Genome-wide association studies found four genetic loci [DHCR7 (related to vitamin D synthesis), CYP2R1 (hepatic

25-hydroxylation), DBP (also known as GC; transport), and CYP24A1 (catabolism)] associated with 1–4% variance in 25(OH)D levels [51]. Proteins encoded by these genes are involved in vitamin D production. These SNPs can be used to perform Mendelian randomization studies and evaluate if genetic variations in 25(OH)D levels are associated with CVD risk. These studies might be limited because only <5% of the variation in 25(OH)D levels can be explained by SNPs. Still, Mendelian randomization studies are considered strong causal evidence as genetically determined variations are in general not confounded and indicate a lifelong exposure [52].

Obesity: Recent bidirectional Mendelian analysis of multiple cohorts suggested that high BMI leads to lower 25(OH)D levels, but no association was seen in the opposite direction [53].

Diabetes mellitus Type 2: Studies investigating relationship between type 2 diabetes and low 25(OH)D levels have conflicting results, with one study showing increased risk but others failed to replicate [43, 54].

Lipid profile: The effects of vitamin D on lipid profile have been conflicting, with one Mendelian randomization study showing high 25(OH)D associated with favorable lipid profile [55]. However, another larger study failed to confirm a causal association [56].

Hypertension: Vimaleswaran et al. showed an 8.1% decrease in odds of hypertension with each 10% increase in genetically determined 25(OH)D levels [57]. Another study, which concentrated on vitamin D-binding protein failed to show association with mean arterial blood pressure [58].

Overall, some Mendelian randomization studies failed to show associations between 25(OH)D levels and risk of ischemic heart disease or myocardial infarction and overall mortality [59, 60]. In a large Mendelian randomization study, genetically low 25(OH)D levels were associated with increased all-cause mortality and cancer mortality but not with cardiovascular mortality [61]. The conflicting evidence in Mendelian randomization studies may stem from the low genetically determined variability in 25(OH)D levels, thus requiring a very large sample size to detect associations.

Randomized Clinical Trials

Observational studies have shown association between the vitamin D deficiency and cardiovascular diseases [62–67]. Possible mechanisms leading to cardiovascular disease include endothelial dysfunction, over activity of the RAAS, vascular noncompliance, inflammation, effects relating to PTH, direct effects of calcium flux affecting myocardial contractility, and increased risk of diabetes [26, 62, 68–70]. However, none of these observational studies prove causality, emphasizing the need for randomized controlled trials. There have been several studies studying the effects of vitamin D supplementation alone or along with calcium to study nonskeletal outcomes. Over the past couple of years, a number of meta-analyses have been published to reanalyze the data and provide better evidence.

A meta-analysis of existing randomized controlled trials to estimate the effects of vitamin D supplements with or without calcium on myocardial infarction or ischemia or cerebrovascular disease, cancer, total fractures, fractures, and mortality was assessed by Bolland et al. [71]. Analyzing data from a total nine trials with 48,647 people for myocardial infarction or ischemic heart disease using a risk reduction threshold of 5% for mortality and 15% for other outcomes, no effect of vitamin D supplementation was seen [71]. When individual outcomes were evaluated separately, the results were similar to the pooled data—myocardial infarction (RR, 1.04; 95% CI, 0.91–1.17), ischemic heart disease or cardiovascular disease (RR, 1.12; 95% CI, 0.78–1.62), stroke (RR, 1.00; 95% CI, 0.88–1.13), and stroke (RR, 1.05; 95% CI, 0.82–1.34) [71].

Ford et al. analyzed 21 studies (n = 13,033) in their systemic review that compared vitamin D to placebo or control and found no significant reduction in the risk event for heart failure (HR, 0.82; 95% CI, 0.58–1.1), myocardial infarction (HR, 0.96; 95% CI, 0.83–1.10), and stroke (HR, 1.07; 95% CI, 0.91–1.29) [72]. Cholecalciferol, calcitriol, ergocalciferol, and vitamin D analogs [doxercalciferol, alfacalcidol, 2-methylene-19-nor-(20S)-1α, 25-dihydroxyvitamin D3, or 1α,25-dihydroxy-2β-(3-hydroxypropyloxy) vitamin D3] were the different forms of vitamin D used in the randomized clinical trials in the meta-analysis [72].

Neither beneficial nor harmful effects are reported in various other meta-analyses comparing interventions with vitamin D and analogs on cardiovascular events including myocardial infarction, heart failure, stroke, or mortality [71–77]. However, a recent collaborative European study in a paper analyzing various meta-analyses of randomized clinical trials (RCTs) on non-skeletal outcomes of vitamin D could not rule out the association from numerous observational studies linking low vitamin D levels to CVD due to lack of RCTs specifically designed to study supplementation effects on CV health especially in patients with low 25(OH)D levels [78]. Thus, current evidence from meta-analyses of vitamin D supplementation does not suggest an effect; however, most studies were performed for other outcomes, used disparate doses and vitamin compounds, and did not evaluate participants specifically with low 25(OH)D levels. Thus, results of currently ongoing clinical trials are highly anticipated [79].

Cardiovascular Risk Factors: Randomized Clinical Trials

Hypertension: Numerous cross-sectional and observational studies have shown inverse association between levels of 25(OH)D levels and blood pressure. However, as observational studies are marked by various confounding factors and do not infer causality, several RCTS have been designed to study vitamin D's effect on blood pressure. The results have been inconclusive so far.

A recent meta-analysis by Golzarand et al. of 30 RCTs with 4744 participants found no effect on systolic blood pressure (SBP) (−0.68 mmHg; 95% CI, −2.19–0.84) and diastolic blood pressure (DBP) (−0.57 mmHg; 95% CI, −1.36–0.22) [80].

However, a subgroup analysis showed significant reduction in both SBP and DBP when a daily dose of >800 IU/day of vitamin D3 was given to subjects ≥50 years for <6 months [80]. Vitamin D at dose ≥800 IU/day increases serum 25(OH)D levels more effectively than doses <800 IU/day. Similar results were also reported by Wu et al. who showed significant reduction in SBP with vitamin D supplementation when compared to calcium and placebo (weighted mean difference (WMD), −2.44; 95% CI, −4.86 and −0.02) [81]. No significant effect on DBP was reported (WMD, −0.02; 95% CI, −4.04 and 4.01) [81]. But this study was limited by small number of trials as they included only 4 RCTs involving 429 individuals of oral vitamin D supplementation in normotensive or hypertensive individuals [81].

A small, statistically significant reduction in DBP (−3.1 mmHg; 95% CI, −5.5 to −0.6) was reported in another meta-analysis [82]. Although a nonsignificant reduction in SBP in vitamin D group was noted when compared to placebo (−3.6 mm Hg; 95% CI, −8.0 to 0.7), subgroup analysis suggested a greater drop in SBP when inactivated form of vitamin D was used rather than activated vitamin D (−6.2 mmHg; 95% CI, −12.32 to −0.04 versus +0.7 mmHg; 95% CI, −4.8 to 6.2) [82]. This was similar to the results reported by Lee et al. in a meta-analysis of 15 RCTs for patients with type 2 diabetes mellitus receiving vitamin D supplementation with a combined effect estimate of −0.16 mmHg (95% CI, −0.30 to −0.02; $p = 0.02$) [83]. A significant reduction in DBP (−1.31; 95% CI, −2.28 to 0.34 mmHg) was reported by Kunutsor et al. in a meta-analysis of 16 trials although the effect was limited to participants with pre-existing cardio-metabolic disease [84]. A nonsignificant reduction in SBP (−0.94; 95% CI, −2.98 to 1.10 mmHg) and DBP (−0.52; 95% CI, −1.18 to 0.14) with heterogeneity and publication bias was noted in pooled meta-analysis [84].

No beneficial effects were reported by Beveridge et al., Pittas et al., and Elamin et al. in their meta-analyses [73, 85, 86]. On the contrary, Manousopoulou et al. reported a significant increase in SBP after vitamin D supplementation (fixed-effects model, standardized mean difference, 0.24; 0.11–0.37; random-effects model, standardized mean difference, 0.24; 0.09–0.39; $I^2 = 23.0$) [87]. Golzarand et al. had also reported an increase in SBP and DBP in subgroup analysis of their meta-analysis, although it was only seen in patients with vitamin D in combination with calcium supplementation and vitamin D supplementation alone in overweight and obese subjects [80]. Thus, the evidence currently is mixed about vitamin D blood pressure-lowering effects.

Diabetes mellitus: An association between low 25(OH)D and impaired glucose tolerance, hemoglobin A1c levels, and diabetes status has been shown in various observational studies. Various RCTs and meta-analyses have been designed to evaluate causality.

A recent meta-analysis by George et al. showed a significant improvement in fasting glucose levels (−0.32 mmol/L; 95% CI, −0.57 to −0.07) and a small improvement in insulin resistance (standard mean difference, −0.25; 95% CI, −0.48 to −0.03) [88]. However, the study was restricted to patients with diabetes or impaired glucose tolerance [88]. In another meta-analysis including 12 RCTs and 1181 overweight

and obese individuals, no effect on glucose concentration, insulin levels, and HOMA-IR values was seen [89]. A subgroup analysis taking supplement dose, time of supplementation, and baseline of 25(OH)D concentration also did not show an effect [89]. Similar results were also reported by another meta-analyses by Haroon et al. with no significant effect on hemoglobin A1c, beta-cell function, and insulin resistance in long-term studies (follow-up >3 months) [90]. However, most of these trials were small, and not all the patients included reported vitamin D deficiency [90].

Conclusion

In this review, we have described animal and human observational studies and randomized clinical trials of vitamin D and cardiovascular disease. Animal and tissue culture studies suggest that vitamin D deficiency may cause cardiovascular disease including its precursors of hypertension and diabetes mellitus. Interestingly, very high vitamin D levels are also associated with vascular calcification in animal models. Thus, there may be a target level that is not too high or too low. Observational studies fairly consistently show an association between low vitamin D levels and cardiovascular disease. However, randomized clinical trials have not conclusively shown a decrease in cardiovascular disease with vitamin D supplementation. Randomized trials have used different doses and formulations of vitamin D, different length of treatment, and different 25(OH)D level entry criteria and often had endpoints that were not primarily cardiovascular disease. Thus, there continues to be a need for well-designed, large randomized clinical trials evaluating the effects of an adequate dose of vitamin D on cardiovascular disease.

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Chapter 9
Vitamin D and Obesity

Dimiter Avtanski, Anabel Garcia, and Emilia Pauline Liao

Obesity: Definition and Epidemiology

Obesity is primarily quantified by using body mass index (BMI) criteria. Individual BMI is calculated as the ratio between the body weight (in kilograms) and the square of body height (in meters). The World Health Organization (WHO) and the National Institutes of Health (NIH) [1] give the following classification based on BMI (note that this classification excludes pregnant women): underweight (BMI < 18.5 kg/m^2), normal weight (BMI between 18.5 and 24.9 kg/m^2), overweight (BMI between 25.0 and 29.9 kg/m^2), class I obesity (BMI between 30.0 and 34.9 kg/m^2), class II obesity (BMI between 35.0 and 39.9 kg/m^2), and class III or extreme obesity (BMI $\geq$ 40 kg/m^2) [2, 3]. It is important to note that the BMI classification should be accepted cautiously, since BMI normally varies among the different ethnicities, populations, individual characteristics, physical activity, height, sex, reproductive status (puberty, pregnancy, menopause), and age. In 2004, a WHO Expert Consultation [4] noted that BMI values should be calculated based on ethnicity-specific equations giving cutoff points for overweight and obesity in different Asian populations. Another inaccuracy of using BMI definition is that fit or

D. Avtanski, PhD (✉)
Endocrine Research Laboratory, Department of Medicine, Lenox Hill Hospital, New York, NY, USA
e-mail: davtanski@northwell.edu

A. Garcia, MD
Division of Endocrinology, Lenox Hill Hospital, New York, NY, USA

E. P. Liao, MD
Division of Endocrinology, Department of Medicine, Hofstra Northwell School of Medicine, Lenox Hill Hospital, Northwell Health, New York, NY, USA
e-mail: eliao@northwell.edu

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very short people (under 5 ft) can often be considered as overweight, because of their higher percentage of heavier muscle mass. Thus, the BMI is a good indicator for evaluating obesity in large population cohorts, but not always an accurate indicator for individuals.

Another criterion for distinguishing between normal and abnormal body weight is based on the waist circumference [5]. The 1997 WHO Expert Consultation on Obesity recognized that abdominal fat can vary significantly within a normal BMI range [2, 6]. Waist circumference is a useful indicator for individuals who, on the basis of the BMI criteria, are considered normal weight or overweight. Compared to BMI, waist circumference has little added predictive value and loses its incremental predictive power in individuals with BMI > 35 kg/m^2. Similarly to BMI, waist circumference cannot be applied to short people (under 5 ft) [5]. One advantage of using waist circumference in addition to BMI is that it can give some additional estimate on the fat distribution throughout the body, as abdominal fat obesity is an independent predictive factor for the development of cardiovascular diseases. Generally, substantially increased disease risk is conferred when waist circumference is >102 cm in men and >88 cm in women, but again, risk also depends on individual characteristics and ethnicity [7–9]. The International Diabetes Federation (IDF) recommends obesity definitions for waist circumferences of >94 cm for Caucasian males and >80 cm for Caucasian women and >90 cm for men and >80 cm for women of South Asian, Chinese, or Japanese origin [10].

An additional criterion for evaluating the state of overweight or obesity is the waist-to-hip ratio (WHR). WHO set up cutoff points for WHR >0.90 for males and >0.85 for females for substantially increased risk of developing metabolic complications [7]. It was estimated that WHR is a better indicator for developing obesity-related complications [11], especially in Asian populations [12].

The reason BMI criterion is commonly used is because of its simplicity. There are a number of other more precise methods to evaluate the amount of body fat, which are based on measuring the total amount of water or potassium, bioelectrical impedance, or dual-energy X-ray absorptiometry (DXA). Disadvantages of these methods include higher cost as well as the need for special equipment which does not exist in all clinics. Although WHO does not set thresholds for defining obesity on the basis of body fat percentage, it states that in Caucasians, overweight status (BMI ≥ 25 kg/m^2) corresponds to body fat percentage of 22% (18–27%) in men and 35% (31–39%) in women [4]. One recent cross-sectional, population-based study [13] sought to identify sex- and age-specific values for body fat percentage based on BMI values of <18.5 kg/m^2 (underweight), 25.0–29.9 kg/m^2 (overweight), and >30.0 kg/m^2 (obesity) by using DXA in 2491 individuals (20–96 years old). The results from the study showed greater prevalence of obesity for men evaluated by the percentage of body fat compared to the BMI criteria as well as lower prevalence of underweight individuals from both sexes evaluated by BMI. The authors concluded that BMI criteria may underestimate obesity levels, especially in white males, and the optimal thresholds for underweight status and obesity will depend on the risk assessment for impaired health and early mortality.

Obesity has become a worldwide epidemic. Since 1960, in the United States, the number of people categorized as overweight or obese has risen dramatically, reaching 54.9% of today's adult population over 20 years old. From 1960 to 1994, the rate of obesity doubled, from 12.8 to 22.5% [14]. In the short period of time between 1980 and 2006, the prevalence of obesity among US Americans increased more than twofold, from 15 to 34% [15, 16]. Today in the United States, 19.5% of the men and 25.0% of the women are considered obese [14].

The main factor for the worldwide epidemic of obesity is the excessive energy uptake and the decreased energy expenditure related to the modern lifestyle [17–19]. Genetic causes, such as mutations of leptin (LEP), leptin receptor (ObR), melanocortin-4 receptor (MC4R), or pro-opiomelanocortin (POMC) genes, are very rare and play insignificant roles in the global sense.

Excessive body weight is a serious factor contributing to higher mortality rates. Overweight individuals, 50–71 years old, have 20–40% higher risk of death, and obese patients have two- to three-fold increased risk of death compared to age-matched lean individuals [20, 21]. Epidemiological studies found that the mortality rate starts to increase significantly when BMI exceeds 25 kg/m^2 and is especially prominent for individuals whose BMI exceeds 30 kg/m^2 [22, 23].

There is a strong correlation between the level of obesity and the risk of cardiovascular diseases (CVD), atherosclerosis, cancer, and type 2 diabetes (T2DM) [24]. Excessive white adipose tissue (WAT) contributes to insulin resistance and consequently produces hyperinsulinemia and elevated IGF-I levels [25]. It was estimated that each additional unit of BMI over 22 kg/m^2 increases the relative risk of developing T2DM by approximately 25% [26]. In this regard, a particular independent risk factor for developing T2DM is abdominal obesity [27]. T2DM is caused by reduced insulin sensitivity in patients with inadequate β-cell reserve. Insulin resistance develops in adipose tissue, muscle, and liver and is usually linked to lipid redistribution and obesity [28]. As a compensatory mechanism to insulin resistance, insulin production by the pancreatic β-cells is initially increased, which is the reason for the higher serum insulin levels observed in T2DM patients, early in the disease.

Adipose cells (adipocytes) play an important role in the pathophysiology of T2DM. One characteristic feature of obesity is disturbed preadipocyte to adipocyte maturation, which leads to chronic inflammation, with increased production of proinflammatory cytokines [29] (Fig. 9.1). Adipocytes and the surrounding macrophages release proinflammatory cytokines such as TNFα, monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, IL-1β, and macrophage migration inhibitory factor (MMIF) [29–31]. Increased release of TNFα and MCP-1 promotes the recruitment and maturation of M1 macrophages to the inflammatory site. These macrophages, in turn, produce additional TNFα, IL-1β, and resistin, thus creating a positive feedback loop that further amplifies the inflammatory process and increases insulin resistance [29, 32]. Animal and human observations show that the number of macrophages in WAT and the expression levels of TNFα in human adipose tissue correlate with the percentage of body fat and are considerably higher in obese individuals [33, 34]. Conversely, weight loss is associated with reduced TNFα levels [35].

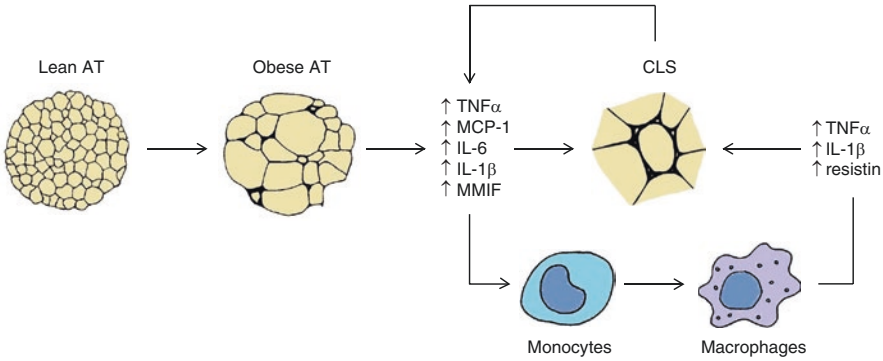

Fig. 9.1 Obesity is an inflammatory disease. Excessive energy intake results in adipocyte hypertrophy (an increase in cellular size), adipose tissue hyperplasia (an increase in adipose mass), and disturbances in vitamin D metabolism. Obesity alters adipocyte maturation and adipose tissue secretion of inflammatory cytokines such as tumor necrosis factor-α (TNFα), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 and interleukin-1β (IL-6, IL-1β), and macrophage migration inhibitory factor (MMIF). This microenvironment attracts more monocytes to the adipose tissue and increases their differentiation into macrophages, which further leads to elevated proinflammatory cytokine production (TNFα, IL-1β, and resistin), formation of crown-like structures (CLS), and chronic inflammation

Chronic inflammation in obesity plays a major role in the development of insulin resistance, hyperinsulinemia, and T2DM [36]. Inflammatory factors released from adipose tissue macrophages activate nuclear factor κ-light chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinase (JNK) inflammatory signaling pathways. Obesity also acts as a chronic stimulus for endoplasmic reticulum (ER) stress (a pathological condition in which unfolded or misfolded proteins are accumulated in the ER) in the peripheral tissues [37–39]. JNK signaling pathway is linked to the ER stress response, which in a chronically active state triggers insulin resistance and T2DM [40]. As a result of the obesity-induced ER stress, insulin receptor substrate (IRS)-1 is phosphorylated resulting in inhibition of insulin receptor signaling [40].

Adipose tissue is an important endocrine organ, producing a variety of chemical substances, namely, (1) cytokines and cytokine-related proteins (as mentioned above), (2) immune-related proteins (e.g., CCL2), (3) fibrinolytic proteins (plasminogen activator inhibitor [PAI]-1), (4) complement and complement-related proteins (adiponectin, complement factor D, CFB, acylation-stimulating protein), (5) lipids and proteins for lipid metabolism or transport (lipoprotein lipase [LPL], cholesterol ester transfer protein [CETP], apolipoprotein E [ApoE], nonessential fatty acids), (6) enzymes involved in steroid metabolism (e.g. cytochrome P450 [CYP]-dependent aromatase, 17β-hydroxysteroid dehydrogenase, 11β-hydroxysteroid dehydrogenase type 1), (7) RAS proteins (angiotensinogen), and (8) variety of other proteins such as resistin [41].

Generally, there are two main types of adipose tissue: WAT and brown adipose tissue (BAT). WAT and BAT are distributed in different anatomical fat deposits (e.g., abdominal, subcutaneous, pericardial) each one serving a different

endocrine role. Additionally, a small amount of brown-like fat cells (the so-called "beige" fat cells) is also present in WAT; beige cells resemble the classical brown adipocytes but possess different characteristics. Recent discoveries started to uncover their eminent role in the conversion of WAT to BAT during physical exercise or cold exposure. Adipose tissue is also present in the bone marrow (bone marrow adipose tissue [MAT]), around the blood vessels (perivascular adipose tissue), and other locations, and each adipose tissue depot possesses different endocrine characteristics.

A distinct characteristic of WAT adipocytes is the single large lipid droplet (for which they are also referred as unilocular cells), unlike BAT adipocytes, which contain multiple lipid droplets (multilocular cells). While WAT is characterized by the expression of genes such as leptin and homeobox protein Hox-C (HOXC) 8 and 9 [42], BAT adipocytes characteristically express uncoupling protein 1 (UCP1), which plays a role in thermogenesis and regulation of energy expenditure.

Mature adipocytes are not capable of proliferating, and they originate from a progenitor self-renewal adipocyte precursor cells (commonly known as preadipocytes) which derive from mesenchymal stem cells (MSCs). Adipogenesis (the formation of mature adipocytes) is a two-step process: (1) preadipocyte generation and (2) differentiation into mature adipocytes [43]. Adipocyte differentiation requires involvement of C/EBP (CCAAT enhancer-binding protein) transcription factor and peroxisome proliferator-activated receptor gamma (PPARγ) [44] and activation of Wnt and Hedgehog signaling pathways [45, 46].

In humans, obesity affects most prominently the abdominal (omental) WAT. There are gender- and age-related differences in the percentage of WAT: the mean normal percentage values for WAT for all ages are 16% for men and 20% for women. Obesity is characterized by WAT adipocyte hypertrophy (an increase in adipocyte size) and hyperplasia (an increase in cell number) [47], and obesity affects WAT adipocyte differentiation, hormonal production, and inflammation. Adipocyte differentiation affects adipose tissue hormonal expression. For example, both preadipocytes and adipocytes produce leptin, but adiponectin is produced mainly by mature adipocytes. Thus, keeping a tight control on adipocyte differentiation is important in maintaining equilibrium in adipose tissue hormonal production. In obese states, after hypertrophy and hyperplasia develop, the excessive WAT mass negatively affects the rate of adipocyte differentiation and thus disturbs the hormone balance favoring the production of leptin. Obesity is also accompanied by recruitment and infiltration of adipose tissue by immune T cells and monocytes [48] which results in increased expression of proinflammatory cytokines such as TNFα, IL-6, and CCL2 and CCL3 [49]. Elevated levels of proinflammatory cytokines, such as TNFα, IL-6, and inducible nitric oxide synthase (iNOS), have direct suppressive effect on insulin signaling pathways [50–52]. Additionally, TNFα induces lipolysis through activation of extracellular signal-regulated kinase (ERK) and JNK signaling pathways [53, 54].

Other adipose tissues also respond to obesity. For example, BAT adipocytes change their mitochondrial function, oxidation, and energy metabolism. In the bone, obesity shifts the differentiation of the MSC (which are precursors of both,

adipocytes and osteoblasts) toward adipocyte lineage. This is confirmed in studies where a negative correlation between relative adipose mass and bone mineral density was observed [55].

Vitamin D and Adipose Tissue

Adipose tissue is the main storage depot for vitamin D (vitamin D_2 and D_3) and serves as an endogenous source of vitamin D during the winter season [56, 57]. Adipose tissue expresses the P450 enzymes 25-hydroxylases (CYP2R1, CYP2J2, CYP27A1, CYP3A4) and 1α-hydroxylase (CYP27B1) [58–60]. Adiposity affects the expression of key enzymes regulating vitamin D synthesis. Obesity in humans is associated with suppressed expression of adipose 25-hydroxylase CYP2J2 and 1α-hydroxylase, but weight loss intervention increases 24-hydroxylase (CYP24A1) levels [60]. Similarly, a mouse study showed lower hepatic mRNA levels of 25-hydroxylases (CYP2R1, CYP27A1, and CYP2J3), higher renal 1α-hydroxylase, and lower renal 24-hydroxylase in diet-induced obesity [61]. Foss [62] hypothesized a hypothalamic involvement and activation of AgRP/NPY and POMC/CART neuronal activation in the metabolic actions of vitamin D. Although it is known that both WAT and BAT adipocytes express VDR [63–65], the function of vitamin D in adipose tissue is still not fully understood. There are contradicting reports demonstrating both inhibition and stimulation of adipogenesis by vitamin D, which could be related to differences in the fat storage among species as well as the stage of adipocyte development [66]. VDR is expressed early in adipocyte development, and it is known to play a role in adipocyte function. In the early stage of adipogenesis, unliganded VDR recruits the nuclear corepressors, NCoR (nuclear receptor corepressor 1), and SMRT (silencing mediator for retinoid and thyroid hormone receptors), thus transcriptionally repressing histone deacetylation [67] and promoting adipogenesis (Fig. 9.2). When bound to a ligand, VDR suppresses C/EBPβ and PPARγ expression resulting in inhibition of adipogenesis [68]. In 3T3-L1 mouse undifferentiated adipocytes overexpressing VDR, the expression of PPARγ is inhibited, and adipocyte differentiation is suppressed [69]. Mouse embryonic PPARγ null stem cells fail to differentiate into adipocytes, instead differentiating to osteoblasts [70]. In the bone, bone marrow stromal cells (BMSCs) are common precursors of osteoblasts and adipocytes, and VDR modulates their early differentiation steps [71, 72]. BMSCs lacking VDR show increased adipogenic potential associated with upregulated expression of dickkopf-related protein 1 (DKK1), a result from activation of the canonical Wnt signaling pathway [73]. CYP27B1 knockout mice which lack 1α-hydroxylase show low leptin levels, hyperphagia, and lean phenotype [74]. Similarly, VDR-knockout (VDRKO) mice demonstrate hypoleptinemia, resistance to high-fat diet-induced obesity, and increase in the expression of UCP1 [74].

By affecting adipocyte maturation and differentiation, vitamin D also affects adipokine secretion and inflammatory responses, but these effects are influenced by a variety of other factors, and the sole role of vitamin D is hard to distinguish.

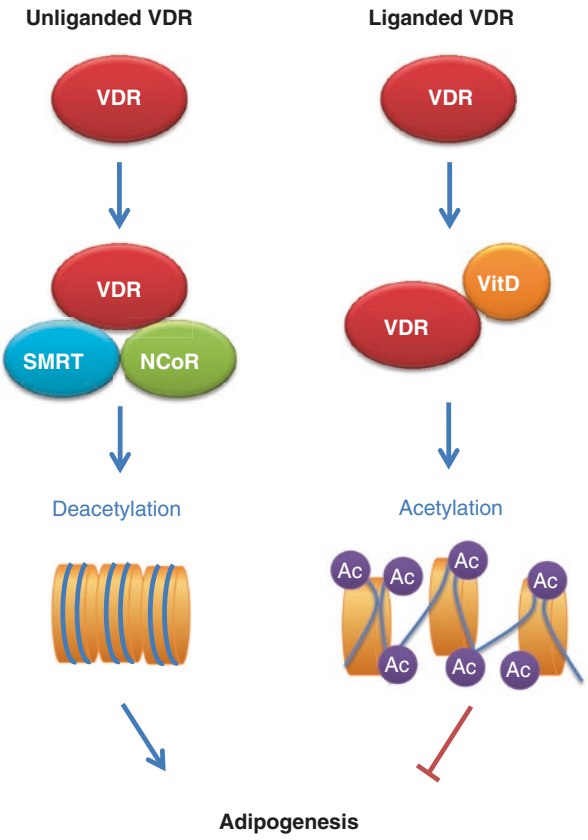

Fig. 9.2 Vitamin D function in adipose tissue. Vitamin D acts on adipocytes through vitamin D receptor (VDR)-dependent and VDR-independent mechanisms. In the early stages of adipocyte development, the unliganded VDR recruits NCoR (nuclear corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) leading to histone deacetylation, but in the mature adipocytes, VDR binds to vitamin D which stimulates histone acetylation, negatively inhibiting vitamin D target gene transcription. Thus, through different mechanisms, vitamin D plays regulatory role on adipogenesis

For example, Lee et al. [75] and Tulk et al. [76] reported proinflammatory actions of vitamin D, while most other studies [77–81] found that vitamin D downregulates the expression of proinflammatory cytokines including TNFα, IL-1, IL-6, IL-8, MCP-1, and PAI-1 [77–81]. A systematic review by Calton et al. [82] aiming to assess the inflammatory actions of vitamin D concluded that calcitriol ($1,25(OH)_2$ vitamin D) suppresses the inflammatory status of cellular models. This includes inhibition of NF-kB signaling [78, 79], which is chronically active in many inflammatory diseases. The mechanism of vitamin D effect on NF-kB involves suppression of toll-like receptors (TLR) expression [82], stabilization of IκBα, and suppression of p65 translocation to the nucleus [78].

In addition, vitamin D displays an inhibitory effect on mitogen-activated protein kinase (MAPK) signaling pathway in human adipocytes, reduces expression of proinflammatory cytokines (IL-6, IL-8, MCP-1, RANTES), and suppresses monocyte migration [83]. Diet-induced obese rats given vitamin D demonstrated reduced inflammatory markers and improved markers of oxidative stress compared to rats who were not given vitamin D [84]. Vitamin D reduces inflammatory signals and

attenuates MCP-1 and adiponectin production in human adipocytes [80]. In other studies, vitamin D increased adiponectin, though calcitriol administration had no effect on adiponectin in human adipocyte cultures [66].

Today, the role of microRNAs as posttranscriptional regulators of the gene function is highly appreciated. Karkeni et al. [85] showed that the expression of microRNAs miR-146a, miR-150, and miR-155 is induced in TNFα-mediated inflammation and that vitamin D downregulates the levels of these three microRNAs. It is worth mentioning that miR-146 was previously found to be induced by NF-κB [86], and deletion of miR-155 in mice prevented diet-induced obesity, concomitantly increasing UCP1 and adiponectin gene expression [87]. Vitamin D was also found to modulate the levels of miR-126-3p, miR-154-5p, and miR-21-5p microRNAs dependently of VDR [88]. Further investigations revealed that VDR activation reduces miR-432-5p, miR-495-3p, and miR-576-5p expression [89]. At the same time, recent study in colon cancer cells under proinflammatory cytokine treatment in vitro demonstrated that VDR is negatively targeted by miR-346 [90].

In addition to adipocytes, adipose tissue macrophages are known to play a role in vitamin D metabolism [65]. Obesity leads to attraction of monocytes and macrophages to adipose tissue and an increase in proinflammatory cytokine production, causing chronic inflammation. Vitamin D deficiency is also associated with increased inflammatory mediators, thus possibly contributing to the inflammatory process. But how these processes affect vitamin D metabolism is still largely unknown.

Obesity and Vitamin D Deficiency

Adipose tissue has a primary importance in regulating systemic energy levels and body temperature, and in this regard, seasonal variations in body weight and body fat mass percentage represent a normal physiological adaptation to the environmental changes. Winter season and lower temperatures provoke an increase in body fat mass, which decreases during the summer season. In an interesting experiment [91], five healthy men (with average age of 21 years) were subjected to warm and cold overnight ambient temperatures in a temperature-controlled research facility for 4 months. During cold acclimation (19 °C), WAT metabolic activity increased by 10%, and BAT volume increased by 42%. In addition, appetite was increased, and satiety was reduced. Adiponectin increased, while leptin decreased. These detected changes were reversed in the warmer months.

Multiple factors are known to play a role for the circannual changes of the adipose tissue, but among them vitamin D is proposed to be a vital one. Vitamin D serves as an environmental sensor detecting the UVB spectrum of the light and then translates environmental changes into metabolic signals. Shorter light duration accompanying the lower temperatures in the winter season is associated with lower circulating vitamin D levels, elevated WAT triglycerides accumulation [62], elevated circulating glucose and triacylglycerols levels, insulin resistance, increase in

the arterial blood pressure, and reduction in the circulating high-density lipoprotein (HDL) cholesterol levels, which are all characteristics of the metabolic syndrome. In this regard, some researchers view obesity as an excessive physiologic response of adapting to lower temperatures [62].

Today, vitamin D insufficiency is considered to affect more than half of the world's population [92, 93]. Evidence suggests that vitamin D metabolism is associated with adiposity and that common obesity increases the risk of vitamin D deficiency, but the mechanisms behind this relationship are still not completely established [94]. In fact, the rise of vitamin D insufficiency coincides with the increase of common obesity worldwide. Multiple clinical trials consistently show a relationship between low vitamin D levels and obesity, with the degree/severity of the vitamin D deficiency correlating with degree of obesity.

Obese individuals who lose weight by diet or surgery show an increase in vitamin D concentrations. A review of 23 studies including over 2000 subjects (mostly female) showed a significant increase in 25-hydroxyvitamin D concentrations (25(OH)D) with reduction of body weight and BMI in the majority of cases [95]. A meta-regression analysis for 17 of these studies demonstrated an increase of 6 nmol/L (2.4 ng/mL) of (25(OH)D) with an average 10 kg weight loss. Similarly, Rock et al. [96] found that women who lost more than 10% of their baseline weight had an increase of 25(OH)D by 5 ng/mL, while women who lost between 5 and 10% of baseline weight increased 25(OH)D by 2.7 ng/mL. In a study of over 400 obese postmenopausal women, Mason et al. [97] also found a proportional increasing 25(OH)D with weight loss of 5–15% of baseline weight.

In a review including 1536 patients achieving weight loss through surgery, mostly Roux-en-Y gastric bypass (RYGB), the authors found a temporary increase of 25(OH)D after surgery; however, vitamin D deficiency was common in long-term follow-up (1–10 years). In one study comparing weight loss from RYGB and duodenal switch (which causes a higher degree of malabsorption), patients undergoing duodenal switch demonstrated a decrease in 25(OH)D, while patients undergoing RYGB showed an increase in 25(OH)D [98]. Patients who lost weight after laparoscopic banding surgery (where malabsorption is not induced) showed stabilization or a decrease in vitamin D levels [99, 100].

Vitamin D status in obese patients who undergo bariatric surgery is more difficult to assess because surgical procedures that cause malabsorption (such as RYBG) also cause vitamin D deficiency. In these patients, the weight loss effect on increasing vitamin D is usually not enough to overcome the intended malabsorption that also causes vitamin D deficiency. Studies have shown that patients who have vitamin D deficiency after bariatric surgery typically require much higher doses of vitamin D for replacement [101].

While obesity contributes to vitamin D deficiency, it is tempting to believe that vitamin D administration will reverse or ameliorate obesity. In a randomized double-blind clinical trial [102] involving 445 healthy, overweight, and obese individuals (both men and women) aged 21–70 years old, a significant inverse correlation between BMI and 25(OH)D levels was found. But 1-year administration of cholecalciferol

(40,000 IU per week) did not result in a change in BMI. Another double-blind study [103] involving 200 healthy overweight subjects receiving cholecalciferol (3332 IU/day) or placebo for 12 months also did not find any significant effect on weight loss. In a different double-blind placebo-controlled study of 77 overweight or obese women [104], cholecalciferol supplementation (25 μg/day) for 12 weeks resulted in significant reduction of body fat mass but no significant change in weight or waist circumference. These results show that although vitamin D deficiency correlates with the level of obesity, improving vitamin D status does not seem to lead to weight loss. In a bi-directional Mendelian randomization analysis [94] comprising data from 21 adult cohorts involving up to 42,024 participants, higher BMI was found to lead to lower vitamin D levels consistently among various age and gender groups, with a 10% increase in BMI resulting in 4.2% reduction in 25(OH)D. However, the effects of vitamin D deficiency increasing BMI were likely small, and thus increasing 25(OH)D is unlikely to reduce weight.

In an interesting study, 19 obese and 19 normal weight subjects were given either 50,000 IU ergocalciferol (vitamin D_2) orally or UVB irradiation (290–320 nm) to increase 25(OH)D. In the irradiation group, the baseline 25(OH)D levels did not differ between obese and nonobese individuals but were significantly increased after UVB irradiation. Obese subjects showed an attenuated response, achieving 57% lower postirradiation 25(OH)D than nonobese subjects. In the oral administration group, baseline 25(OH)D were lower in obese subjects. After administration of 50,000 IU ergocalciferol, both, obese and nonobese individuals, showed significant increases, but the peak vitamin ergocalciferol concentrations did not differ between them. The authors found a significant correlation between BMI and peak ergocalciferol concentration after oral vitamin D and between BMI and vitamin D_3 concentrations after UVB irradiation. The study indicates that oral vitamin D is more bioavailable than cutaneously produced vitamin D, despite obese persons theoretically having larger surface area for cutaneous production [105].

Different hypotheses are proposed to explain the relationship between adiposity and vitamin D deficiency. Aside from direct biochemical connections between signaling pathways involved in vitamin D and adiposity, there are a variety of other factors that play additional roles. Increased urbanization following industrial revolution globally promoted obesity incidence and led to decreased sun exposure and vitamin D deficiency. Furthermore, obese individuals experience social stigma and are less likely to expose their skin to the sun. Vitamin D deficiency is also suggested to be influenced by other factors such as reduced activation accompanied with increased catabolism, sequestration of vitamin D in the adipose tissue, and volumetric dilution based on body weight. Release of vitamin D from adipocytes (the mechanism of which is not known) may also contribute. One study involving 44 normal weight and 67 obese male individuals given vitamin D supplementation for 1 year demonstrated reduced catecholamine-induced release of vitamin D and altered activity of vitamin D-metabolizing enzymes in adipocytes from the obese group [106].

Biopsies of subcutaneous and omental fat from 21 obese (mean BMI = 44) and 15 control (BMI = 23) women showed that vitamin D concentrations in both fat

specimens were significantly correlated with serum 25(OH)D in obese and control women. Interestingly, the 25(OH)D in obese and control women were similar at baseline, which could be due to most (71%) of the obese women taking vitamin D supplements compared to only two control women taking vitamin D supplements. Total body vitamin D stores were significantly higher in the obese women compared to control women, suggesting that lower 25(OH)D is due partly to volumetric dilution and that a large amount of adipose tissue (as seen in obese persons) becomes a sink for vitamin D [107].

Conclusions

Incidences of both obesity and vitamin D deficiency have been increasing dramatically in the last few decades, and obesity and vitamin D are interrelated. Though the underlying mechanisms of this association are not known, it seems obesity contributes to vitamin D deficiency, as reducing adiposity increases vitamin D levels. There is less evidence for the converse relationship (that vitamin D deficiency contributes to obesity), though vitamin D regulates adipocyte differentiation and impacts its secretory functions. Vitamin D also has anti-inflammatory effects that may attenuate inflammation associated with obesity. While weight loss reverses some of the vitamin D deficiency related to obesity, vitamin D supplementation does not facilitate weight loss. More studies are needed to elucidate the nature of the relationship between vitamin D and adipose tissue.

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Chapter 10
Vitamin D in Male and Female Reproduction

Anindita Nandi

Vitamin D Physiology and Metabolism

Vitamin D is an essential steroid hormone that is predominantly generated in the epidermis from its precursor 7-dehydrocholesterol to previtamin D (cholecalciferol), after exposure to ultraviolet B radiation [1, 2]. Vitamin D can also be obtained from food, such as dairy products and egg yolk. Cholecalciferol binds to vitamin D-binding protein (VDBP) and transports it to the liver where it is hydroxylated by the mitochondrial enzyme CYP27A1 or the microsomal enzyme CYP2R1 to 25-hydroxyvitamin D. In the proximal tubule of the kidney, further hydroxylation by CYP27B1 leads to conversion to the physiologically active form 1,25(OH)2D3.

Cholecalciferol (vitamin D3) is absorbed through the gastrointestinal tract into the lymphatics. It ultimately enters the circulation, primarily bound to vitamin D-binding protein (VDBP). Eighty-eight percent of vitamin D is bound to VDBP, 0.03% is in free circulation, and the rest is bound to albumin. Mice with VDBP deficiency have an increased rate of vitamin D toxicity [1]. VDBP acts as a reservoir for vitamin D and helps to transport it through circulation. In target tissues, the mitochondrial enzyme CYP24A1 can inactivate the vitamin D with further hydroxylation steps. Formation of active vitamin D is controlled by circulating factors such as parathyroid hormone (PTH), calcium, 1,25(OH)2D3, and phosphaturic fibroblast growth factor 23 (FGF23) [1, 3–6] (Fig. 10.1).

Within target cells, vitamin D is released from VDBP and binds to vitamin D receptors (VDR) present in the cytoplasm. This complex is transported into the nucleus where it forms a heterodimer with retinoid X receptor. Further conformational changes and binding to co-activators results in a transcriptional regulatory complex that can bind to the vitamin D response element (VDRE) in the promoter

A. Nandi, MD
Icahn School of Medicine, Graduate School of Biomedical Sciences, New York, NY, USA
e-mail: anindita.nandi@mssm.edu

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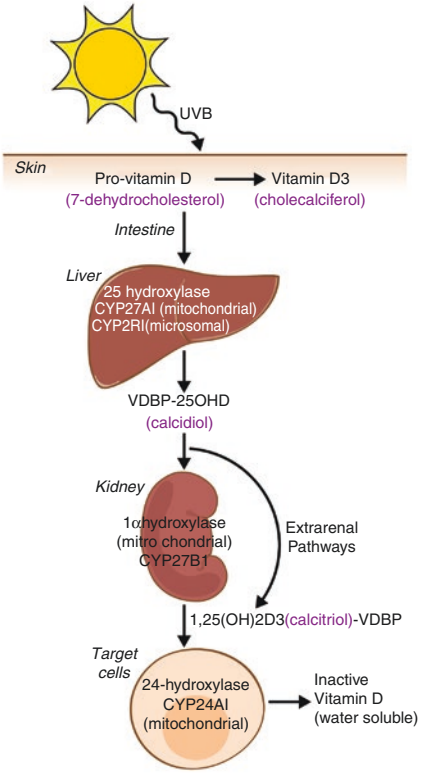

Fig. 10.1 The major source of vitamin D is the skin. Here the precursor of vitamin D (7-dehydrocholesterol) is transformed into cholecalciferol. A small fraction of active vitamin D precursor is obtained from the intestine. In the liver, mitochondrial CYP27A1 and microsomal CYP2R1 regulate the conversion of vitamin D into 25-hydroxyvitamin D. Bound to vitamin D-binding protein (VDBP), it undergoes 1α-hydroxylation by CYP27B1 in the kidney. In target cells, 1,25(OH)2D3 upregulates CYP24A1, which transforms vitamin D into its inactive form. J Gregory ©2015 Mount Sinai Health System

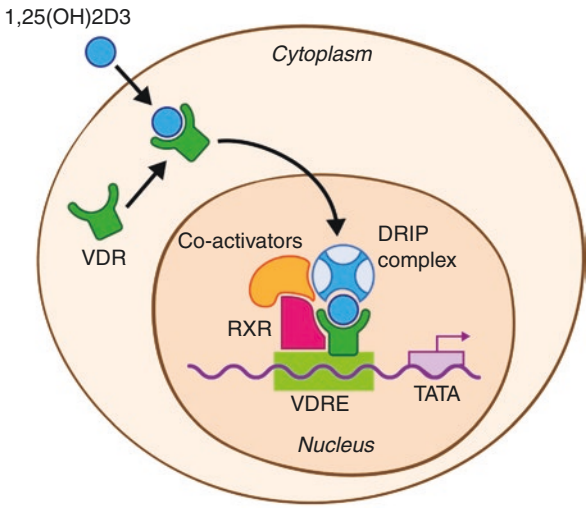

Fig. 10.2 Once 1,25(OH)2D3 reaches the target cell, it binds to the cytoplasmic vitamin D receptor (VDR). VDR bound to 1,25(OH)2D3 is then transported into the nucleus. Here, it complexes with co-activators such as retinoid X receptor (RXR) and the DRIP complex. This transcriptional unit binds to the vitamin D response element (VDRE) in the promoter region of genes, in order to regulate transcription. J Gregory ©2015 Mount Sinai Health System

region of genes and regulate transcription (Fig. 10.2). There is an additional binding pocket on the VDR protein that can mediate more rapid non-genomic effects [2–4, 7].

Vitamin D deficiency is defined as a concentration of 25(OH)D between 21 and 29 ng/mL and deficiency as below 20 ng/mL [8, 9]. Currently vitamin D deficiency is prevalent throughout populations, including women during pregnancy and those with darker skin pigmentation, such as African Americans, Latin Americans, or those of Asian descent [10–13]. Ethnic differences in polymorphisms of the vitamin D-binding protein gene may also play a role in such differences between groups [14].

The classical role of vitamin D involves calcium and phosphorus metabolism through actions on the major target organs: intestines, bone, and parathyroid glands [6, 15]. In recent years, VDR expression has been confirmed in a number of noncanonical tissues, including immune cells, the pancreas, the cardiac system, and skeletal muscle. Accordingly, vitamin D deficiency has been correlated with various clinical syndromes, ranging from infectious diseases like tuberculosis to autoimmune diseases such as type 1 diabetes and Hashimoto's [2, 16, 17]. VDR has been identified in cardiac myocytes, fibroblasts, and vascular cells, suggesting a potential role in cardiovascular disorders such as hypertension, myocardial hypertrophy, and peripheral vascular disease. Importantly, there is a high prevalence of vitamin D deficiency in type 2 diabetes, prediabetes, and metabolic syndrome [18, 19]. More recently there has been an increased interest in the role of vitamin D in reproductive function [20].

Vitamin D and Reproductive Function

Genetic Alterations in Vitamin D and Reproductive Function

There has now been a significant body of literature suggesting that vitamin D may play a role in reproductive function in both males and females. Let us first review the effects of genetic manipulation of the VDR gene. The phenotype of the VDR knockout mouse shows both male and female reproductive dysfunction. Male mice have decreased sperm count as well as decreased motility. Histological abnormalities of the testis are noted. Female mice present with uterine hypoplasia and impaired folliculogenesis. In addition, aromatase activity is significantly decreased compared to wild-type mice: by 24% in the ovary, by 58% in the testis, and by 35% in the epididymis [21]. Interestingly, testosterone levels in males remain normal. These mice have elevated LH and FSH levels indicating hypergonadotropic hypogonadism, emphasizing the importance of peripheral gonadal actions of vitamin D. The Leuven VDR-ablated (*Vdr*−/−) mice were established as a model of vitamin D-resistant rickets. In this mouse model of inactivated vitamin D signaling, testicular morphology is normal. There also appears to be normal production of testosterone and estradiol. There are changes noted in ERβ and Erα expression in the testes. In testicular tissue, there is decreased ERβ expression. In epididymal samples, there

is altered expression of ERα [22]. The significance of these changes to reproductive function has not been fully elucidated. In humans, children with 1,25(OH)2D3-resistant rickets secondary to a lack of VDR showed a normal testosterone response [15, 23]. This conglomeration of data continues the debate as to the true significance of vitamin D action in peripheral gonadal function.

Vitamin D and Male Reproduction

In addition to the genetic data presented above, expression analyses have also supported a role for vitamin D in the male reproductive tract. Reproductive function is mediated by the hypothalamic-pituitary-gonadal axis in both males and females. Both VDR and vitamin D metabolizing enzymes have been localized throughout central organs in this system, such as the hypothalamus and the pituitary gland. In males, peripheral organs include the reproductive tracts such as the seminiferous tubules, the testes, and developing spermatogonia. Expression has been noted in subsections of testicular cells, including Sertoli and Leydig cells, germ cells, and spermatozoa. This pattern of expression is conserved throughout species such as the rat [21, 24], the mouse [25, 26], the rooster [27], the ram [28], and humans [29].

Vitamin D Expression in the Male Reproductive System

In the brain, VDR and 1α-hydroxylase are most highly expressed in the substantia nigra and the hypothalamus. Immunohistochemistry has shown that VDR is expressed in the nucleus, while 1α-hydroxylase is expressed mainly in the cytoplasm [30]. Gene and protein expression of VDR in the pituitary gland has also been confirmed [31, 32]. The pituitary gland is a key regulator of the reproductive axis. LH and FSH produced by the anterior pituitary result in the stimulation of testosterone production by the testes and maturation of spermatogonia. The pituitary transcription factor-1 (Pit-1) gene is highly expressed in the pituitary gland. It activates prolactin and growth hormone gene transcription. It is also important in the development of the anterior section of the pituitary gland. Immunoprecipitation assays have confirmed that the VDR complex binds to the promoter region of the *Pit-1* gene, where it can modulate downstream gene expression [33, 34].

The presence of VDR in human sperm was initially described in 2006. Evaluation of sperm from fertile men has shown expression of VDR predominantly in the head and nucleus. There is also some localization in the neck region. The molecular weight of the VDR has been noted to be 50 kDa [35]. Nuclear localization has been confirmed with electron microscopy studies [36]. Binding to VDR is time and temperature dependent. Labeling studies have demonstrated specificity of binding of 1,25(OH)2D3 to the VDR receptor [37]. Western blot studies have also indicated the presence of the mitochondrial enzyme CYP24A1 within human sperm, suggesting

local regulation of 1,25(OH)2D3 concentrations [38]. The vitamin D-inactivating enzyme CYP24A1 is regulated by vitamin D. Its expression in human sperm is noted at the annulus.

The sperm maturation process involves a tightly regulated set of developmental steps that propagates the formation of mature spermatids from spermatogonia. It is plausible that vitamin D action plays a role in this maturation process. VDR is expressed in human gonocytes, immature Sertoli cells, and Leydig cells as early as gestational week 16. Interestingly, in human Sertoli cells, VDR is expressed mainly in fetal or immature cells, but in mice they are also seen in mature Sertoli cells. The role of vitamin D in developmental physiology however remains to be fully elucidated [39]. In mature sperm, VDR expression has been noted primarily in the head and nucleus, with lesser expression levels in the neck region. Labeling studies have confirmed binding of 1,25(OH)2D3 to the VDR receptor in the human sperm [40]. Vitamin D metabolizing enzymes such as the mitochondrial CYP24A1 have been co-localized with VDR in the mature human sperm, suggesting local modulation of vitamin D action in male gonads [36]. Interestingly, subfertile men have lower levels of CYP24A1 expression than fertile men [37].

Similar co-localization of vitamin D metabolizing enzymes and VDR are noted throughout the male reproductive tract. Enzymes such as 1α-hydroxylase (CYP27B1) and the vitamin D-inactivating enzyme (CYP24A1) are often co-localized with VDR. Moreover, CYP27B1 and 25-hydroxylase (CYP2R1) are expressed at greater levels in the testis in comparison to other tissues. CYP2R1 is expressed in both germ cells and Leydig cells. Defective spermatogenesis in men is associated with decreased expression of this peptide in the testes. CYP2R1 expression is actually higher in the testis than in the liver [41]. The coordinated expression of VDR and its metabolizing enzymes supports the concept of local modulation of vitamin D activation that may differ from systemic vitamin D levels.

Effects of Vitamin D Deficiency in Animal and Human Studies

Association studies have confirmed a link between vitamin D and decreased male fertility in both animal and human models. In murine studies, vitamin D deficiency during weaning of offspring results in a 45% decrease in successful matings when compared to vitamin D replete counterparts. Moreover, in mating studies involving vitamin D-deficient male rats, there is a 73% decrease in successful matings [42]. Targeted deletion of 1α-hydroxylase in mice leads to a phenotype of decreased sperm count and mobility in the male 1α(OH)ase(−/−) genotype. These findings were accompanied by downregulation of cyclin E and CDK2 and upregulation of p53 and p21 expression. However, unexpectedly, correction of serum calcium and phosphorus levels in these mice reversed the defective reproductive phenotype, suggesting that the processes are electrolyte dependent [43].

In a recent human cross-sectional study involving 2299 men referred for coronary angiography, a positive association between vitamin D and testosterone level

was noted [44]. In a case-control study, including 122 type 2 diabetic males, hypogonadal men (n = 51) had significantly lower 25(OH)D levels than men without hypogonadism (n = 71), 20.1 ± 6.58 vs. 24.0 ± 5.6 ng/mL, $p < 0.01$ [45]. Molecular studies have shown altered expression patterns in vitamin D metabolizing enzymes, such as CYP24A1 in subfertile men [37]. Vitamin D also appears to modulate testicular peptides such as anti-mullerian hormone (AMH) and inhibin B. Inhibin B affects the function of Sertoli cells and spermatocytes in the adult testes and serves as a marker for spermatogenesis. AMH is produced by immature Sertoli cells. Its function is to aid in the development of the male reproductive tract. A recent report showed a positive association between vitamin D and AMH [46].

In addition, several studies support an association between vitamin D and sperm quality and function. In a cross-sectional evaluation of healthy men, vitamin D deficiency was correlated with reduced sperm motility and percentage of normal morphology. Administration of 1,25(OH)2D3 has been shown to increase sperm motility and acrosin activity, suggesting that it is important in developing fertilization capacity. Calcitriol has also been shown to decrease triglyceride content in sperm, affecting its functional ability [47]. In fact, a Danish study suggests that higher levels of vitamin D can show a lower sperm count and decrease in percentage of normal morphology [48].

Randomized clinical trials with 25(OH)2D supplementation are the gold standard for showing whether there is a causal effect of vitamin D on male reproductive function. It appears that there may be an ideal or optimal dose of vitamin D for male fertility. Studies in normal mice with vitamin D-deficient diets showed decreased testicular function. However, while replacement with low doses of vitamin D improved these parameters, higher doses caused a worsening of function [49]. A similar pattern is seen with male sperm. Exposure of normozoospermic samples to low concentrations of vitamin D leads to protein phosphorylation and sperm survival, but higher concentrations failed to show such an effect [35]. In a Danish study, higher levels of vitamin D are linked to a lower sperm count and decrease in percentage of normal morphology [48].

Vitamin D in Germ Cell Tumors

A final topic in male reproduction is the potential importance of vitamin D in male germ cell differentiation. Testicular germ cell tumors are the most prevalent solid tumor in young men [40]. These tumors are derived from the male reproductive tract. It has been found that both VDR and its metabolizing enzymes have a high level of expression carcinoma in situ of these tissues. The level of expression decreases when there are more dedifferentiated forms of embryonic carcinoma [37]. It is plausible that the antiproliferative effects of vitamin D are mediated through cell cycle regulators such as p21, p27, p63, or p73. In vitro studies have shown a decreased need for cisplatin with the concomitant use of 1,25(OH)2D3. Interestingly, cisplatin is part of the treatment regimen for testicular cancer. This raises the

possibility that addition of vitamin D can decrease toxicity from testicular cancer treatment. However, in vivo studies in mouse models have not been able to duplicate this effect [40].

Vitamin D and Female Reproduction

The female reproductive system, as the male reproductive system, is composed of central regulators including the hypothalamus and the pituitary gland and peripheral organs such as the ovary, fallopian tubes, uterus, and during pregnancy the placenta. VDR expression has been noted throughout the female reproductive tract (Fig. 10.3) [50]. In addition, both in vitro and in vivo studies suggest a role for vitamin D in female reproductive function and ovarian physiology. Cultured follicles from the rhesus monkey have shown increased preantral follicle survival with exposure to low vitamin D levels in culture and improved folliculogenesis and oocyte development when exposed to high levels of vitamin D [51, 52]. This data suggests that there is a dose-dependent effect of vitamin D on ovarian tissues, a mode of action seen with other steroid hormones.

In other studies, primary cultures of granulosa cells incubated with and without vitamin D show a differential effect on anti-mullerian hormone (AMH) signaling and steroidogenesis. In female reproductive physiology, anti-mullerian hormone (AMH) is a marker of ovarian reserve. AMH inhibits recruitment of primordial follicles into folliculogenesis [53]. A functional vitamin D response element (VDRE) has been noted in the promoter region of the AMH gene [54]. Although genetic differences in VDBP polymorphisms can affect such data, in vitro exposure of granulosa cells to the active 1,25-dihydroxyvitamin D3 metabolite bypasses this step in cellular transport.

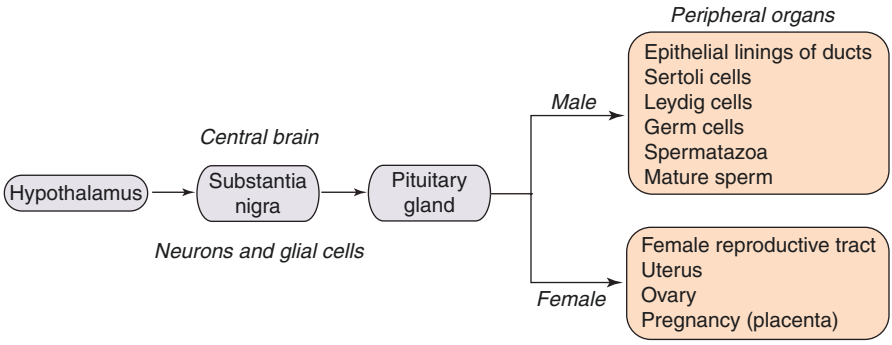

Fig. 10.3 Vitamin D receptor (VDR) is localized in both central and peripheral organs of the male and female reproductive tracts. In the brain it is found in neurons and surrounding glial cells. In the male peripheral reproductive tract, it is found in the ducts, Sertoli and Leydig cells, germ cells, developing spermatozoa, and mature sperm. In females, VDR is found throughout the reproductive tract, in the uterus and the ovary. During pregnancy, VDR expression is noted in the placenta

In animal models, disruption of the murine *VDR* gene results in uterine hypoplasia and defective folliculogenesis. A decrease in aromatase activity and gene expression is also noted. Interestingly, in these mice elevated LH and FSH levels suggest the importance of VDR action in peripheral rather than central tissues of the female reproductive tract. Female reproductive function in a second murine model with *VDR* disruption, the Leuven *VDR*-ablated mouse, has not been well studied. However, in male reproductive tissues, aberrant expression of genes in the estrogen signaling pathway is noted [22].

Association studies in humans have also supported a correlation between vitamin D and markers of ovarian reserve, such as AMH. Women with HIV who have low vitamin D levels are also noted to have decreased AMH levels in circulation [52]. Moreover, seasonal changes in AMH levels have been correlated with vitamin D status [55]. A direct role for vitamin D is suggested, as supplementation with 1,25(OH)2D3 is able to reverse the seasonal decrease in AMH levels [46]. Other studies, however, contradict these results. A prospective study evaluating 1,25(OH)2D3 replacement in vitamin D-deficient premenopausal women did not show an increase in AMH levels ($p = 0.6$) [50]. Other studies, similarly, have not supported an association between vitamin D and AMH [56].

Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age. Previously coined "diabete de femme a barbe" or "diabetes of the bearded woman," this diagnosis closely links insulin dysregulation, reproductive dysfunction, and hirsutism. Fifty to seventy percent of women with PCOS have metabolic disturbances, such as insulin resistance. They also have a higher incidence of metabolic syndrome, hypertension, hyperlipidemia, diabetes, and obesity [57]. Interestingly, 67–85% of women with PCOS have serum concentrations of vitamin D25 less than 20 ng/mL [58]. Other studies have not found a difference in vitamin D levels in women with and without PCOS [59].

It is difficult to assess whether vitamin D action in women with PCOS directly affects the hypothalamic-pituitary-gonadal axis or whether its effects are mediated through changes in the insulin signaling pathway. Vitamin D deficiency in women with PCOS predicts more severe metabolic disturbances, including obesity, homeostatic model assessment-insulin resistance (HOMA-IR), and BMI [60]. In multivariable analyses, vitamin D and BMI were independent predictors of HOMA-IR, quantitative insulin sensitivity index (QUICKI), and metabolic syndrome [50]. In a study population, divided into three groups by vitamin D status, insulin resistance was greatest in the patients with most severe vitamin D deficiency [61].

Despite this data, an independent association between vitamin D and the reproductive manifestations of PCOS is not well established. While some studies have shown an inverse relationship between vitamin D and androgens such as testosterone and DHEAS [60], most studies have not confirmed a direct relationship between

vitamin D and reproductive parameters after adjustment for confounding factors. For example, in a substudy of the Pregnancy in PCOS I (PPCOS I) trial, 25(OH)D levels did not support an association with this parameter.

In addition to association studies, clinical trials with vitamin D replacement have demonstrated an effect on both metabolic and reproductive parameters in women with PCOS. One study evaluated the effects of treatment with the vitamin D3 analogue (alfacalcidol) for 3 months in 15 patients with PCOS. This regimen resulted in an increase in the first phase of insulin secretion and improvement in lipid profiles [62]. A similar study with 11 patients did not show improvement in glucose or insulin levels after 3 weeks of treatment, but did show a significant decrease in HOMA-IR. Interestingly, androstenedione, total and free testosterone, and DHEAS levels were not altered [63]. A larger study of 46 patients did observe a decrease in fasting glucose, stimulated glucose, and C-peptide levels. There was as well a significant decrease in estradiol and triglyceride levels. However, in a subsection of women with improvement in menstrual irregularities, there was no change noted in androgen level profiles [64].

There is some interest in the effect of advanced glycation end products (AGEs) and anti-mullerian hormone (AMH) in the presentation of PCOS. AMH levels are elevated in women with PCOS, suggesting abnormal ovarian folliculogenesis. sRAGE is a soluble receptor for advanced glycation end products (AGEs). In diabetes, AGEs have been implicated in a pro-inflammatory response. The soluble receptor for AGE (sRAGE) binds AGE and prevents its deposition and adverse effects. In animal models, vitamin D supplementation has decreased the deposition of AGEs in the vascular system. One study evaluated the effects of 1,25(OH)2D3 supplementation in women with PCOS (cases with PCOS = 22 and controls without PCOS = 45). In women with PCOS, vitamin D3 increased sRAGE levels ($p = 0.03$) and decreased AMH levels ($p < 0.001$) [63].

A different study, performed in Australia, looking at the seasonal increase in vitamin D levels did not find an effect on circulating AMH levels [65]. The patient population studied may reflect a different genetic background than those in the earlier study. Genetic polymorphisms in components of the vitamin D transport system have shown gradations in the presenting phenotype of PCOS. For example, the rs757343 single-nucleotide polymorphism (SNP) of the VDR gene correlates with severity of reproductive irregularities in such patients [66]. It is plausible that the variable results in AMH levels in a different cohort of patients similarly represent genetic variations in components of the vitamin D metabolism pathway.

Endometriosis and Leiomyoma

Vitamin D may have anti-immunogenic properties. Inflammation may be important in the pathogenesis of endometriosis. In gross animal experiments, treatment with 1,25(OH)2D3 improved in endometriosis parameters. In murine models, elocalcitol, a selective vitamin D receptor agonist, inhibits lesion development [67].

1,25(OH)2D3 supplementation also reduces the size of endometriosis . This is also accompanied by lower levels of VEGF and matrix metalloproteinase-9 [68, 69]. In prospective human studies, dairy food intake has been associated with a lower risk for endometriosis [70, 71].

The etiology of uterine fibroids (UF) is not well known. There does again seem to be an association between vitamin D and UF and leiomyoma cells. Recent studies have shown an association between polymorphisms of the vitamin D receptor gene and uterine leiomyomas after adjustment for age, geographic region, and ethnicity [72]. Other studies have corroborated such associations between vitamin D and both presence and volume of UF [73, 74].

Experiments with in vitro and in vivo models support the associations noted in clinical studies. Administration of vitamin D has been shown to decrease myometrial and leiomyoma cell proliferation in cell culture studies. Growth inhibition in cell lines by 12% has been demonstrated. Moreover, the inhibition has been shown to be dependent on concentration of vitamin D, with higher levels exerting a greater effect [75]. In vivo, murine studies such as those in Eker rats showed significant reductions in leiomyoma tumor size when treated with 1,25(OH)2D3 [76].

Investigations into the mechanism of action of 1,25(OH)2D3 against leiomyoma cell proliferation have identified a number of candidate downstream target genes. Uterine fibroids have high levels of metalloproteinase activity. Treatment of the immortalized human uterine fibroid cell line (HuLM) with 1,25(OH)2D3 reduces mRNA and protein levels of MMP-2 and MMP-9 in a concentration-dependent manner [77]. TGF-β3 increases expression of several extracellular matrix proteins that may play a role in tissue fibrosis. Incubation of HuLM cells with 1,25(OH)2D3 suppresses TFG-β3-induced expression of fibronectin, collagen type 1 protein, and plasminogen activator inhibitor-1. There is also a reduction in phosphorylation of Smad2 and Smad3 [78]. In HuLM cells, expression of cell cycle regulators such as proliferating cell nuclear antigen (PCNA), BCNA, BCL-2, BCL-w, cyclin-dependent kinase (CDK) 1, and catechol-O-methyltransferase (COMT) is also negatively affected by incubation with 1,25(OH)2D3 [79].

In Vitro Fertilization

Recent data indicate that global rates of infertility have increased from 42.0 million in 1990 to 48.5 million in 2010 [80–82]. Studies evaluating whether vitamin D plays a role in the success rate of in vitro fertilization procedures have found mixed results. Studies do consistently support a strong correlation between serum and follicular fluid (FF) levels of vitamin D and procedure outcomes [50, 81]. In 84 infertile women treated with IVF, clinical pregnancy was achieved more frequently in patients with higher FF levels of vitamin D. This association was maintained after multivariable regression analysis adjusted for confounders such as age, BMI, ethnicity, and numbers of embryos transferred [83].

Other human studies have given more variable results. In one prospective cohort study with 84 infertile women undergoing in vitro fertilization, multivariable regression analysis adjusting for age, body mass index, ethnicity, and number of embryos transferred showed that 25(OH)D is an independent predictor of successful implantation and clinical pregnancy [81]. Other studies have corroborated this effect, with a positive association between vitamin D levels and fertilization rates (p, 0.003) [83]. The Pregnancy in PCOS I (PPCOS I) trial is a randomized clinical trial evaluating the percentage of women achieving live birth when treated with metformin, clomiphene citrate, or both. A sub-analysis of this study has shown a significant decrease in live birth with vitamin D levels below 30 ng/mL, odds ratio 0.58 (0.35–0.92). An increasing trend in successful live birth has also been found with increasing levels of vitamin D. These positive associations were maintained after adjustment for confounding factors [84]. The majority of studies, however, fail to detect a significant correlation between vitamin D and outcomes of IVF cycles [81, 85–90]. In one study of 101 women, the quality of retrieved embryos was not significantly better in the group with vitamin D sufficiency than in the group with insufficiency [50]. There may be some ethnic differences in the association of vitamin D and fertility outcomes [91]. Not surprisingly, a meta-analysis concluded that there is insufficient evidence for vitamin D supplementation in IVF procedures [92].

Pregnancy Outcomes

Studies reviewing the cumulative literature on the potential role for vitamin D in pregnancy outcomes have shown a correlation between vitamin D deficiency and adverse outcomes such as gestational diabetes, preeclampsia, preterm birth, and low birth weight. A recent meta-analysis of the literature, reviewing 3357 studies, showed an increased odds ratio (OR) for gestational diabetes (1.85, 95% CI 1.18–1.89), preeclampsia (1.79, 95% CI 1.25–2.58), and low birth weight (1.85, 95% CI 1.52–2.26) in the setting of vitamin D deficiency [93–96].

Gestational Diabetes

Human studies involving gestational diabetes support an association between GDM and vitamin D levels as well as its metabolizing enzymes, CYP27B1 and CYP24A1. Both mRNA and protein expression of the metabolizing enzymes were higher in the placenta from patients with GDM versus normal pregnancies. VDR expression, however, was not significantly different between the two groups [97]. In a study with 234 women with gestational diabetes and 168 controls patients, only the group with severe vitamin D deficiency showed a significant association. Lower vitamin D levels in the first trimester of pregnancy were not only associated with increased risk for GDM but also with indices of insulin resistance in the second trimester [98].

Preeclampsia

Preeclampsia is another complication of pregnancy that is associated with insulin resistance. This condition often results in infants that are small for gestational age (SGA) at birth. Several studies have supported an inverse correlation between vitamin D levels and development of preeclampsia. Vitamin D levels in early pregnancy were lower in women with preeclampsia compared to women without preeclampsia ($p < 0.01$), after adjusted analyses [99]. This association was supported in a study that evaluated 25(OH)D concentrations at 14 weeks in cases and controls [100]. Serum 25(OH)D levels, later in pregnancy at weeks 24–26, were also significantly lower in women with preeclampsia than normal controls [101]. In the Collaborative Perinatal Project, vitamin D levels above 50 nmol/L were found initially to have a protective effect on the development of preeclampsia. However, this effect was not sustained after adjusted analyses [102]. An association between vitamin D levels and low SGA has also been noted. Women in the lowest quartile of 25(OH)D level had a higher risk for SGA infants [103]. Other studies have not found a relationship between vitamin D and risk of preeclampsia [104–107]. In one study, an increase in vitamin D levels to greater than 30 mmol/L gives a lower risk for developing preeclampsia (OR 0.22, p 0.002). However, after multivariable adjusted analyses, the association between vitamin D and pregnancy-induced hypertension did not persist [108].

Few studies have evaluated potential downstream modulators of vitamin D action in preeclampsia. In one such study, placental growth factor was negatively correlated with preeclampsia. As preeclampsia is a result of endothelial dysfunction, factors such as soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor, intracellular adhesion molecule-1 (ICAM-1), and vascular adhesion molecule-1 (VCAM-1) may play a role in its pathogenesis. In a prospective cohort study of 697 women, lower vitamin D levels were associated with higher odds of developing preeclampsia. Vitamin D levels at 12–18 weeks, but not at 24–26 weeks, were negatively correlated with ICAM-1 levels. No association was noted with sFlt-1 and VCAM-1. In a recent study, interleukin-6 (IL-6), a pro-inflammatory marker with increased expression in metabolic syndrome and diabetes, has been significantly associated with preeclampsia [109]. In this study, vitamin D level was correlated with preeclampsia, but no direct measure of association was evaluated between vitamin D and IL-6 [110].

Randomized controlled clinical trials have evaluated vitamin D supplementation in pregnancy. Safety of daily supplementation with 2000–5000 IU of vitamin D has been established [109]. Clinical trials with vitamin D supplementation in pregnancy have shown conflicting results in terms of birth weight and postnatal growth [111–115]. Several studies have focused on the metabolic outcomes of vitamin D supplementation in pregnancy. A randomized trial with 54 women with GDM showed that vitamin D supplementation led to a significant decrease in fasting glucose ($p < 0.001$), insulin ($p = 0.01$), and HOMA-IR levels ($p < 0.001$), with a significant increase in the QUICKI ($p = 0.003$) [116]. A trial in pregnant women with 9 weeks of supplementation, with 400 IU of vitamin D versus placebo, showed higher vita-

min D levels, but also significantly lower CRP and insulin levels, with significantly higher QUICKI in the vitamin D-treated group than in the placebo group (−1.4 μg/mL vs. + 1.5 μg/mL, $p = 0.01$; −1.0 μIU/mL vs. +2.6 μIU/mL, $p = 0.04$; +0.02 vs. −0.02, $p = 0.006$) [117]. Other studies have not confirmed improvements in metabolic parameters with vitamin D supplementation [118].

Maternal-Fetal Interface

The importance of vitamin D in pregnancy may in part result from its function in the gestational uterine decidua, at the maternal-fetal interface. The decidual tissue is formed from the maternal endometrium with invasion of fetal-derived trophoblasts. Close cell-to-cell interactions facilitate the early stages of maternal-fetal exchange of nutrients and wastes. It is also a source of secretory products such as hormones and growth factors [119]. There is evidence of 1,25(OH)2D3 production in both the maternal and fetal tissues. Both VDR and CYP27B1 expression have been noted in the placenta and the decidua. Moreover, gene expression appears to be induced in pregnancy. Expression of the catabolic enzyme (CYP24A1), however, seems to be decreased in gestation [120–123].

This utero-placental unit is an area where maternal immune function shifts toward tolerance to accept the fetus. The interplay and specificity of subsets of immune cells within the interface are thus important in pregnancy progression. 40% of the decidual stromal population is made of leukocytes: uterine natural killer (uNK) cells, macrophages, CD4+ and CD8+ T lymphocytes, and antigen-presenting cells (APCs) [120]. Studies have suggested an immunomodulatory role for vitamin D in the placenta. Vitamin D has been correlated with induction of monocyte cathelicidin [119]. Purification of decidual cells shows that adherent cells demonstrate greater 1,25(OH)2D3 levels [124]. Macrophages in the decidua consist of the pro-inflammatory variety (M1) and anti-inflammatory and pro-tolerogenic variety (M2) cells. Loss of placental vitamin D, in *Vdr* and *Cyp27b1* gene knockout mice, leads to an exaggeration of pro-inflammatory cells [125]. Furthermore, in vitro studies have shown that 1,25(OH)2D3 inhibits differentiation and maturation of dendritic cells [126]. At the time of implantation, there is a prominence of uNK cells. Numbers of uNK cells have been shown to be altered in preeclampsia. In peripheral blood, 1,25(OH)2D3 inhibits NK activation [118]. uNK cells treated with 1,25(OH)2D3 show a decrease in cytokine production and increase in antimicrobial peptides [126].

Cells of humoral immunity are also found at the maternal-fetal interface. T cells have been noted to express VDR. Moreover, the expression level of VDR increases with T cell proliferation [127]. Vitamin D seems to promote a shift from Th1 to Th2 cytokine profile [119]. Decidual B cells are low in number during human pregnancy compared to T cells. As with T cells, 1,25(OH)2D3 seems to suppress proliferation and immunoglobulin production [126, 127]. 1,25(OH)2D3 can also inhibit differentiation of plasma cells and class-switched memory cells [128]. A recent study in 183 pregnant women showed that both the peripheral T cell and B cell populations differed depending on vitamin D levels [129].

Recurrent Miscarriages

Two recent studies have implicated the importance of vitamin D levels in maintaining successful pregnancies. Alteration in vitamin D-related gene and protein expression has been noted in patients with recurrent pregnancy loss. Both VDR mRNA and protein levels in chorionic villi and decidua were evaluated with immunohistochemistry, confocal laser scanning microscopy, Western blot, and quantitative real-time PCR. The evaluations revealed decreased VDR mRNA and protein expression in villi and decidual tissues ($p < 0.001$). Serum levels of VDR were also noted to be lower in women with recurrent pregnancy loss ($p = 0.009$). Immunohistochemistry demonstrated that the decrease in levels was seen in trophoblasts and epithelial and glandular cells ($p < 0.005$) [130].

As noted in the section above, vitamin D may have a modulatory role in the immune architecture of the endometrium and placenta. In women with recurrent pregnancy loss, irregularities of cellular immunity have been noted in women with concurrent vitamin D deficiency. The percentage of CD19+ B cells, NK cytotoxic cells, and TNFα-expressing cells in 99 women with recurrent miscarriages were decreased in those with vitamin D levels below 30 ng/mL ($p < 0.05$). Furthermore, when 1,25(OH)2D3 supplementation was given to the women with recurrent pregnancy loss, a decrease in immune dysregulation was observed [131]. These data strongly suggest a causative relationship between vitamin D insufficiency and immune dysfunction in the gestational uterus, a role that may be important in the maintenance of a successful pregnancy.

Direct Versus Indirect Effect of Vitamin D

After review of current data, it remains in debate whether vitamin D has a direct role in male and female fertility or an indirect one. An experiment done with vitamin D-deficient mice showed that fertility improves with vitamin D replacement. However, fertility can also be restored in these mice with a high-calcium diet, despite persistent vitamin D deficiency [131]. In addition, treatment of VDR null mice with calcium showed partial correction of hypogonadism and an increase in aromatase activity [132]. In other murine models, correction of calcium and phosphate balance in 1α(OH)ase(−/−) mice has led to normalization of reproductive function [43]. It has been shown that exposure to 1,25(OH)2D3 led to release of intracellular calcium through VDR-mediated action [38].

Another possible indirect effect of vitamin D on reproduction may be through the regulation of the estradiol concentration within male reproductive organs. The concentration of estradiol is reported to be 100 times higher in the testes than in circulation, suggesting a potentially important role for estrogen in male reproductive function [40]. As previously mentioned, the Leuven *VDR* null mouse shows an altered ER expression pattern [133]. A second mode of alteration in estrogen levels in the testis can be through modulation of the aromatase (CYP19A1) gene, which

is responsible for conversion of testosterone to estradiol. A copy of the VDRE is present in the promoter region of the CYP19A1 gene. Moreover, treatment with 1,25(OH)2D3 leads to induction of aromatase expression in immature rat Sertoli cells in vitro [40].

Lastly, as noted in the sections above, vitamin D action on male and female reproduction may be indirectly mediated through its effects on insulin signaling and metabolic dysregulation. This is possible in diseases such as PCOS, where there is a known link between poor metabolic control and worsening of symptoms. In these diseases, there is also a concurrent link between vitamin D and the degree of metabolic dysregulation. Vitamin D may also exert its reproductive effects through alterations in subsets of immune cells in reproductive tissues such as the uterine decidua or the placenta.

Conclusion

Vitamin D expression is ubiquitous in both male and female central and peripheral reproductive organs. Moreover, in in vitro, in vivo, and in human studies, there are strong correlations between hypovitaminosis D and reproductive abnormalities. However, not all the studies have been adjusted for confounding factors. Thus, the true significance of vitamin D in reproductive function remains elusive. The ultimate effect of vitamin D is mediated by the binding of the active hormone to the VDRE in promoter regions of genes. This modulates downstream genes that are involved in maintaining the hypothalamic-pituitary-gonadal function. Ultimately, it is not known whether the effects of vitamin D are direct effects on genes involved in reproduction or whether vitamin D acts through secondary messengers such as calcium, estradiol signaling, insulin pathway, or immune modulation. Co-localization of vitamin D and its metabolizing enzymes suggests local variations in vitamin D levels. Questions remaining in understanding the role of vitamin D in reproductive function require controlled prospective randomized controlled studies evaluating effects of vitamin D supplementation on both male and female reproductive dysfunction.

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Chapter 11
Muscle Weakness and Falls

Giovanni Iolascon, Antimo Moretti, Letizia Stefano, and Francesca Gimigliano

Background

Recent years have seen a growing trend of scientific interest in the extraskeletal effects of vitamin D system and in its role on muscle function. The hypothesis that vitamin D was related to muscle activity was born from the observation that children with severe insufficiency of vitamin D, suffering from rickets, experienced a severe reduction in muscle strength as already described centuries ago by Glisson [1]. Historically, the first exhaustive report of muscle impairment due to rickets was provided by Hagenbach-Burckhardt in 1904 [2] and subsequently by Bing [3], who coined the term "myopathia rachitica" to describe the severe muscle weakness caused by many factors such as nonuse, periosteal pain, and neural involvement. To better understand the pathophysiological mechanisms of muscle damage, Peitsara [4] formulated a theory based on the key role of alterations of phosphoric ester concentration in muscle tissue. However, he did not point out the role of secondary hyperparathyroidism due to vitamin D deficiency on skeletal muscle, as it was already known that primary hyperparathyroidism could lead to significant muscle weakness. Patten et al. [5] first described the neuromuscular impairment during hyperparathyroidism, characterized by muscle atrophy mainly of type II fibers, without pathognomonic alterations of primary myopathy (necrosis or degeneration of muscle fibers and proliferation of endomysial connective tissue). In these patients, the electromyography (EMG) showed a decrease in duration and amplitude of

G. Iolascon, MD (✉) • A. Moretti, MD • L. Stefano, MD
Department of Medical and Surgical Specialties and Dentistry,
University of Campania "Luigi Vanvitelli", Naples, Italy
e-mail: giovanni.iolascon@gmail.com; letizia-stefano@libero.it

F. Gimigliano, MD, PhD
Department of Mental and Physical Health and Preventive Medicine,
University of Campania "Luigi Vanvitelli", Naples, Italy

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motor unit action potentials, with polyphasic potentials after a voluntary muscle contraction, suggesting a reduced number of motor units. The discovery of vitamin D receptors (VDRs) is a cornerstone of the study of the effects of vitamin D in different tissues [6]. In 1985, VDRs were also identified in cultured myoblasts, myotubes, and rat muscle cells, suggesting a predominant role of vitamin D on myoblast proliferation and differentiation [7]. The expression of VDRs was later described also in several human tissues including the smooth muscle, cardiac muscle, liver, lungs, bowel, gonads, and skin [8]. In 1986, Costa et al. performed the first description of this receptor in human skeletal muscle cells [9], and, more recently, Pojednic et al. [10] identified the VDR and its gene into the nucleus of human myocytes.

Molecular Mechanisms of Vitamin D Action in Skeletal Muscle

As in other target tissues, vitamin D exerts its effects on muscle by long- and short-term mechanisms that involve genomic and non-genomic pathways, respectively [11].

In the first scenario, vitamin D stimulates muscle cell proliferation and differentiation through gene transcription in myoblasts, resulting in increased synthesis of specific muscle proteins, such as myosin and calcium-binding protein (CBP). This mechanism involves the direct binding of vitamin D activated by the complex VDR/retinoid receptor (RXR) to specific DNA sequences, known as vitamin D response elements (VDREs), resulting in the regulation of transcription [12]. There are recognized different VDR domains involved in the genomic effects of vitamin D: the ligand-binding domain (LBD), connective hinge, DNA-binding domain (DBD), and A/B domains [13, 14]. Vitamin D regulates VDR activation inducing conformational modifications of LBD responsible for the stability and selectivity of the interactions with the RXR. It has been hypothesized that the dynamic view of the interaction between vitamin D and its receptor might explain the selective biological action of this hormone in different tissues.

The long-term effects of vitamin D on muscle calcium uptake depend on RNA and protein synthesis, such as CBP, via the activation of nuclear VDR (nVDR) [15]. Moreover, vitamin D affects the synthesis of muscle proteins, including calmodulin-binding component of the myoblast cytoskeleton [16]. Genomic pathway is involved also in the regulation of phosphate metabolism in muscle cell precursors. Phosphate in the form of ATP or inorganic phosphate is necessary for structural and metabolic needs of the cell, and exposure to vitamin D stimulates phosphate uptake in the cells [17].

Different phenotypic expressions of the vitamin D-VDR interaction in skeletal muscle tissue can be linked to receptor polymorphisms. This research topic is effectively investigated in VDR knockout mice. In these murine models, in addition to osteomalacia and growth retardation with secondary hyperparathyroidism [18], a reduction of 20% in muscle fiber diameter compared to wild type at 3 weeks of age was observed, with alterations of differentiation, development, and maturation of muscle cells [19].

Recently, additional information has been obtained from the analysis of VDR polymorphisms, which may be associated with various muscle clinical features, including strength [20]. In a cross-sectional study performed on non-obese women, the BsmI polymorphism, a VDR restriction fragment length polymorphism, was associated with significant differences in quadriceps and handgrip strength [21]. Specifically, women with bb allele showed quadriceps strength (+23%) and handgrip strength (+7%) significantly higher than those with BB allele, but the influence of this polymorphism on the VDR action remains unknown.

It should be highlighted that there are still conflicting evidences about VDR expression in mature muscle cells. Wang et al. [22], using highly specific antibodies versus VDR, did not confirm the receptor expression in skeletal, smooth, and cardiac muscle cells, suggesting only an indirect effect of vitamin D on muscle mediated by still unknown receptors. Moreover, authors suggested that muscle impairment related to vitamin D deficiency is attributable to metabolic changes, such as hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism, that affect skeletal muscle. On the other side, Chen et al. published in the same year a study demonstrating that VDR gene knockout mice developed a phenotype characterized by smaller muscle fibers, probably linked to a prolonged expression of immature muscle-specific genes [23]. More recently, Girgis et al. [24], using polymerase chain reaction (PCR), Western blot, and immunohistochemistry, showed the existence of VDR in mice skeletal tissue, both in vitro (C2C12 myotubes culture) and in vivo (quadriceps muscle). Moreover, authors claimed that the VDR expression progressively decreased with age. Shortly afterward, Girgis et al. using the same in vitro models demonstrated dose- and time-dependent increases in the expression of both VDR and its target gene CYP24A1 after 1,25(OH)2D treatment [25]. These findings were confirmed in a study performed on cloned myoblasts from healthy volunteers [10]. These cells were exposed to 1,25(OH)2D3 for 18 h. PCR was used to quantify the amount of VDR and CYP24A1 before and after treatment, and an increased expression of both of them in a dose-dependent manner was found.

The dynamic view of vitamin D-VDR interaction could play a role also in regulating non-genomic pathway [26]. This short-term mechanism is implicated in fast regulation of second messenger calcium-mediated system linked to signal transduction. Non-genomic action, downstream of nVDR and/or membrane VDR (mVDR) complexed to caveolin 1, includes the activation of intracellular signaling molecules such as PKC, PI3K, MAPK, CaMKII, and PLA2 [27]. According to Boland, 1,25(OH)2D3 non-genomic mechanism influences muscle function by acting on the voltage channels SOC/TRPC3 (SOC, store-operated Ca2+; TRPC3, transient receptor potential canonical 3), via regulation of intracellular calcium levels, thus affecting the excitation-contraction coupling of the skeletal muscle fibers [28, 29]. In summary, vitamin D seems to exert its biological effects on skeletal muscle through two synergistic mechanisms: on muscle contraction in response to intracellular calcium fluxes (fast response) [30] and on muscle mass and strength (long-term response) [31].

Recently, a third mechanism by which vitamin D affects muscle function has been hypothesized—inhibiting the transdifferentiation of myogenic precursors into adipogenic cells thus reducing intra- and intermuscular adipose tissue (IMAT) stor-

age. IMAT increase [32, 33] might play a role in the decline of muscle function and physical performance, particularly during aging [34]. Moreover, the excess of adipose tissue could induce the secretion of pro-inflammatory cytokines that adversely affects the skeletal muscle [35] with consequent inhibition of protein synthesis. Phillips et al. suggested that some pro-inflammatory cytokines, such as TNF-α and IL-6, might induce apoptosis of muscle fibers, especially those of type II [36]. Hence, vitamin D improves muscle function also through an indirect mechanism of action, reducing both myosteatosis and inflammatory response [37].

The Role of Vitamin D in Muscle-Bone Cross Talk: A Biological Perspective

It has been hypothesized that vitamin D might modulate the cross talk between bone and muscle. Evidence suggested that the interaction between muscle and bone seems to be due to an extremely complex two-way communication system including muscle and bone chemokines [38]: myokines, such as pro-inflammatory cytokines, myostatin, FGF-2, IGF-1, Tmem119, and the osteoglycin [39], and osteokines including PGE2, Wnt3a, osteocalcin, and sclerostin [40]. The osteoglycin produced by muscle cells under the control of vitamin D is able to modulate the osteoblastic activity [41]. Myostatin, a potent inhibitor of muscle fiber growth [42], can also exert its effects on bone mass and fracture healing process, regardless of changes in muscle strength. It was observed that low levels of myostatin were associated with a higher bone mass [43] and that treatment with vitamin D analogues reduced myostatin in muscle cells in vitro [44].

Vitamin D inhibits the synthesis of myostatin by muscle cells [45], and it is able to double the size of myotubes in C2C12 cells [46]. Moreover, a recent study suggested that even the muscle production of vascular endothelial growth factor (VEGF) and IGF-1 was stimulated by the 25(OH)D, and it is well known that both factors have potential beneficial effects on bone [24]. The IL-6, produced by the muscle as a result of muscle contraction and/or exercise, stimulates bone resorption, thus reducing the bone strength. Vitamin D acts by regulating the inflammatory response by reducing the levels of IL-6 [47].

Osteocalcin, produced by osteoblasts and classically considered as a marker of bone formation, has potential actions on skeletal muscle, particularly concerning mitochondrial function and insulin sensitivity [48]: its gene contains a VDRE, suggesting that vitamin D controls directly its expression [49].

Sclerostin, secreted by mature osteocytes present in the mineralized bone matrix, is a potent inhibitor of bone formation. Mutations in the SOST gene encoding sclerostin cause the onset of sclerosteosis, a clinical condition characterized by increased bone formation, considerable bone mass, and neurological deficits caused by nerve entrapment [25]. The production of sclerostin by osteocytes increases during bed rest and decreases in response to weight-bearing activities and muscle contractions [50], and an increase in circulating sclerostin levels was observed in men treated with vitamin D, but not in women [51].

Vitamin D Status and Musculoskeletal Health

It is well known that the elderly population has low serum levels of 25(OH)D and reduced expression of VDR in several tissues, including skeletal muscle [52]. It was hypothesized that the vitamin D endocrine system dysregulation plays a pivotal role in the age-related muscle wasting.

Vitamin D deficiency is a very common condition in older individual especially in those who are institutionalized, and the reduction in the levels of 25(OH)D seems to be itself a risk factor for institutionalization [53]. Hypovitaminosis D in this population occurs because of the reduced skin's ability to synthesize cholecalciferol, as well as to the decreased food intake and absorption of vitamin D, the reduced sun exposure, and the use of protective filters [54]. In the elderly, a vitamin D deficiency prolonged over time results in a reduction of type II fibers and BMD. The loss of muscle mass, strength, and physical performance increases risk of falls that, along with the skeletal fragility, results in higher risk of osteoporotic fractures [55] (Fig. 11.1). All these factors, in turn, may contribute to the onset of reduced mobility, a sedentary lifestyle, and a state of "frailty" [56]. The final result is a vicious circle, since frailty per se can determine or exacerbate a state of hypovitaminosis D, due to the reduction of outdoor activities and therefore of sunlight exposure [57].

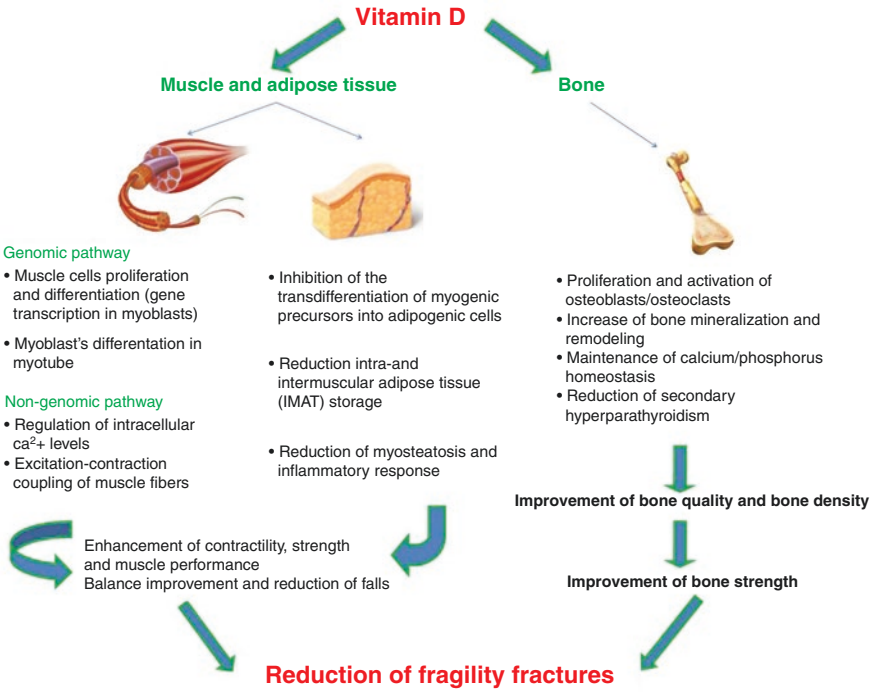

Fig. 11.1 Selective biological actions and clinical implications of vitamin D endocrine system on skeletal muscle, adipose tissue and bone

Frailty is a multifactorial syndrome characterized by impairments in multiple physiological systems and reduced strength and endurance, a general state of decay that leads the individual to an increased risk of falls, disability, vulnerability to depending on others, hospitalization, and death [58]. Sarcopenia is, therefore, a component of the fragile phenotype. According to the most widely used definition of the European Working Group on Sarcopenia in Older People (EWGSOP), sarcopenia is the loss of muscle mass combined with the strength or muscular performance reduction, measured by different methods [59]. A prevalence of sarcopenia of 5–13% in patients aged 60–70 years and 11–50% in octogenarians was estimated [60]. This condition is characterized by a prevalent loss of type II fibers that seems to be associated to a significant loss of motor units [61]. Moreover, about 25% of all motor neurons innervating muscle fiber type II are lost during aging [62], as suggested by the increased serum concentration of C-terminal fragment of agrin, a component of the neuromuscular junction, in sarcopenic patients [63].

However, the age-related muscle wasting is only in part due to the loss of muscle mass. It has been recently coined the term "dynapenia," in relation to the concept of "skeletal muscle function deficits" (SMFD), characterized by the reduction of age-related muscular performance, due to a loss of strength in the presence of normal muscle mass [64]. In particular, three new terms that characterize the manifestations of SMFD have been proposed: mixed SMFD resulting from reduction in muscle mass, strength, and physical performance, sarcopenic SMFD characterized by an alteration of muscle mass and muscle performance, and dynapenic SMFD characterized by a loss of muscle strength and performance regardless of muscle mass.

Moreover, the controversial role of the adipose tissue on the musculoskeletal unit should be taken into account, and several studies investigated the concept of "sarcopenic obesity," a clinical condition characterized by a relative excess of adipose tissue in the presence of reduced skeletal muscle mass [65–67]. The "inclusive" expression of dysmobility syndrome has been proposed, comprising sarcopenia, osteoporosis, obesity, and mobility limitations, all factors that identify the "fragile" phenotype and which are able to identify the increased risk of fall and fracture [68]. In a cohort study of 4551 nonfrail women, there was an increased risk of incident frailty or mortality in those with vitamin D levels <20 ng/mL [69]. Also, in older men, vitamin D deficiency and secondary hyperparathyroidism are associated with frailty [70] and increased mortality [71].

In a population of Korean women aged ≥50 years, the serum levels of 25(OH)D were significantly associated with sarcopenia, regardless of obesity, body composition, serum PTH, diet, and hormone replacement therapy, and vitamin D levels <10 ng/mL were associated with an increased risk of sarcopenia by 1.46-fold [72, 73]. Many studies investigated the association between sarcopenia and osteoporosis. For a sarcopenic woman, the OR of having osteoporosis can range from 1.80 [74] to 12.9 [75], compared to non-sarcopenic women. Moreover, sarcopenia was correlated with an increased risk of falls [76] and of vertebral [76] and hip fractures [77]. Furthermore, increased serum levels of 25(OH)D were associated with a better functional recovery after rehabilitation in women with hip fractures, also without a significant improvement of ALM [78].

Vitamin D Status on Muscle Strength and Performance

The influence of vitamin D status on muscle performance has been widely investigated in several studies. Visser et al. [79] performed an observational prospective 3-year study on elderly subjects with serum levels of 25(OH)D lower than 10 ng/mL at baseline compared to subjects with normal serum levels of 25(OH)D (>20 ng/mL). The authors demonstrated that older people with vitamin D deficiency and secondary hyperparathyroidism had a doubled risk to develop muscle wasting. The NHANES III study, conducted on 4100 outpatient older adults [80], investigated the correlation between serum levels of vitamin D and muscle function, measured using the 8-m walking test and the "sit-to-stand" test [81]**.** Both tests measure muscular strength and performance of the lower limbs simulating some activities of daily living.

Physical performance tests have been shown to be proportional to the vitamin D status, irrespective of gender, level of physical activity, race (Caucasians, African Americans, and Mexican Americans), and calcium intake. These findings suggest that higher blood concentrations (30–40 ng/mL) seem to be associated with better functional outcome. These results were also confirmed by other studies, which showed that subjects with hypovitaminosis D have a reduced muscle function than those with normal vitamin D status.

Mastaglia et al. [82] reported that, in a sample of 54 older women, serum levels of 25(OH)D above 50 nmol/L were associated with a higher strength in the lower limbs (KES 13.4 ± 2.7 kg vs. 11.6 ± 2.5 kg in patients with vitamin D deficiency, $p < 0.03$). The Osteoporosis Prospective Risk Assessment (OPRA) study [83], performed on 986 elderly Swedish women, showed that patients with serum concentrations of 25(OH)D below 30 ng/mL had a significant decrease in gait speed, balance, and the flexion-extension isokinetic strength of the knee, compared with women with serum 25(OH)D above 30 ng/mL. Individuals included in the InCHIANTI study [84] with vitamin D deficiency (<30 ng/mL) showed a significant reduction of grip strength. In this study subjects with serum 25(OH)D below 10 ng/mL also experienced a lower short physical performance battery (SPPB) score that included gait speed, ability to perform sit-to-stand test, and balance assessment. The Progetto Veneto Anziani (Pro.V.A) study [85], which included 2694 community-dwelling elderly women (1597 women and 1097 men, of which 40% of women and 20% of men had serum 25(OH)D below 20 ng/mL), demonstrated that vitamin D deficiency was related to lower scores in the 6-min walking test (6MWT) and to reduced muscle strength, irrespective of gender. On the other side, the IMPROveFALL study including elderly patients with a history of falls (230 men and 370 women) demonstrated that higher serum 25(OH)D levels were associated with better outcomes in terms of Timed Up and Go (TUG) test and sit-to-stand test, particularly in male subjects who showed a double speed in performance testing [86]. Also the Longitudinal Aging Study Amsterdam (LASA), including 979 elderly Dutch men and women, suggested that, in 3-year period, the physical performance scores improved proportionally to serum levels of 25(OH)D [87]. However, other studies

did not confirm the association between vitamin D status and patients' functional outcomes. In a study performed on elderly people with high vitamin D deficiency prevalence (80%), any association between overall physical performance and blood levels of 25(OH)D was not demonstrated. However, the authors hypothesized that these findings might be due to reduced VDR expression in skeletal muscle, commonly occurring in older people [88]. Also in an Hawaiian female population [89], with reduced number of falls, high nutritional intake of vitamin D and high levels of sun exposure, and consequently high serum concentration of 25(OH)D (32 ng/mL), no correlation between vitamin D status and skeletal muscle function, incidence of falls, and performance in activities of daily living (ADL) was reported. Ceglia et al. [90] in a population of about 1000 males (mean age, 47 years) had not reported any association between blood levels of 25(OH)D and muscle performance.

It was conceivable that a threshold of serum 25(OH)D might influence the muscle function. The Health, Aging, and Body Composition Study (Health ABC) [91] prospectively analyzed a large population of elderly subjects ($n = 2641$) for 4 years, defining a threshold of 32 and 28 ng/mL of serum 25(OH)D needed to achieve optimal muscle performance and muscle strength, respectively, regardless of race, gender, body weight, and period of the year. The authors also reported that the decline of both physical performance and muscle strength over time was not affected by baseline vitamin D status. In another study it was demonstrated that postmenopausal women with vitamin D deficiency (<30 ng/mL) experienced a significant decrease in appendicular muscle strength, physical performance, and higher frequency of fragility fractures, compared with an age-matched control group with normal vitamin D status [92].

In our opinion vitamin D plays a key role in maintaining muscle function, particularly in the elderly population with vitamin D deficiency, muscle weakness, decreased physical performance, and balance impairment. The clinical implication of the link between hypovitaminosis D and muscle function, particularly regarding the proximal antigravity muscles of the lower limbs, is that children affected by rickets have difficulty in standing and walking abilities [93], while older people with osteomalacia are also at increased risk of falls and fractures.

It is mandatory to perform a comprehensive assessment of skeletal muscle function in elderly. Therefore, we recommend a multidimensional approach to age-related muscle impairment combining assessment of muscle mass, muscle strength, and physical performance. Muscle atrophy should be investigated through the measurement of appendicular lean mass adjusted for body mass index (BMI) (ALM_{BMI}) performed by DXA, considered as the gold standard both in research and clinical practice [59]. The ALM_{BMI} cut points for low muscle mass are <0.789 for men and <0.512 for women [94]. Muscle weakness should be assessed by handgrip strength, measuring the maximum value (in kilograms) of three consecutive trials of the upper dominant limb (with an interval of 1 min after each measurement) through a handheld Jamar dynamometer [94, 95]. The cutoffs for identifying patients with muscle weakness are 16 kg for women and 26 kg for men [94]. Lower limb muscular strength can be measured with isokinetic or isometric technique by the KES, performed by isokinetic testing machine or handheld dynamometer, respectively.

Table 11.1 Clinical approach to skeletal muscle assessment

Parameter	Outcome measure	Tool
Muscle mass	ALM_{BMI}	DXA
Muscle strength	Handgrip strength Knee extension strength	Jamar handheld dynamometer Isokinetic testing machine Handheld dynamometer
Physical performance	Gait speed Sit to stand Balance	SPPB TUG

The mean value (in kilograms) of three consecutive measurements is assumed for each patient [96–98]. The assessment of physical performance should be done through the SPPB [99] or in alternative by the TUG [100], which consists in the measurement of time taken to get up from a chair and walk a distance of 3 m and then back to the chair (Table 11.1).

Efficacy and Effectiveness of Vitamin D and its Metabolites for Muscle Weakness

Data about vitamin D or its hydroxylated derivative administration to improve muscle function are conflicting. Some randomized controlled trials (RCTs) demonstrated that vitamin D supplementation at different doses is able to increase the appendicular muscle strength [101, 102]. In 2011, a systematic review investigated the effect of vitamin D supplementation on muscle strength, suggesting a dose-response relationship. In the 17 RCTs examined, there was no significant effect of cholecalciferol supplementation on muscle strength in patients with serum 25(OH) D above 10 ng/mL, while a significant effect on the lower limb proximal muscle strength was observed in patients with severe vitamin D deficiency [103]. Similar data were derived from a meta-analysis of 13 RCTs including older patients. A daily supplementation of vitamin D (800–1000 IU) was associated with discrete benefits in terms of muscle strength and balance [104]. However, Pfeifer et al., in an 8-month double-blind controlled study including elderly subjects with low serum 25(OH)D levels, demonstrated that the group treated with calcium and cholecalciferol (1000 mg + 800 IU per day) showed significant improvements in both KES and physical performance [105]. In 2013 Ceglia et al. [106] carried out an RCT to investigate the efficacy of oral supplementation with high doses of vitamin D (4000 IU daily) for 4 months on muscle mass (measured as total mass and fiber cross-sectional area, FCSA) in elderly women with hypovitaminosis D (9–24 ng/mL) and impaired physical performance (SPPB ≤ 9). The results showed an increased FCSA (+10%) with additional benefits in terms of VDR concentration (+30%) in muscle biopsies of individuals receiving vitamin D supplementation.

Concerning the efficacy of vitamin D administration on muscle strength, a recent meta-analysis demonstrated that this intervention improved overall muscle strength,

particularly at lower limbs [107]. However, a previous systematic review [108] reporting discordant results about the effects of cholecalciferol supplementation on muscle strength, balance, and gait speed in older people pointed out that the combination of vitamin D and calcium appears to be more effective than vitamin D or calcium supplementation alone. On the other hand, Latham and colleagues [109] previously claimed that there was insufficient evidence to support the benefits of vitamin D supplementation in improving functional performance in elderly population. Another RCT including healthy older men evaluated the effect of daily cholecalciferol and calcium supplementation (1000 IU + 500 mg) for 6 months on muscle strength and physical performance [110] and reported no significant differences in handgrip strength, quadriceps muscle strength, and SPPB, compared with the control group (calcium supplementation alone). Similar results were reported also in a recent systematic review and meta-analysis [111], suggesting that vitamin D and/or calcium supplementation did not improve muscle function in community-dwelling older individuals.

However, vitamin D supplementation appears to enhance the effectiveness of many other interventions, including exercise and/or nutraceutical use, in improving skeletal muscle function, as suggested by several studies. In 2006 [112], Bunout et al. investigating the effects of an exercise program and vitamin D supplementation in elderly individuals with vitamin D deficiency demonstrated significant improvement in physical performance. More recently, Rondanelli et al. [113] showed that combined daily dietary supplementation of whey protein and essential amino acids, vitamin D, and physical activity was able to significantly increase lean body mass (LBM), skeletal muscle mass, and strength in sarcopenic elderly.

However, it is to point out that high-dose, intermittent vitamin D therapy seems to be ineffective or deleterious. In fact high doses of vitamin D did not lead to significant improvement of physical performance, as suggested by Glendenning et al. [114]. In this study performed on 689 older women receiving 150,000 IU of cholecalciferol every 3 months up to 9 months, authors did not report statistically significant differences in terms of muscle strength or muscle performance compared to placebo group. The authors hypothesized that infrequent high-dose administration of cholecalciferol had no beneficial effect on muscle metabolism due to differential actions on gene regulation. However, this dosing regimen probably did not allow maintenance of high serum 25(OH)D levels for a long period, as supported by Gupta et al. [115]. Furthermore, doses higher than 500,000 IU should be avoided, because serious adverse effects have been reported, including increased number of falls (15%) and risk of fracture (26%), particularly in the first 3 months from the end of the treatment and also in the presence of blood levels of 25(OH)D > 30 ng/mL [116, 117]. It has been suggested that higher serum levels of 25(OH)D resulting from these megadose administration might lead to increased expression of 24-hydroxylase, which converts the active metabolite in the inactive form, causing a rapid degradation of the dose with a consequent vitamin D deficiency [118].

Vitamin D Status and Risk of Falls

Undoubtedly, among the extraskeletal effects of vitamin D supplementation, fall risk prevention is provided of the most relevant scientific evidences. These beneficial effects are probably mediated by the action of vitamin D on muscle function. A large prospective 1-year cohort study including 1231 older people demonstrated that lower serum levels of 25(OH)D (<10 ng/mL) were significantly associated with multiple falls, irrespective for confounders such as age, gender, region, season, weight, BMI, and comorbidities [119]. Moreover, authors pointed out that reduced muscle function was a predictive factor of falling in patients with hypovitaminosis D. It should be emphasized that growing evidence highlights how the risk of falling in subjects with vitamin D deficiency might be due to indirect mechanisms, including cognitive and cerebellar impairments [120]. Eriksson et al. claimed that, in nursing home residents, the percentage of older individuals reporting one or more falls is higher in those who also received a diagnosis of dementia (62% vs. 41%) [121]. Moreover, a systematic review demonstrated that lower serum concentrations of 25(OH)D or reduced vitamin D intake was associated with impaired cognitive function tests and/or increased frequency of dementia [122]. Vitamin D seems to play a role in cognitive function. The presence of VDR was also observed in the cerebellar tissue, and its expression is reduced in patients with Alzheimer's dementia [123]. These findings suggested that vitamin D deficiency might be linked to cognitive impairment, particularly in executive functions and speed of processing information, key factors in postural control strategies and reaction patterns to the fall [124]. Furthermore, hypovitaminosis D might be a risk factor for orthostatic hypotension, contributing to the deterioration of autonomy in ADL in older people with a close connection with increased risk of mortality and morbidity [125]. To better understand the role of vitamin D deficiency on cognitive impairments and the risk of falling, it would be necessary to perform randomized trials.

Vitamin D Efficacy and Effectiveness for Fall Prevention

In 2012, a Cochrane review analyzing the efficacy of vitamin D analogues alone or in combination with calcium to prevent or reduce the risk of falling [126] reported no statistically significant differences between the treatment and control groups about the rate of falls in elderly individuals (RR 0.87, 95% CI 0.70–1.08). However, more recent data support vitamin D administration in older people with hypovitaminosis D or history of falls [127, 128]. According to a previous study performed by Bischoff-Ferrari et al., vitamin D supplementation with or without calcium is effective in improving both the proximal muscle strength and the physical performance, resulting in a fall risk reduction of 22% compared to single administration of calcium or placebo [129]. A meta-analysis performed by Bischoff-Ferrari et al.

in 2009 [130], investigating the efficacy of vitamin D supplementation with or without calcium on the prevention of falls in the elderly, concluded that supplementation of 700 to 1000 IU per day of vitamin D reduced the risk of falls of 19% in this population. Moreover, authors claimed that lower dosages did not reduce significantly this risk, presumably because of the failure to reach serum concentrations of 25(OH) D above 24 ng/mL. A reanalysis of the data of the same trials, referring to the recommendations of the Institute of Medicine (IOM), showed that high doses of vitamin D (700–1000 IU/day) reduced the risk of falls by 34% [131]. Pfeifer et al. [105] demonstrated that a daily combined supplementation of calcium and vitamin D at a dose of 1000 mg + 800 IU was associated with a 27% reduction in the risk of a first fall after 1 year of treatment and 39% after 20 months. Bischoff-Ferrari et al. [132], in 2003, observed a reduction of 49% of falls in elderly women hospitalized in a Geriatrics unit receiving a vitamin D supplementation of 800 IU/day. According to the available evidence, it might be plausible that the beneficial effects of vitamin D on muscle strength and the degree of physical performance are greater than those exerted on bone mineral metabolism [80]. The US Preventive Services Task Force had set as a grade B recommendation a daily intake of vitamin D at 600 IU for adults aged between 51 and 70 years and 800 IU for older people (>70 years) to reduce falls [133]. In 2014, the American Geriatrics Society workgroup on vitamin D supplementation in elderly subjects proposed as a minimum objective the attainment of serum 25(OH)D concentration of at least 30 ng/mL, particularly for patients with comorbidities, frailty, and with a higher risk of falls and fracture. The workgroup recommended a daily vitamin D intake of 4000 IU, while 1000 IU was proposed as the lowest effective daily dose to reduce the risk of falling, combined with daily calcium intake from 500 to 1200 mg [128]. The optimal 25(OH)D concentrations should be certainly higher than 30 ng/mL, but probably also higher than 40 ng/mL, in particular to improve muscle function. Serum 25(OH)D levels between 30 and 40 ng/mL may give additional benefits over those performed on target tissues, such as bone, kidney, and bowel, and to reach these serum concentrations, a vitamin D supplementation of at least 2000 IU/day is required [134, 135]. It has been shown that calcifediol increased effectiveness, in terms of knee extension strength, muscle performance measures, and higher and faster increase in 25(OH)D levels compared with the native form [136]. More recently Jetter et al. demonstrated that calcifediol is two to three times more potent in increasing 25(OH)D compared to vitamin D3. There was an increase of 28% and 123%, respectively, after the first and after the last daily dose and an increase of 67% and 178% after the first and the last weekly dose. Moreover, all participants receiving calcifediol reached the 30 ng/mL of serum 25(OH)D threshold after about 2 weeks of treatment, whereas only 70% of women treated with vitamin D3 reached the threshold after more than 2 months [137]. Another double-blind RCT [138] including two groups receiving 20 μg of cholecalciferol (800 IU) or 20 μg of calcifediol reported an increase of serum 25(OH)D three times higher, a double improvement in muscle strength, improvement in gait speed (+18%), and balance in calcifediol group versus vitamin D3 group at 6 months. In contrast, a recent RCT evaluating the effectiveness of high doses (24000–60,000 IU) of vitamin D to improve lower limb function and limit

functional decline after 1 year of treatment did not show improvement in muscle function of the lower limbs. Interestingly, an even higher fall risk, compared to the control group, was reported in the higher-dose groups, even though serum concentration of 25(OH)D exceeded 30 ng/mL [139].

Data published in the literature are often contradictory and inconclusive probably because the elderly population is extremely heterogeneous. Usually, community-dwelling older individuals are younger and have better health conditions than those who are institutionalized [140]. Therefore, it would be more correct to distinguish between these groups in the analysis of the effectiveness of dietary, pharmacological, or rehabilitation approaches to prevent the muscle wasting and the decrease of functional performance [107, 141]. Furthermore, the reported trials have heterogeneous study designs, concerning the type and duration of the interventions, and outcome measures, particularly for the assessment of muscle strength and function.

Conclusions

The field of vitamin D effects on skeletal muscle health is receiving more and more attention. We are learning that this hormone, as well as other steroids, functions in target tissues by binding to a specific and dynamic intracellular receptor that is then transferred to the nucleus to facilitate gene transcription for several proteins, including calcium and phosphorus transport proteins, many of which are still relatively unknown. The detection of VDR on skeletal muscle increased the knowledge on the mechanisms of action of vitamin D in this tissue. Therefore, the role of this hormone in muscle health remains an exciting research area with substantial clinical implications. The association between vitamin D deficiency and age-related muscle wasting, such as sarcopenia, has a considerable impact in terms of social and healthcare costs. Preclinical and clinical data suggest that vitamin D supplementation might contribute to maintenance or enhancement of muscle function. Furthermore, this hormone is inexpensive, safe, and, according to available evidences, particularly effective in patients with vitamin D deficiency in terms of serum 25(OH)D increase, improvement of muscle function, and prevention of falls.

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Chapter 12
Vitamin D and the Central Nervous System: Development, Protection, and Disease

Samantha Roman and Ellen M. Mowry

Abbreviations

Aβ	Amyloid-β
AD	Alzheimer disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood-brain barrier
CDMS	Clinically definite multiple sclerosis
CIS	Clinically isolated syndrome
CNS	Central nervous system
DVD	Developmental vitamin D
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NSC	Neural stem cell
PD	Parkinson disease
RRMS	Relapsing-remitting multiple sclerosis
SPMS	Secondary progressive multiple sclerosis
VDR	Vitamin D receptor

Vitamin D is a neurologically active secosteroid essential for the proper development and functioning of the central nervous system (CNS). Despite the expanse of knowledge in the last several decades, many questions still remain about the role vitamin D plays within the CNS. This chapter will review the current understanding of how vitamin D is involved in CNS development and protection as well as the risk,

S. Roman, MD • E.M. Mowry, MD MCR (✉)
Johns Hopkins School of Medicine, Baltimore, MD, USA
e-mail: emowry1@jhmi.edu; sroman6@jhmi.edu

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prognosis, and treatment of several neurodegenerative disorders. Throughout this chapter, "vitamin D levels" or "vitamin D status" will refer to the total serum concentration of 25-hydroxyvitamin D, the inactive circulating vitamin D metabolite, while "vitamin D" will refer specifically to 1,25-dihydroxyvitamin D_3, the active vitamin D_3 metabolite, unless otherwise specified.

The Presence of Vitamin D in the Nervous System

Vitamin D acts similar to other steroid hormones at the cellular level. Following ligand binding, the vitamin D receptor (VDR) forms homodimers or heterodimers with the retinoid X receptor, which bind to vitamin D response elements on target genes to directly regulate gene expression [1]. Notably, while the VDR is found at the plasma membrane in renal and liver cells, it is predominantly a nuclear receptor in the CNS, likely reflecting its role in regulating gene transcription, rather than calcium homeostasis [2]. In the 1980s, the earliest studies implicating a role for vitamin D in the nervous system demonstrated the ability to cross the blood-brain barrier (BBB) and bind to receptors in the CNS [3, 4]. Mapping of VDR expression in the brain using immunohistochemical techniques demonstrated a wide distribution throughout sensory, motor, and endocrine-autonomic regions of the rodent brain, findings which were confirmed in the late 1990s with VDR-targeted antibodies [5, 6].

Later studies revealed a similar pattern in the human CNS, demonstrating the presence of VDR and 1α-hydroxylase, the enzyme that catalyzes the synthesis of 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3, in neurons, glial cells, and pericytes throughout most areas of the human brain [7, 8]. Pericytes, cells that surround CNS vasculature and contribute to the BBB function, also express 25-hydroxylase, the enzyme required to synthesize 25-hydroxyvitamin D_3. Pericytes are able to upregulate 25-hydroxylase, 1α-hydroxylase, and VDR expression in response to inflammatory stimuli, not only indicating the presence of a vitamin D paracrine/autocrine pathway within the CNS but also the existence of a local, coordinated mechanism to respond to tissue injury [8].

The Neuroprotective Role of Vitamin D in the Central Nervous System

In vitro and, to a lesser extent, animal studies show vitamin D provides protection against a variety of CNS insults, including ischemia, reperfusion injury, glutamate excitotoxicity, and oxidative damage [9–12]. While there is evidence vitamin D regulates expression of various BBB efflux transporters, thus decreasing exposure to toxins, most of the neuroprotective effects are attributed to immunomodulation [13]. Vitamin D reduces production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNFα and increases expression of anti-inflammatory signals like

IL-10 in vitro, reducing inflammation and decreasing microglial activation [14, 15]. Microglia are the innate immune cells of the CNS, and chronic microglial activation, leading to chronic inflammation, has been implicated in many neurodegenerative disorders [16]. Vitamin D also modulates the adaptive immune response, promoting a more regulatory T cell immunophenotype and reducing B cell immunoreactivity [17, 18].

Vitamin D and Neurodevelopment

In addition to its immunomodulatory actions, vitamin D signaling also triggers prodifferentiation and antiproliferative effects, providing potential protection from CNS malignancies and playing an important role in neurodevelopment [1, 19, 20]. Neural stem cells (NSCs) are pluripotent cells with the ability to differentiate into neurons, astrocytes, and oligodendrocytes and are vital to CNS development and repair [21]. NSCs constitutively express VDR and, in response to vitamin D signaling, upregulate VDR expression, creating a positive feedback loop [22]. Administration of vitamin D not only protects NSCs from neurotoxic insults, but enhances expression of neurotrophic factors, including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and neurotrophin 3 (NT3), which promote differentiation of NSCs into neurons and oligodendrocytes [22, 23]. Oligodendrocytes, the cells responsible for myelination in the CNS, are vital to CNS repair and require VDR signaling in order to differentiate from progenitor cells [24].

Vitamin D likely plays an important role in early brain development, as VDR is expressed almost immediately after neural tube closure. Throughout in utero development, VDR continues to be expressed in the CNS, most prominently in neuroepithelium and actively differentiating areas of the brain, spinal cord, and dorsal root ganglia [25]. Research utilizing the developmental vitamin D (DVD)-deficient animal model has helped elucidate some of the important roles vitamin D plays in brain development. DVD-deficient rats are born to mothers who are vitamin D deficient throughout gestation. They are given vitamin D supplementation at birth and typically reach normal serum vitamin D levels within 2–3 weeks [26].

Alteration in Brain Morphology

Although there is evidence that VDR is at least partly autoregulated by available vitamin D levels in adult models, DVD-deficient rats have normal VDR expression at birth, indicating in utero VDR expression does not depend on vitamin D availability [2, 27]. Genomic studies have demonstrated the binding of VDR by vitamin D results in alterations of many genes, some of which ultimately function to inhibit cell proliferation [1]. Consistent with this data, DVD-deficient rat pups have an

increased number of immature and mitotic neural cells at birth in the hippocampus, hypothalamus, and basal ganglia, without a corresponding increase in apoptosis. As a result, these pups have altered brain morphology when compared to healthy pups, including 30% longer cortical hemispheres, 200% larger lateral ventricles (seemingly due to a thin neocortex rather than excess cerebrospinal fluid), and decreased expression of neurotrophic factors in the CNS, including nerve growth factor (NGF), GDNF, and $p75^{NTR}$ [26]. These findings are interesting, given that vitamin D deficiency has been linked to schizophrenia, for which a hallmark radiologic finding is enlarged lateral ventricles [28].

Long-Lasting Effects of Vitamin D Deficiency In Utero

Maternal vitamin D deficiency not only affects early brain development in offspring but can also result in long-lasting changes in the structure and function of the adult rat brain. For example, the enlarged lateral ventricles and reduced NGF expression persist into adulthood for DVD-deficient offspring, even after reintroduction of vitamin D through supplementation [29]. As adults, these animals exhibit abnormal expression of genes involved in cytoskeleton maintenance (MAP2, NF-L) and neurotransmission (GABA-Aα4), as well as abnormal expression of proteins involved in synaptic plasticity and mitochondrial function [29, 30]. There may be a critical period in late pregnancy during which vitamin D deficiency results in abnormal adult phenotypes in rats [31]. While most of the research using an animal model of maternal vitamin D deficiency has been performed with rats, the few studies using mice have observed several opposite morphological brain changes at birth, including decreased length, head size, and lateral ventricle volume [32]. This discrepancy highlights the physiologic differences between animal models and limitations to generalizing results to humans.

Outcomes in Humans

Human studies evaluating the effect of maternal vitamin D deficiency on offspring have typically utilized large prospective cohorts and have focused on whether an association exists between prenatal maternal vitamin D levels and various markers of neurocognitive development in children. One study found no significant independent relationship between third trimester maternal vitamin D level and cognitive or psychological outcomes in children over a 9-year follow-up period [33]. However, another found in multivariate models that maternal vitamin D status in the first half of pregnancy was positively and linearly associated with mental and psychomotor development at 14 months of age [34]. Two additional studies found a positive relationship in multivariate models between second trimester maternal vitamin D level and language development, with nearly twofold higher rates of developmental

language difficulties at 5 and 10 years in children whose mothers were vitamin D deficient (<46 nmol/L) [35, 36].

Studies investigating neonatal vitamin D levels from cord blood samples have shown little to no evidence for an association with cognitive development, intelligence, or behavior [37, 38]. Research evaluating neurocognitive outcomes in adolescence and beyond are limited in humans, but thus far one study found no association between third trimester maternal vitamin D level and diagnosis of attention-deficit hyperactivity disorder (ADHD), clinical depression, or standardized exam scores over a 22-year period [39]. While the results of human studies are somewhat inconsistent, they suggest that sufficient maternal vitamin D levels may be important early in pregnancy for proper language development in offspring.

Vitamin D Status and Brain Volume

Unlike in rodents, maternal vitamin D status and offspring brain morphology has not been studied in humans; however, several papers have been published assessing brain volume in adults relative to vitamin D status. A 2014 meta-analysis of nine animal and cross-sectional human studies found serum vitamin D levels were positively associated with brain volume and negatively associated with lateral ventricle size. However, two subsequent cross-sectional studies showed the opposite relationship—in both young and elderly adults, higher serum vitamin D levels were associated with decreased brain volume [40–42]. The authors hypothesized a smaller brain volume may be due to the antiproliferative, pro-apoptotic effects of vitamin D or earlier bone maturation leading to skull maturation at a smaller size. Yet, without prospective data to guide our understanding of causality in this relationship, it remains unclear if and how vitamin D levels affect brain size.

Cognition

There is currently a limited understanding of the relationship between vitamin D levels and cognitive function. Animal studies demonstrate deficient vitamin D levels correlate with increased markers of oxidative and nitrosative stress in the brain, consistent with changes seen at the cellular level in the brain with cognitive impairment [43]. Genetic studies of polymorphisms in the VDR gene provide some evidence that alterations in the VDR may influence overall longevity, cognitive performance, and susceptibility to both age-related cognitive decline and depressive symptoms in older adults [44–47].

Multiple epidemiological studies have been conducted in an attempt to determine whether a relationship exists between vitamin D levels and cognition. However, as with brain volume and most of the neurodegenerative disorders discussed in the following section, it is difficult to infer causality between vitamin D deficiency and

cognitive decline given that the majority of studies evaluating these associations are observational, cross-sectional studies. A limited number of prospective studies evaluating cognitive decline among healthy, older adults have been published with conflicting outcomes. One study demonstrated a significantly higher relative risk of cognitive decline over 6 years among vitamin D-deficient elderly adults, while another showed no significant association between the two [48, 49]. A later study with both cross-sectional and prospective components demonstrated a correlation between vitamin D levels and cognitive performance in participants over 65 years of age, with vitamin D levels being predictive of cognitive performance 7–13 years later [45]. Few randomized controlled trials (RCTs) evaluating vitamin D and cognition have been conducted, although a very recent study involving older adults without dementia showed high-dose vitamin D supplementation (4000 IU/day) over 18 weeks improved nonverbal memory [50]. More RCTs are needed to clarify the relationship between cognition and vitamin D, which remains an area of active research.

CNS Disorders

As our understanding of the role vitamin D plays in immune function and inflammation expands, researchers and medical providers are gaining insight into how vitamin D is involved in the development, course, and treatment of specific neurologic diseases. There is strong evidence that links vitamin D deficiency to multiple sclerosis and a growing body of research associating it with Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis as well. Particularly notable is that these four disorders, while varied in many clinical aspects, share common pathophysiological characteristics including increases in oxidative stress, inflammation, mitochondrial dysfunction, and cell death. Throughout the remainder of this chapter, we will summarize the existing literature regarding each of these diseases and the potential role of vitamin D in disease risk, prognosis, and potential treatment options.

Parkinson Disease

Parkinson disease (PD) is a neurodegenerative disease in which specific destruction of dopaminergic neurons in the substantia nigra occurs with accumulation of α-synuclein cytoplasmic inclusions called Lewy bodies, resulting in movement abnormalities such as tremor, bradykinesia, rigidity, and postural instability. Vitamin D has been implicated in the development and proper functioning of dopaminergic neurons, and research is ongoing to elucidate the specific role vitamin D plays in the development of PD [51].

Risk

Patients with PD tend to have higher rates of vitamin D deficiency than age-matched healthy controls and patients with AD, which has also been associated with low vitamin D levels [52–54]. However, causality has yet to be determined; while vitamin D deficiency may be a risk factor for PD, having PD may also increase the risk of vitamin D deficiency, with immobility reducing sunlight exposure or leading to malnutrition. Most of the existing data are cross-sectional, and although two recent prospective cohort studies were published on this topic, the results are varied. Data from the Mini-Finland Health Survey, a 29-year cohort with 50 incident PD cases, indicated a relative risk of 0.33 in participants with baseline vitamin D levels ≥50 nmol/L compared to <25 nmol/L [55]. However, evaluation of data from the Atherosclerosis Risk in Communities (ARIC) study, a 17-year cohort with 67 incident PD cases, found no significant relationship between baseline vitamin D level and incident PD [56]. Notably, the former study had nearly double the follow-up duration and significantly lower baseline vitamin D levels across the sample population than the latter.

Genetic studies in many diseases linked to vitamin D, including PD, have investigated specific VDR single nucleotide polymorphisms (SNPs). Polymorphisms are genetic variants that can affect gene expression or protein composition, creating the potential to cause or increase risk of developing a disease. Some of the common VDR polymorphisms studied in neurodegenerative disorders include *TaqI*, *BsmI*, and *ApaI*, which are found in exons 8 and 9 in a region with unknown function, and *FokI*, which is found in exon 2 and involves a T to C change within a start codon, yielding a longer VDR protein [57]. A meta-analysis in 2014 found no association between *BsmI*, *ApaI*, or *TaqI* VDR polymorphisms and PD risk, but two meta-analyses the following year including the *FokI* polymorphism found an association between *FokI* and PD [58–60]. Specifically, the *FokI* C allele, resulting in the longer VDR protein, was associated with an increased risk of PD (OR 1.41, 95% CI 1.14–1.75), which was even greater with the homozygous CC versus TT *FokI* genotype (OR 2.45, 95% CI 1.52–3.93) [60].

Prognosis

Several studies have evaluated vitamin D as a prognostic indicator of PD severity, with varied results. While one evaluation of Chinese patients with PD observed lower serum vitamin D levels with longer disease duration and greater disease severity, a similar study of Iranian patients showed no association [61, 62]. Gatto et al. found the *FokI* C allele correlated with faster cognitive decline; on the contrary, Suzuki et al. observed milder disease in patients with the *FokI* CC genotype [63, 64]. Despite differences in patient population and analyses, the methodology of these studies remains limited by their cross-sectional design and thus an inability to determine the causative direction of any relationship that may exist.

Treatment

Several studies utilizing a preclinical mouse model of PD have suggested vitamin D may have a therapeutic benefit by decreasing inflammation, protecting dopaminergic neurons from glutamate neurotoxicity, and promoting recovery of dopaminergic functioning in injured nigrostriatal neurons [65–67]. Studies with rotenone-induced neurotoxicity PD models illustrate the ability of vitamin D to induce autophagy, an intracellular degradation process, which is hypothesized to be abnormal and potentially contribute to pathophysiology in PD [68–70]. The only double-blind RCT in humans testing the efficacy of vitamin D as a PD therapy found 1200 IU daily vitamin D_3 supplementation slowed PD progression over 12 months, but that the effect was modified by the *FokI* polymorphism [71]. Patients with the wild-type TT *FokI* genotype had a strong, consistent response to vitamin D supplementation, while patients with the heterozygous *FokI* genotype had a moderate response, and patients with the CC *FokI* genotype had no response, experiencing a similar level of clinical deterioration as the placebo group. No association was seen with the *BsmI*, *Cdx2*, *ApaI*, *TaqI*, and *GC1* polymorphisms [71]. While further research must be done to evaluate the utility of vitamin D as a PD treatment, these results suggest genotype influences the response to vitamin D and highlights the importance of precision medicine in disease prognosis and treatment.

Alzheimer Disease

Alzheimer disease (AD) is a neurodegenerative disease characterized by progressive loss of memory and cognitive function. The pathophysiology is marked by brain accumulation of amyloid-β (Aβ) plaques and hyper-phosphorylated tau protein neurofibrillary tangles, which are neurotoxic, leading to mitochondrial dysfunction, increased oxidative stress, persistent microglial activation, and a chronic inflammatory state [72, 73].

Risk

Patients with AD have been shown to have significantly higher rates of vitamin D deficiency than healthy, age-matched controls [74]. Several large, prospective cohorts have been studied to evaluate the relationship between vitamin D status and Alzheimer disease, with conflicting findings. Data from the Cardiovascular Health Study and the Copenhagen City Heart Study both showed evidence of an association between vitamin D deficiency and increased risk of all-cause dementia and AD [75, 76]. Data from the Framingham Heart Study found an association between lower vitamin D levels and decreased cognitive function and hippocampal volume, but no association with incident all-cause dementia or AD [77]. As with PD, it is

impossible to determine whether low vitamin D levels are a cause or effect of AD. In fact, the pathophysiology of AD may directly reduce vitamin D availability and action in the CNS, as the presence of Aβ suppresses VDR expression while simultaneously increasing expression of 24-hydroxylase (CYP24A1), the enzyme required for vitamin D catabolism [78].

Genetic studies evaluating VDR polymorphisms as potential AD risk factors have found a possible association between *ApaI* and AD, but limited or no evidence for an association with *FokI*, *TaqI*, or *BsmI* [79–81]. Where an association is found to exist, the potential risk conferred by the VDR polymorphism seems to be ethnicity-dependent [82]. Surprisingly, genetic studies have demonstrated that the apolipoprotein E (ApoE) ε4 allele, a known genetic risk factor for AD, is associated with higher vitamin D levels in both mice and humans [83]. Further investigation showed the vitamin D deficiency prevalent among patients with AD is predominantly found in patients without the ε4 allele, indicating vitamin D deficiency may be a larger contributing risk factor in these patients [84].

Prognosis

There is some evidence that vitamin D deficiency is associated with accelerated cognitive decline in elderly patients, but these results are from a general population [85]. Little research has been done focusing on vitamin D as a prognostic factor specifically in AD.

Treatment

A major goal in treating AD is decreasing Aβ levels and pro-inflammatory cytokines. Encouraging results have demonstrated that vitamin D supplementation reduces Aβ levels, amyloid plaque formation, and inflammation in animal models of AD [73, 86]. Vitamin D supplementation also results in upregulation of Mdr1a/P-gp, a blood-brain barrier efflux transporter implicated in Aβ secretion, effectively reducing the amount of Aβ in the CNS [13, 87]. In a study utilizing a mouse model of AD, P-gp induction with vitamin D treatment prior to Aβ plaque formation resulted in lower levels of soluble and insoluble Aβ in the CNS, while P-pg induction after plaque formation resulted in decreased soluble Aβ, but had no effect on the insoluble Aβ already incorporated into plaques [88]. These results suggest vitamin D supplementation may play a preventative as well as therapeutic role in limiting Aβ plaques formation and progression of AD.

In humans, there has only been one RCT to date evaluating vitamin D supplementation in AD patients. In that study, the authors did not find an improvement in cognition or level of disability after 8 weeks of 1000 IU daily oral vitamin D_2 [89]. Unfortunately, until more large RCTs are conducted, and perhaps with varied doses, it will be unclear whether vitamin D supplementation has a therapeutic benefit for patients with AD.

ALS

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease in which progressive destruction of upper and lower motor neurons occurs over months to years, eventually leading to death. The mechanism of damage is multifactorial, including glutamate excitotoxicity, free radical damage, mitochondrial dysfunction, autoimmune inflammation, and accumulation of intracellular calcium leading to caspase-mediated apoptosis [90, 91].

Risk

There is relatively weak evidence that vitamin D deficiency plays a significant role in ALS risk, and research specifically investigating the association of vitamin D deficiency or VDR polymorphisms with ALS is limited [92]. The earliest study analyzing VDR polymorphisms in ALS patients did so in the context of evaluating susceptibility to lead exposure, the primary ALS risk factor being studied, and no significant association was found [93]. Only one other study, which was published in 2016, addressed VDR polymorphisms as a potential ALS risk factor. The authors of that study found the A allele of the *ApaI* VDR polymorphism, which encodes a larger protein than the C allele, was significantly more common among ALS patients than healthy controls and may potentially be one genetic risk factor. That study did not find associations between ALS and the *BsmI*, *TaqI*, or *FokI* VDR polymorphisms [91].

Prognosis

Research studies evaluating vitamin D levels and ALS progression have had conflicting results. Camu et al. proposed serum vitamin D levels be used as a prognostic factor in ALS after completing a retrospective analysis showing ALS patients with severe vitamin D deficiency progressed four times more rapidly than patients with normal vitamin D levels and survived a median of 29.5 months compared to 52.8 months [94]. Additionally, vitamin D deficiency in the mouse model of ALS has been noted to worsen motor performance and exacerbate pathophysiology in the spinal cord [95, 96]. However, in contrast to these findings, a prospective study with 125 ALS patients found that higher vitamin D levels were actually associated with worse clinical outcomes, although this association was weak ($p = 0.06$) [97]. A more recent retrospective analysis concluded that ALS prognosis is not associated with vitamin D levels at all, but rather age at onset and the presence or absence of bulbar features [98]. Thus, whether vitamin D levels have any prognostic value in ALS remains to be determined.

Treatment

The ability of vitamin D to potentiate neurotrophic factors in motor neurons, as well as to rescue motor neurons from Fas-induced cell death in vitro, provided encouraging results that vitamin D may be helpful in the treatment of ALS [94]. Furthermore, studies utilizing the mouse model of ALS indicate vitamin D supplementation at a dose ten times an "adequate" intake improves motor performance, although supplementation at higher doses (50 times the "adequate" intake) fails to provide additional benefit and increases the risk of vitamin D_3 toxicity [99, 100]. In a retrospective review of human ALS patients, those given 2000 IU vitamin D_3 supplementation by their personal physicians had slightly slower decline at 9 months than patients without supplementation, but this difference was not observed at 3, 6, and 12 months; it is also not clear how these patients may have systematically differed from those patients who were not recommended to take supplements [101]. Despite functional improvements with vitamin D supplementation in both mouse and human studies, no significant effect on disease onset, progression, or survival has been documented [102]. Although it has been determined that supplementation of 2000 IU daily is safe for ALS patients, due to the small number of existing studies and limitations in study design, it remains unknown whether vitamin D supplementation is truly beneficial in slowing the progression of ALS.

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by CNS inflammation and demyelination causing, for most, relapsing and remitting neurologic symptoms which, for a subset of patients, eventually transitions to a progressive deterioration, with a fraction of individuals experiencing only progressive symptoms from disease onset. Of all the neurodegenerative disorders in which vitamin D deficiency has been implicated, the most research and strongest evidence exists for MS.

Risk

Environmental risk factors seem to play a predominant role in the development of MS, demonstrated by a pattern of higher MS prevalence at latitudes farther from the equator, migration studies showing risk depends on area of residence, and twin studies showing only 30% concordance among monozygotic twins [103–106]. A groundbreaking study in 2006 demonstrated a significant inverse relationship between serum vitamin D levels and MS risk in adult Caucasian patients, with a

particularly strong effect for vitamin D status prior to 20 years of age [107]. Vitamin D levels early in life may be particularly important, as maternal vitamin D deficiency in the first trimester as well as low neonatal vitamin D levels increases the risk of developing MS in adulthood [108, 109]. Furthermore, low sun exposure in adolescence has been shown to correlate with earlier age at MS onset, and the migration studies that have been done indicate the risk of MS is greatest with relocation to high-prevalence regions in the first two decades of life [105, 110]. However, a study investigating vitamin D status and evidence of cumulative sunlight exposure showed that while higher vitamin D levels were associated with a decreased risk of developing MS, interestingly, cumulative sunlight exposure had an independent, protective association that was even stronger than vitamin D level [111]. These results do raise the question about whether it is truly vitamin D levels, or UV light exposure (which may have immunomodulatory properties independent of vitamin D), that are truly important in MS [112].

A large body of literature has been published evaluating potential genetic risk factors of MS, and while the HLA locus DRB1*1501 is a known risk factor, case-control investigations into vitamin D enzyme and receptor polymorphisms have been less conclusive [110]. A large meta-analysis summarizing much of the data suggested the *ApaI* and *FokI* polymorphisms may be significant risk factors for MS, with the *TaqI* T allele exerting a protective effect, but also noted individual study results varied by methodology [113]. In a prospective cohort analysis, patients with the homozygous wild-type *FokI* TT genotype had an 80% reduced MS risk with 400 IU daily vitamin D supplementation (unclear formulation). In an interesting parallel to the response seen in PD patients with the same genotype, vitamin D supplementation in MS patients with the altered CC *FokI* genotype did not seem to confer any protection against disease development [71, 114].

Prognosis

Vitamin D status appears to influence disease course after a clinically isolated syndrome (CIS), a single MS-like episode prior to meeting MS diagnostic criteria, as well as after diagnosis of clinically definite MS (CDMS). Patients with vitamin D levels ≥50 nmol/L at the time of CIS diagnosis have lower risk of conversion from CIS to CDMS, decreased radiological evidence of disease, and less disability than patients with levels <50 nmol/L [115]. Furthermore, higher vitamin D levels are associated with preserved gray matter volume in CIS patients, which is significant given that gray matter atrophy correlates to greater levels of disability in patients with MS [116]. Lower vitamin D levels are associated with an increased relapse rate in CIS as well as CDMS, which is a consistent finding in both pediatric-onset and adult-onset MS [115–121]. In fact, for every 10 nmol/L higher serum vitamin D level, patients with MS may experience up to a 12% reduction in risk of relapse [120]. Furthermore, vitamin D has been implicated in progression from relapsing-remitting MS (RRMS) to secondary progressive MS (SPMS). SPMS patients tend to have lower vitamin D levels, and a retrospective analysis demonstrated that

patients with a faster progression to SPMS also had lower vitamin D levels at the time of their MS diagnosis [122].

Prevention and Treatment

Studies evaluating the efficacy of vitamin D as both a preventative and therapeutic agent in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), have shown promising results. Vitamin D protects neurons from T cell-mediated killing in vitro, and when administered to mice prior to EAE induction reduces disability, CNS inflammation, and axonal damage [123, 124]. In a cuprizone-mediated demyelination mouse model, high-dose vitamin D injected intraperitoneally compared to placebo results in faster clearance of damaged myelin followed by greater numbers of mature oligodendrocytes and higher levels of remyelination [125]. As an alternative to the immunosuppressive therapies currently used to treat MS, some researchers are now focusing on development of a vaccine to prevent development of MS in the first place. One such vaccine, consisting of vitamin D and myelin oligodendrocyte glycoprotein (MOG) injected intraperitoneally, effectively prevents development of EAE in mice, while neither vitamin D nor MOG independently achieves a similar result [126].

In humans, research has not yet focused on MS vaccination, but does suggest vitamin D supplementation is beneficial. RCTs have demonstrated short-term vitamin D_3 supplementation up to 10,400 IU daily is safe, tolerable, and effective in increasing serum vitamin D levels in patients with MS [127]. Interestingly, Caucasian women with MS experience a smaller increase in plasma vitamin D levels than healthy controls when given the same oral dose [128]. Additionally, a gender disparity exists in response to vitamin D supplementation; females may experience a stronger anti-inflammatory effect than males do, which has been attributed to potential synergy between vitamin D and estradiol signaling [129]. Nonetheless, administration of high-dose vitamin D (10,400 IU) daily to both male and female MS patients results in significant changes to immunophenotype in vivo, including a reduction in circulating IL-17-producing $CD4^+$ T cells [127].

Administration of vitamin D to improve symptoms and progression of MS is currently an active area of research. Preliminary results from the SOLAR trial presented at the 2016 ECTRIMS (European Committee for Treatment and Research in Multiple Sclerosis) conference indicate high-dose (14,007 IU/day) vitamin D_3 supplementation did not affect disease progression, or the proportion of patients free of disease activity,[1] which was the authors' primary endpoint, over a 4-year follow-up period. However, vitamin D supplementation did significantly decrease the number of active MS lesions seen on MRI, one of the authors' secondary endpoints [130]. Other data suggest that high-dose vitamin D supplementation may result in improved mental health and quality of life for patients with MS [131]. Results from additional ongoing trials of vitamin D supplementation and MS are pending [132].

[1] Disease activity was defined as relapse, Expanded Disability Status Scale (EDSS) score progression, new gadolinium-enhancing T1 lesions, or new or enlarging T2 MRI lesions.

Conclusion

After several decades of research, vitamin D is now recognized as a neurologically active secosteroid with a role in CNS development and function. However, a comprehensive understanding of the far-reaching downstream effects of vitamin D signaling is currently unavailable, and many questions remain regarding the role of vitamin D in human neurologic disease. Research suggests vitamin D likely plays a role in the pathogenesis and progression of MS, while an association between vitamin D and the risk, prognosis, or treatment of AD, PD, and ALS is less apparent. Looking to the future, additional prospective studies and placebo-controlled randomized clinical trials are necessary to determine the relationship between vitamin D and neurologic diseases.

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