

# The Nonskeletal Effects of Vitamin D: An Endocrine Society Scientific Statement

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Significant controversy has emerged over the last decade concerning the effects of vitamin D on skeletal and nonskeletal tissues. The demonstration that the vitamin D receptor is expressed in virtually all cells of the body and the growing body of observational data supporting a relationship of serum 25-hydroxyvitamin D to chronic metabolic, cardiovascular, and neoplastic diseases have led to widespread utilization of vitamin D supplementation for the prevention and treatment of numerous disorders. In this paper, we review both the basic and clinical aspects of vitamin D in relation to nonskeletal organ systems. We begin by focusing on the molecular aspects of vitamin D, primarily by examining the structure and function of the vitamin D receptor. This is followed by a systematic review according to tissue type of the inherent biological plausibility, the strength of the observational data, and the levels of evidence that support or refute an association between vitamin D levels or supplementation and maternal/child health as well as various disease states. Although observational studies support a strong case for an association between vitamin D and musculoskeletal, cardiovascular, neoplastic, and metabolic disorders, there remains a paucity of large-scale and long-term randomized clinical trials. Thus, at this time, more studies are needed to definitively conclude that vitamin D can offer preventive and therapeutic benefits across a wide range of physiological states and chronic nonskeletal disorders. (*Endocrine Reviews* 33: 456–492, 2012)

- I. Introduction
- II. Distribution, Structure, and Function of the Vitamin D Receptor
  - A. Background
  - B. VDR distribution
  - C. VDR structure
  - D. Role of coactivators and corepressors
  - E. Plasticity of the VDRE
  - F. Nongenomic actions of vitamin D
- III. Vitamin D and the Skin
  - A. Introduction
  - B. Proliferation, differentiation, barrier function of skin
  - C. Coactivators and corepressors of vitamin D in skin
  - D. Hair follicle phenotype, 1,25-(OH)<sub>2</sub>D independence, molecular interactors, and targets
  - E. Translational studies and clinical trials of vitamin D and skin
- IV. Vitamin D and Its Relationship to Obesity and Diabetes Mellitus
  - A. Introduction
  - B. Observational studies of the relationship of vitamin D to obesity and the metabolic syndrome
  - C. Randomized trials of vitamin D in obesity, type 2 diabetes mellitus
  - D. Conclusions
- V. Vitamin D for the Prevention of Falls and Improvement in Quality of Life
  - A. Introduction
  - B. Observational studies of vitamin D and falls
  - C. Randomized trials of vitamin D on falls
  - D. Effects of vitamin D supplementation on pain and quality of life
- F. Conclusions

Abbreviations: BMI, Body mass index; CI, confidence interval; CV, cardiovascular; CVD, CV disease; 1,25D-MARRSBP, 1,25-(OH)<sub>2</sub>D membrane-associated rapid response steroid-binding protein; DRIP, VDR-interacting protein; HAT, histone acetyl transferase; HR, hazard ratio; IFN- $\gamma$ , interferon- $\gamma$ ; LBD, ligand-binding domain; LEF1, lymphoid enhancer-binding factor-1; LPS, lipopolysaccharide; mTB, *Mycobacterium tuberculosis*; NR, nuclear receptor; NOD, nonobese diabetic; 1,25-(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; OR, odds ratio; PRR, pattern recognition receptor; RCT, randomized clinical trial; RR, relative risk; RXR, retinoid X receptor; SRC, steroid receptor coactivator; TLR, Toll-like receptor; UCP, uncoupling protein; VDR, vitamin D receptor; VDRE, vitamin D response element.

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- E. Conclusions
- VI. Vitamin D and Cancer
  - A. Introduction
  - B. Total cancer and cancer mortality: research findings
  - C. Vitamin D and the risk of site-specific cancers
  - D. Other site-specific cancers
  - E. Conclusions
- VII. Vitamin D and Cardiovascular Disease
  - A. Introduction
  - B. Studies of hypertension and lipids
  - C. Studies of other CVD endpoints
- VIII. Vitamin D and Immune Function
  - A. Introduction
  - B. Clinical observations and trials
  - C. Conclusions
- IX. Vitamin D, the Placenta, and Maternal/Fetal Health
  - A. Introduction
  - B. Biological plausibility
  - C. Animal data
  - D. Observational and association studies
  - E. Randomized interventional trials
  - F. Conclusions
- X. Summary and Future Direction

## I. Introduction

The 100th anniversary of the identification of a factor whose deficiency was linked to the development of rickets, and was later found to be cholecalciferol, is approaching. Painstaking work by a number of laboratories over the last nine decades convincingly demonstrated that cholecalciferol not only was essential for skeletal health but also was a hormone mediating nonclassical tissue effects across a wide range of homeostatic functions. The physiology of vitamin D from its synthesis in the skin to its active form, 1,25-dihydroxyvitamin D [ $1,25-(\text{OH})_2\text{D}$ ], was fully defined by the mid-1970s (Fig. 1, A and B). However, the cloning of the vitamin D receptor (VDR) did not occur until 1987, and its subsequent identification in virtually all tissues spurred further basic and clinical studies and led to a much greater appreciation of the physiological role of vitamin D (Fig. 2). At the same time, interest in vitamin D as a therapeutic modality for the prevention of chronic diseases grew exponentially. Indeed, in a 2-month span during the summer of 2011, there were more than 500 publications centered on vitamin D, most of which were related to its relationship to nonskeletal tissues. However, the results from those studies, as well as others, are confounded and difficult to interpret. In this Scientific Statement we seek to outline the evidence that defines the effects of vitamin D on epidermal, neuromuscular, cardiovascular (CV), metabolic, immunological, maternal/fetal, and neoplastic tissues. Before reviewing the evidence in these areas, we first present an overview of the VDR

because this molecule represents the final common pathway through which vitamin D works on nonskeletal tissues. We next critically evaluate the literature for each organ system, beginning with the biological plausibility of an association, followed by utilization of the available evidence from observational studies and randomized trials, to delineate the strength of associations between serum 25-hydroxyvitamin D [ $25(\text{OH})\text{D}$ ] and/or dose of vitamin D supplementation and tissue-specific outcomes.

Several reviews of the skeletal effects of vitamin D have recently been published, including The Endocrine Society's Clinical Practice Guideline on vitamin D deficiency and the complete Institute of Medicine (IOM) Report on Calcium and Vitamin D (1–3). It is important to note that after the publication of these two summaries, our work took on additional significance, particularly in relation to nonskeletal effects of vitamin D, as the controversy surrounding the definition of a target serum level of  $25(\text{OH})\text{D}$  reached new heights. Importantly, this Scientific Statement represents the first comprehensive evaluation of both the basic and clinical evidence related to the effects of vitamin D on nonskeletal tissues.

## II. Distribution, Structure, and Function of the Vitamin D Receptor

### A. Background

Vitamin D is a steroid hormone, and the active metabolite,  $1,25-(\text{OH})_2\text{D}$ , is the ligand for a transcription factor and intracellular receptor called the “vitamin D receptor.” The VDR is widely distributed across many tissues. Indeed, cells lacking the VDR are the exception rather than the rule, and this widespread distribution underlies the potential myriad of physiological actions for vitamin D. Not surprisingly, most if not all effects of  $1,25-(\text{OH})_2\text{D}$  are mediated by the VDR acting primarily by regulating the expression of genes whose promoters contain specific DNA sequences known as vitamin D response elements (VDRE). The VDR works in partnership with other transcription factors, the best-studied of which is the retinoid X receptor (RXR), and a number of coactivators and corepressors that provide context, tissue, and target gene specificity. However, some actions of  $1,25-(\text{OH})_2\text{D}$  are more immediate and may be mediated by a membrane-bound VDR that has been less well characterized than the nuclear VDR. Our understanding of the mechanism by which VDR regulates gene expression has increased enormously over the past few years.

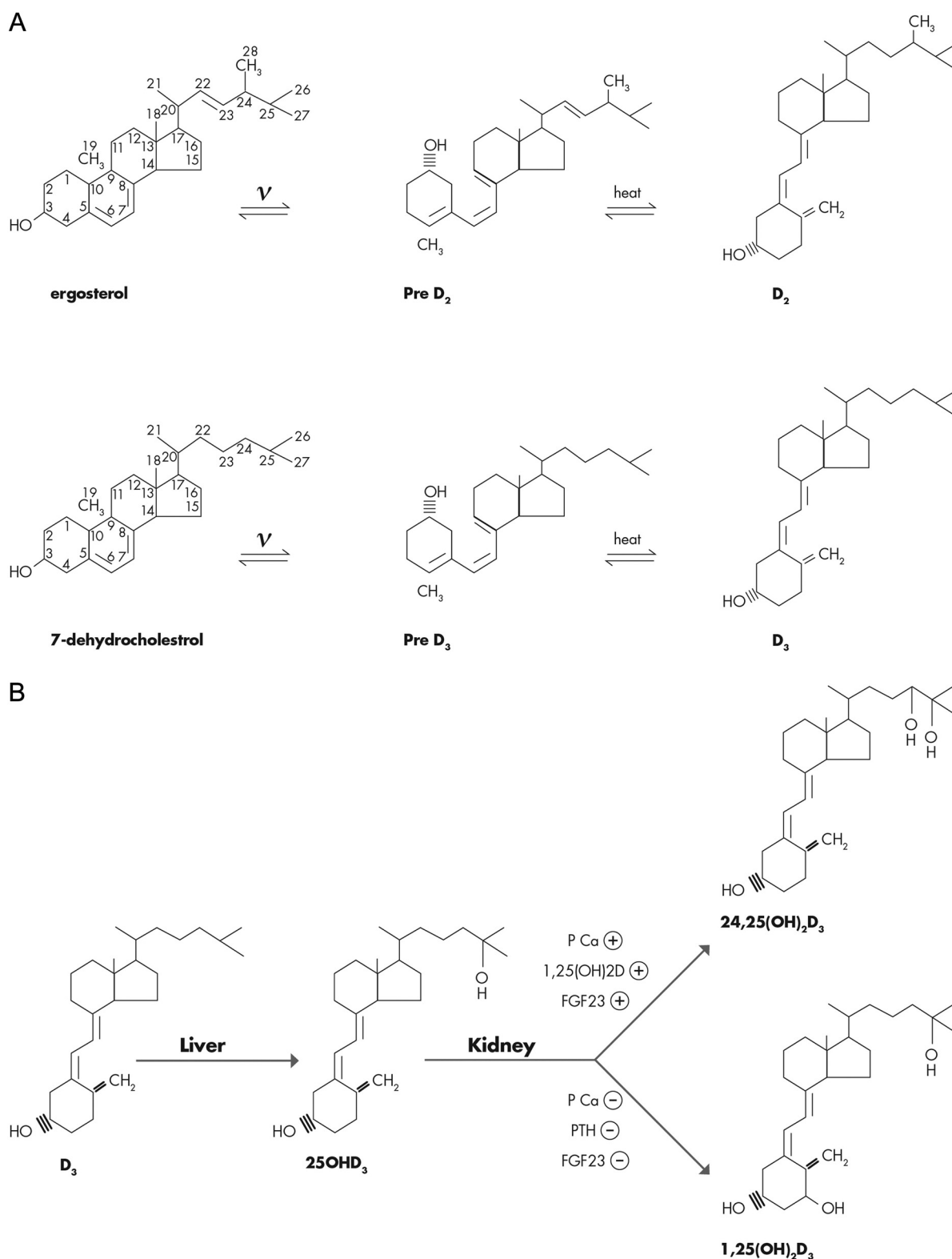
**Figure 1.**

Figure 1. A, Production of vitamin D from the skin via ultraviolet radiation (290–330 nm) in a nonenzymatic manner. B, The synthesis of vitamin D metabolites including the inactive form, 24,25-dihydroxyvitamin D, and the active form, 1,25-(OH)<sub>2</sub>D. This process is controlled at several levels, including the liver, kidney, and peripheral tissues, and is regulated by systemic hormones including PTH, 1,25-(OH)<sub>2</sub>D, and FGF23. Calcium and phosphorus are also major modulators of 1 $\alpha$ -hydroxylase and 24,25-hydroxylase activity through their effects on PTH and FGF23. FGF 23, Fibroblast growth factor 23.

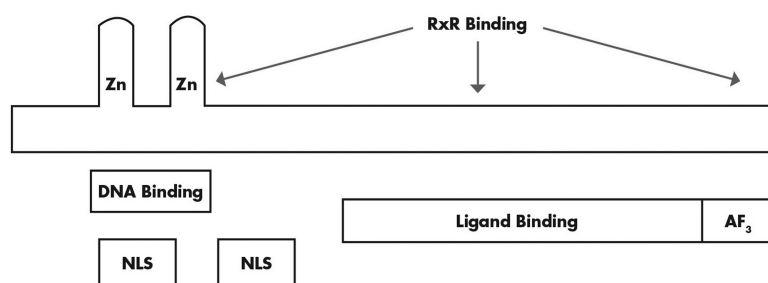
**Figure 2.**

Figure 2. Model of the VDR. The N-terminal region is short relative to other steroid hormone receptors. This region is followed by two zinc fingers, which constitute the principal DNA-binding domain. NLS are found within and just C-terminal to the DNA-binding domain. The LBD makes up the bulk of the C-terminal half of the molecule, with the AF-2 domain occupying the most C-terminal region. The AF-2 domain is largely responsible for binding to coactivators such as the SRC family and DRIP in the presence of ligand. Regions on the second zinc finger and within the LBD facilitate heterodimerization with RXR. Corepressor binding is less well characterized but appears to overlap that of coactivators in helices 3 and 5, a region blocked by helix 12 in the presence of ligand. NLS, Nuclear localization signals.

## B. VDR distribution

The VDR was discovered in 1969 [although only as a binding protein for an as-yet unknown vitamin D metabolite subsequently identified as 1,25-(OH)<sub>2</sub>D]; this binding protein was eventually cloned and sequenced in 1987 (4–6). It is a member of a large family of proteins (more than 150 members) that includes receptors for the steroid hormones, T<sub>4</sub>, the vitamin A family of metabolites (retinoids), and a variety of cholesterol metabolites, bile acids, isoprenoids, fatty acids, and eicosanoids. A large number of family members have no known ligands and are called orphan receptors.

VDR is widely, although not universally, distributed throughout different tissues of the body (7). Many of these tissues were not originally considered targets for 1,25-(OH)<sub>2</sub>D. The discovery of VDR in many cell types along with the demonstration that 1,25-(OH)<sub>2</sub>D altered the function of these tissues has markedly increased our appreciation of the protean effects of 1,25-(OH)<sub>2</sub>D. Inactivating mutations in the hereditary vitamin D resistant rickets result in hereditary vitamin D resistant rickets (8). An animal model in which the VDR has been deleted has the full phenotype of severe vitamin D deficiency, indicating that VDR is the major mediator of vitamin D action (9). The one major difference is the alopecia seen in hereditary vitamin D-resistant rickets and VDR knockout animals, a feature not associated with vitamin D deficiency, suggesting that the VDR may have functions independent of 1,25-(OH)<sub>2</sub>D, at least in hair follicle cycling.

## C. VDR structure

The VDR is a molecule of approximately 50–60 kDa, depending on species. The basic structure is shown in Fig. 2.

The VDR is unusual in that it has a very short N-terminal domain before the DNA-binding domain when compared with other nuclear hormone receptors. The human VDR has two potential start sites. A common polymorphism (Fok 1) alters the first ATG start site to ACG. Individuals with this polymorphism begin translation three codons downstream such that in these individuals the VDR is three amino acids shorter (424 aas *vs.* 427 aas). This polymorphism has been correlated with reduced bone mineral density in some studies, whereas other genome-wide association studies have not found a strong signal for polymorphisms in the VDR gene and bone mass or fractures (10). The most conserved domain in VDR from different species and among the nuclear hormone receptors

in general is the DNA-binding domain. This domain comprises two zinc fingers. The name derives from the cysteines within this stretch of amino acids that form tetrahedral complexes with zinc in a manner that creates a loop or finger of amino acids with the zinc complex at its base. The proximal (N-terminal) zinc finger confers specificity for DNA binding to the VDRE, whereas the second zinc finger and the region following provide at least one of the sites for heterodimerization of the VDR to the RXR. The second half of the molecule is the ligand-binding domain (LBD), the region responsible for binding 1,25-(OH)<sub>2</sub>D, but that also contains regions necessary for heterodimerization to RXR. At the C-terminal end is the major activation domain, AF-2, which is critical for the binding to coactivators such as those in the steroid receptor coactivator (SRC) and VDR-interacting protein (DRIP, also known as Mediator) families (11). In mutation studies of the homologous thyroid receptor, corepressors were found to bind in overlapping regions with coactivators in helices 3 and 5, a region blocked by helix 12 (the terminal portion of the AF-2 domain) in the presence of ligand (12). Deletion of helix 12 promoted corepressor binding while preventing that of coactivators (12).

The LBD for VDR has been crystallized, and its structure solved (13). It shows a high degree of structural homology to other nuclear hormone receptors. It comprises 12 helices joined primarily by  $\beta$ -sheets. The ligand 1,25-(OH)<sub>2</sub>D is buried deep in the ligand-binding pocket and covered with helix 12 (the terminal portion of the AF-2 domain). Assuming analogy with the unliganded LBD of RXR $\alpha$  and the ligand-bound LBD of RAR $\gamma$ , the binding of 1,25-(OH)<sub>2</sub>D to the VDR triggers a substantial movement

of helix 12 from an open position to a closed position, covering the ligand-binding pocket and putting helix 12 in position with critical residues from helices 3, 4, and 5 to bind coactivators (14). Some coactivator complexes such as DRIP bridge the gap from the VDRE to the transcription machinery at the transcription start site. Other coactivator complexes with histone acetyl transferase (HAT) activity such as the SRC family facilitate the opening of the chromatin structure, allowing transcription to occur. Although these two coactivator complexes are essential for VDR function, their interaction with each other remains unclear (11). Both will be discussed further in *Sections II.D. and II.E.*

#### D. Role of coactivators and corepressors

Nuclear hormone receptors including the VDR are further regulated by protein complexes that can be activators or repressors (15, 16). The role of corepressors in VDR function has been demonstrated but is less well studied than the role of coactivators (17). One such corepressor, hairless, is found in the skin and may regulate 1,25-(OH)<sub>2</sub>D-mediated epidermal proliferation and differentiation as well as 1,25-(OH)<sub>2</sub>D-independent VDR regulation of hair follicle cycling (18–20). The SRC family of coactivators has three members, SRC 1–3, all of which can bind to the VDR in the presence of ligand [1,25-(OH)<sub>2</sub>D] (21). These coactivators recruit additional coactivators such as CBP/p300 and p/CAF that have HAT, an enzyme that appears to help unravel the chromatin allowing the transcriptional machinery to do its job. The domain in these molecules critical for binding to the VDR and other nuclear hormone receptors is called the nuclear receptor (NR) box. The NR box harbors a central LxxLL motif where L stands for leucine and x for any amino acid. Each SRC family member contains three well-conserved NR boxes in the region critical for nuclear hormone receptor binding. The DRIP complex of coactivators comprises 15 or so proteins, several of which contain LxxLL motifs (22). However, DRIP205 is the protein critical for binding the complex to VDR. It contains two NR boxes. Different NR boxes in these coactivators show specificity for different nuclear hormone receptors (23). Unlike the SRC complex, the DRIP complex does not have HAT activity (11). Rather the DRIP complex spans the gene from the VDRE to the transcription start site linking directly with RNA polymerase II and its associated transcription factors. DRIP and SRC appear to compete for binding to the VDR. In keratinocytes, DRIP binds preferentially to the VDR in undifferentiated cells, whereas SRC2 and SRC3 bind in the more differentiated cells in which DRIP levels have declined (24). Thus, in these cells DRIP may regulate the early stages of 1,25-(OH)<sub>2</sub>D-induced differ-

entiation, whereas SRC may be more important in the later stages, although overlap in gene specificity is also observed (25–27). SMAD3, a transcription factor in the TGF- $\beta$  pathway, has been found to complex with the SRC family members and the VDR, enhancing the coactivation process (28). Phosphorylation of the VDR may also control VDR function (29). Furthermore, VDR has been shown to suppress  $\beta$ -catenin transcriptional activity (30), whereas  $\beta$ -catenin enhances that of VDR (31). Thus, control of VDR activity may involve crosstalk between signaling pathways originating in receptors at the plasma membrane and in the nucleus.

#### E. Plasticity of the VDRE

VDR acts in concert with other nuclear hormone receptors, in particular RXR (32). Unlike VDR, RXR has three forms— $\alpha$ ,  $\beta$ , and  $\gamma$ —and all three are capable of binding to VDR with no obvious differences in terms of functional effect. RXR and VDR form heterodimers that optimize their affinity for the VDRE in the promoters of the genes being regulated. RXR appears to be responsible for keeping VDR in the nucleus in the absence of ligand (33). VDR may also partner with other receptors including the thyroid receptor and the retinoic acid receptor (34, 35), but these are the exceptions, whereas RXR is the rule. The VDR/RXR heterodimers bind to VDRE, which typically comprise two half sites, each with six nucleotides separated by three nucleotides of nonspecific type; this type of VDRE is known as a DR3 (direct repeats with three nucleotide spacing). RXR binds to the upstream half site, whereas VDR binds to the downstream site (36). However, a wide range of VDRE configurations have been found (31). 1,25-(OH)<sub>2</sub>D is required for high-affinity binding and activation, but the RXR ligand, 9-cis retinoic acid, may either inhibit (37) or activate (38) 1,25-(OH)<sub>2</sub>D stimulation of gene transcription. A DR6 has been identified in the phospholipase C- $\gamma$ 1 gene that recognizes VDR/retinoic acid receptor heterodimers, and a DR4 has been found in the mouse calbindin 28k gene (34, 39). Inverted palindromes with seven to 12 bases between half sites have also been found (31). Furthermore, the half sites of the various known VDRE show remarkable degeneracy. The G in the second position of each site appears to be the only nearly invariant nucleotide. 1,25-(OH)<sub>2</sub>D can also inhibit gene transcription through its VDR. This may occur by direct binding of the VDR to negative VDRE that in the PTH and PTHrP genes are remarkably similar in sequence to positive VDRE of other genes (40, 41). However, inhibition may also be indirect. For example, 1,25-(OH)<sub>2</sub>D inhibits IL-2 production by blocking the NFATp/AP-1 complex of transcription factors from activating this gene (42) through a mechanism not yet clear. Similarly,

1,25-(OH)<sub>2</sub>D inhibits CYP27B1 in some cells by an indirect mechanism (43). In the latter case, this has been shown to involve the role of two DNA methyl-transferases in the inhibitory complex that in the presence of 1,25-(OH)<sub>2</sub>D serve to methylate CpG sites in the CYP27B1 promoter (44). Thus, a variety of factors including the flanking sequences of the genes around the VDRE and tissue-specific factors play a large role in dictating the ability of 1,25-(OH)<sub>2</sub>D to regulate gene expression.

#### F. Nongenomic actions of vitamin D

A variety of hormones that serve as ligands for nuclear hormone receptors also exert biological effects that do not appear to require gene regulation and may work through membrane receptors or the VDR situated outside of the nucleus, rather than their cognate nuclear hormone receptors. Examples include estrogen, progesterone, testosterone, corticosteroids, and thyroid hormone (45–49). 1,25-(OH)<sub>2</sub>D has also been shown to have rapid effects on selected cells that are not likely to involve gene regulation and that appear to be mediated by a distinct receptor which is likely on the membrane receptor. Similar to other steroid hormones, 1,25-(OH)<sub>2</sub>D has been shown to regulate calcium and chloride channel activity, protein kinase C activation and distribution, and phospholipase C activity in a number of cells including osteoblasts, liver, muscle, and intestine (50–54). A putative membrane receptor for 1,25-(OH)<sub>2</sub>D—*i.e.*, 1,25-(OH)<sub>2</sub>D membrane-associated rapid response steroid-binding protein (1,25D-MARRSBP), also known as endoplasmic reticulum stress protein 57—has been purified from the intestine, cloned, and sequenced, and blocking antibodies have been prepared that block the rapid actions of 1,25-(OH)<sub>2</sub>D (55–58). More recently, a mouse null for 1,25D-MARRSBP in the intestine has been developed and shown to lack the rapid response of intestinal cells to 1,25-(OH)<sub>2</sub>D (59). However, these rapid actions of 1,25-(OH)<sub>2</sub>D appear to require the VDR (ineffective in VDR null mice), which suggests that 1,25D-MARRSBP and VDR cooperate in mediating these acute actions of 1,25-(OH)<sub>2</sub>D but without the need for new protein synthesis. In the latter case, analogs of 1,25-(OH)<sub>2</sub>D that do not support genomic actions of 1,25-(OH)<sub>2</sub>D do support these nongenomic actions, which suggests that the membrane VDR may have a different three-dimensional structure with a different binding pocket for its activating ligands.

### III. Vitamin D and the Skin

#### A. Introduction

The skin is unique in that it is the only organ system identified thus far that is able to synthesize all the critical

components of the vitamin D-signaling pathway. The skin is capable of synthesizing the vitamin D prohormone in response to UV radiation, it expresses the hydroxylases required to generate 25(OH)D and 1,25-(OH)<sub>2</sub>D, as well as the nuclear VDR that mediates the effects of the active hormone on target gene expression. CYP24A1, which inactivates 1,25-(OH)<sub>2</sub>D by 24-hydroxylation, is also expressed in the skin. The evolutionary importance of the autocrine and paracrine actions of vitamin D in skin is exemplified by the observation that, in *Xenopus*, the highest levels of VDR are expressed in the skin (60).

The liganded VDR exerts prodifferentiation and anti-proliferative effects on epidermal keratinocytes (61). These actions are critical for expression of proteins that are involved in formation of the cornified envelope, which is an important contributor to the epidermal barrier. In addition, the liganded VDR is important for production of lipids that play a role in barrier function. In contrast, the effects of the VDR on cyclic regeneration of the hair follicle are 1,25-(OH)<sub>2</sub>D-independent and may involve interactions with a distinct group of coregulators (62), such as hairless.

#### B. Proliferation, differentiation, barrier function of skin

Like calcium, 1,25-(OH)<sub>2</sub>D exerts antiproliferative and prodifferentiative effects on skin keratinocytes (63). Although *in vitro* investigations demonstrate that the effects of 1,25-(OH)<sub>2</sub>D and calcium partially overlap, it is not known whether they exert these effects by regulating the same target genes and pathways. However, studies in keratinocytes isolated from VDR knockout mice demonstrate normal acquisition of markers of keratinocyte differentiation in response to calcium, but not 1,25-(OH)<sub>2</sub>D (64). Investigations in mice lacking the VDR demonstrate impaired keratinocyte differentiation after the second week of life, which correlates with the development of impaired calcium absorption and hypocalcemia (65). However, this impaired differentiation is not observed in VDR knockout mice in which normal calcium levels are maintained by a special diet; thus, calcium and 1,25-(OH)<sub>2</sub>D may have redundant roles in keratinocyte differentiation *in vivo*. Similarly, the impaired keratinocyte differentiation in mice lacking the vitamin D 1 $\alpha$ -hydroxylase CYP27B1 is lessened by maintenance of normal mineral ion levels (61).

Epidermal keratinocytes are in contact with a basal lamina that separates the epidermis from the underlying dermis. Proliferation of these basal keratinocytes results in differentiation of cells that give rise to the population of keratinocytes that are present in the external or upper layers. These more differentiated keratinocytes are characterized by a specific profile of gene expression that cor-

relates with their function: to provide a barrier that prevents water loss and contributes to host defense against environmental pathogens and toxins. As cells differentiate from basal to spinous layer keratinocytes, expression of keratins 5 and 14 decreases, and they start to express keratins 1 and 10 as well as involucrin. In addition to these proteins, lipids produced by these differentiating keratinocytes form the cornified layer. The production of glucosylceramides, which also contribute to the physical epidermal barrier, is decreased in mice lacking the VDR. The impaired lipid barrier observed in the VDR null mice and mice lacking CYP27B1 is not rescued by normalization of mineral ion homeostasis (27); thus, calcium and the VDR do not exert overlapping effects on lipid barrier formation.

In addition to contributing to the formation of a physical barrier, the VDR regulates genes involved in host defense. Disruption of the epidermal barrier results in exposure of the dermis and underlying structures to infectious agents. Activation of Toll-like receptors (TLR) activates vitamin D signaling in keratinocytes and monocytes by activation of CYP27B1 and induction of VDR expression (66). In humans, this leads to induction of cathelicidin, a peptide involved in host defense, as well as enhancing TLR expression in a positive feedback loop (67). This feature of the epidermal barrier also requires the ligand-dependent effects of  $1,25\text{-(OH)}_2\text{D}$  (66) (see *Section VIII*).

### C. Coactivators and corepressors of vitamin D in skin

Investigations directed at identifying the molecular basis for the differing gene expression profiles associated with keratinocyte differentiation revealed that the VDR associates with a different set of nuclear receptor coactivators, depending on the state of keratinocyte differentiation (68). In proliferating keratinocytes, the VDR interacts with the DRIP/Mediator complex. Impairing expression of DRIP 205/Med1 or Med21, key components of this coactivator complex, leads to an increase in proliferation accompanied by impaired acquisition of markers of keratinocyte differentiation. The DRIP/Mediator complex is critical for responsiveness of keratinocytes to both calcium and  $1,25\text{-(OH)}_2\text{D}$ , which suggests that these two prodifferentiation agents converge on a common molecular pathway to exert their effects.

Keratinocyte differentiation is characterized by a decrease in the expression of proteins that make up the DRIP/Mediator complex and an increase in expression of SRC3. The VDR-SRC3 interaction is critical for induction of proteins and lipids that contribute to formation of the epidermal barrier (27). *In vitro* knockdown of SRC3 or of VDR in keratinocytes leads to a similar reduction in the expression of lipids that contribute to epidermal barrier function.

### D. Hair follicle phenotype, $1,25\text{-(OH)}_2\text{D}$ independence, molecular interactors, and targets

The observation that humans and mice with mutations in the VDR develop alopecia, whereas those with mutations in CYP27B1 do not, was the first indication that the actions of the VDR on the hair follicle do not require  $1,25\text{-(OH)}_2\text{D}$ . The availability of mice with ablation of the VDR or CYP27B1 provided invaluable tools for dissecting the effects of the VDR in the hair follicle (69–73). The VDR is expressed by the outer root sheath and hair bulb keratinocytes of the hair follicle, as well as by the sebaceous gland. During embryogenesis, the hair follicle develops in response to reciprocal signaling between dermal cells, which give rise to the dermal papilla, and the epidermal placode, which then invaginates to form the hair follicle. Postnatally, the hair follicle goes through cycles of growth, characterized by proliferation of cells from the bulge, which lies below the sebaceous gland and is thought to contain keratinocyte stem cells. The end of this proliferative anagen phase is characterized by the formation of a mature hair shaft. This is followed by catagen, characterized by apoptosis of the keratinocytes that lie below the bulge (74). This is thought to bring the dermal papilla in close proximity to the bulge, during the telogen phase, to permit reciprocal communication that results in the initiation of a new anagen phase. In humans, the hair cycle can last from months to years, depending on the location of the hair follicle, and is thought to contribute to the differing lengths of hair on various parts of the body. In mice, hair cycles occur approximately every 4 wk. Studies in mice lacking the VDR demonstrate that development of hair follicles proceeds normally, but hair cycles are absent after the morphogenic period (64). In contrast to epidermal keratinocytes, where calcium and  $1,25\text{-(OH)}_2\text{D}$  play redundant roles in the regulation of proliferation and differentiation, normocalcemia was unable to prevent the defect in postmorphogenic hair cycles. This suggested that the actions of the VDR that maintain hair cycling differed from those required for keratinocyte differentiation.

Hair reconstitution assays, in which the hair follicle is reconstituted by implantation of morphogenic dermal papilla cells and keratinocytes into a nude mouse host, demonstrated that keratinocytes lacking the VDR were unable to support postmorphogenic hair cycles, whereas the absence of the VDR in the dermal papilla had no untoward effects (75). Transgenic expression of the VDR in the keratinocytes of VDR null mice prevented alopecia, demonstrating that the effects of the VDR in keratinocytes are critical for the maintenance of cutaneous homeostasis (76). Furthermore, expression of a VDR transgene with a mutation that prevents  $1,25\text{-(OH)}_2\text{D}$  binding and transactivation also prevents alopecia, demonstrating that the

effects of the VDR on the hair follicle are 1,25-(OH)<sub>2</sub>D-independent (62). Mice with deletion of the first zinc finger and AF-1 domain of the VDR phenocopy mice that express no VDR protein, demonstrating that this region of the receptor is critical for cutaneous integrity (69).

In addition to the absence of postmorphogenic hair cycles, the VDR null mice develop lipid-laden dermal cysts with epidermal markers and expansion of sebaceous glands, which suggests that an abnormality in the stem cells gives rise to these cells. The effect of VDR ablation on keratinocyte stem cell number and function was examined. A progressive decline in keratinocyte stem cell number was observed with age in the VDR null mice; however, at 28 d, when the number of these cells is normal, the keratinocyte stem cells are unable to form colonies *in vitro* or regenerate a hair follicle *in vivo*, demonstrating a functional abnormality in the keratinocyte stem cells as well (77).

Studies directed at identifying molecular partners of the unliganded VDR that play a role in the regulation of keratinocyte stem cell function demonstrated that, in contrast to investigations demonstrating that the liganded VDR impairs canonical Wnt signaling (30, 78), the unliganded VDR is essential for canonical Wnt signaling in keratinocytes. Absence of the VDR impairs expression of a Wnt reporter in primary keratinocytes as well as that of Wnt target genes in keratinocytes *in vitro* (77, 79). The effect of VDR ablation on Wnt target gene expression *in vivo* is dependent upon the age of the mice examined and the stage of the hair cycle (79, 80).

In keratinocytes, the unliganded VDR interacts with lymphoid enhancer-binding factor-1 (LEF1) but not with other effectors of the canonical Wnt signaling pathway, including  $\beta$ -catenin or Tcf3. Interactions of the VDR with LEF1 were mapped to the first zinc finger of the DNA-binding domain, an interesting finding based on the alopecia observed in mice lacking this region of the VDR (79). The importance of LEF1 in maintenance of the hair follicle is evidenced by the alopecia observed in mice lacking LEF1 and the hair loss, accompanied by the development of lipid-laden dermal cysts, in mice with keratinocyte-specific expression of a dominant negative LEF1 transgene (81). Whether impairment of VDR/LEF1 interactions underlies the alopecia observed in VDR null mice remains to be determined. However, the interaction of liganded VDR with  $\beta$ -catenin appears to have different effects on the hair follicle than the unliganded VDR (31, 82), which suggests that, in the absence of 1,25-(OH)<sub>2</sub>D, the VDR may recruit LEF1 and/or other comodulators to regulate hair cycling.

Other transcriptional regulators that interact with the VDR have been shown to be critical for the maintenance of the postmorphogenic hair follicle. Mice with keratino-

cyte-specific ablation of RXR- $\alpha$ , the dominant RXR isoform in skin, develop a phenotype analogous to that seen in the VDR null mice, including progressive alopecia and the formation of lipid-laden dermal cysts (83). The phenotype, which also includes an inflammatory response, is more extensive than that of the VDR null mice, suggesting that ablation of RXR- $\alpha$  impairs the action of additional nuclear receptor heterodimerization partners. Ablation of the nuclear receptor corepressor hairless also leads to the development of alopecia with severe skin wrinkling and lipid-laden dermal cysts (84, 85). Interestingly, the interactions of the VDR with LEF1, RXR- $\alpha$ , and hairless do not involve the AF-2 region, which is required for interactions with classical nuclear receptor comodulators (19).

The hedgehog pathway also plays a role in the hair follicle. Absence of hedgehog signaling impairs hair follicle development, whereas activation of this pathway postnatally induces anagen both in wild-type and, to a lesser extent, in VDR null mice (80, 86). The VDR interacts directly with effectors of hedgehog signaling by binding to the regulatory regions of the GLI1 and Sonic hedgehog genes (79) and regulating their expression (87). Like the expression of Wnt target genes, the effect of VDR ablation on expression of genes in the hedgehog pathway depends upon the stage of the hair cycle, suggesting that the VDR may exert differential effects in epidermal *vs.* hair follicle keratinocytes (79, 80). Consistent with this hypothesis is that, in addition to being dysregulated in the skin of VDR null mice at the time of anagen, these genes are induced by the canonical Wnt signaling pathway. It remains to be determined whether VDR-LEF1 interactions are critical for induction of this pathway in the postnatal hair follicle.

## E. Translational studies and clinical trials of vitamin D and skin

The antiproliferative and prodifferentiation effects of 1,25-(OH)<sub>2</sub>D on keratinocytes led to an interest in its therapeutic potential for the treatment of skin disorders. Many investigations have examined the effects of vitamin D analogs on psoriasis, a disorder associated with keratinocyte hyperproliferation. Although these studies do suggest that topical treatment with combined glucocorticoids and vitamin D metabolites are superior to either alone, large, double-blind, placebo-controlled clinical trials demonstrating the effects of active vitamin D metabolites are required.

Exposure to UV light increases vitamin D synthesis as well as the risk of skin cancers. Investigations in animal models demonstrate that the VDR attenuates cutaneous malignancies. Mice lacking the VDR are more susceptible to skin cancers induced by either chemical carcinogens or UV radiation (87–89). Interestingly, mice lacking

CYP27B1, the enzyme required for  $1\alpha$ -hydroxylation of vitamin D metabolites, are not more susceptible to chemically or UV-induced tumors. Thus, the effects of the VDR on prevention of skin cancer in this model do not require  $1,25\text{-(OH)}_2\text{D}$ . Whether this is due to direct target gene regulation or is a reflection of the role of the VDR in regulating keratinocyte stem cell function remains to be determined.

## F. Conclusions

All the elements of the vitamin D regulatory system are present in skin, and studies in humans and animals with mutations in key elements of this system support the biological role of vitamin D in regulation of the skin barrier and hair follicles.  $1,25\text{-(OH)}_2\text{D}$  is strongly prodifferentiative and antiproliferative for keratinocytes, thereby supporting the use of topical and oral vitamin D in skin disorders such as psoriasis. Moreover, mice lacking the VDR gene are more susceptible to skin cancers induced by UV radiation. However, there are no large-scale, randomized, placebo-controlled clinical trials demonstrating that vitamin D metabolites are superior to other types of treatment for various proliferative skin disorders or for the prevention of skin cancer.

## IV. Vitamin D and Its Relationship to Obesity and Diabetes Mellitus

### A. Introduction

Low serum levels of  $25\text{(OH)D}$  have been linked through observational studies to the pathophysiology of obesity, diabetes mellitus, and the metabolic syndrome. A number of mechanisms are plausible (90, 91). First, the VDR is highly expressed in adipocytes and is responsive to activation by  $1,25\text{-(OH)}_2\text{D}$  (92–94). Second, vitamin D is fat soluble and can be stored in adipose tissues, although questions remain about the dynamics of its reentry into the circulation and subsequent fate (91, 95). Third, large cohort studies have shown that an increased percentage body fat and high body mass index (BMI) are strongly and inversely correlated with serum  $25\text{(OH)D}$  concentrations, particularly in Caucasians (96, 97). Fourth, in rodent models, vitamin D modulates insulin synthesis and secretion (98, 99). Importantly,  $1,25\text{-(OH)}_2\text{D}$  regulates calcium trafficking in  $\beta$ -cells *in vitro* and in mouse models (100, 101). There is also strong evidence that  $1,25\text{-(OH)}_2\text{D}$  modulates intracellular ionized calcium signaling in the adipocyte, which in turn promotes increased lipogenesis and decreased lipolysis, possibly through the inhibition of uncoupling protein-2 (UCP2) (92, 100).

Thus, it is plausible that vitamin D could play a role in the pathogenesis of the metabolic syndrome and other obesity syndromes. However, *in vivo* data from mouse models add to the complexity of that relationship. For example, VDR null mice exhibit atrophy of adipose tissue in mammary and prostate glands (94, 102). And decreased overall fat mass, reduced serum leptin, and increased energy expenditure have been demonstrated in VDR $^{-/-}$  mice (102–104). These changes, which are age dependent, are accompanied by an increase in UCP1 gene expression and a lean phenotype (104). In fact, recently, de Paula *et al.* (92) showed that VDR $+/+$  heterozygous mice also demonstrate a modest but significant lean phenotype. However, the mechanisms responsible for the remarkable changes in energy expenditure in VDR $^{-/-}$  mice have not been fully clarified (100). Notwithstanding the mouse data, there remains an evidence gap in regard to the precise physiology of vitamin D in adipocyte physiology, but the clinical data that obesity consistently is associated with low  $25\text{(OH)D}$  levels lie in sharp contrast to the animal models in which absence of vitamin D is related to increased resting energy expenditure. Despite this paradox, there have been several observational and controlled trials of vitamin D in preventing or treating obesity and type 2 diabetes mellitus.

### B. Observational studies of the relationship of vitamin D to obesity and the metabolic syndrome

Numerous observational studies (mostly cross-sectional, but some longitudinal) demonstrate a consistent association of low serum  $25\text{(OH)D}$  levels with diabetes, prediabetes, metabolic syndrome, obesity, and fat content (adiposity) (105–107). This relationship is noted in adults and in children, in both sexes, and in various ethnic backgrounds (97, 108–114). Pittas *et al.* (115) performed a prospective cohort analysis of the Nurses Health Study in women followed for 20 yr relative to serum  $25\text{(OH)D}$  levels and glucose intolerance. They found that total vitamin D and calcium intake was inversely associated with the risk of type 2 diabetes. Moreover, women who consumed three or more dairy servings per day were at a lower risk of developing diabetes compared with those consuming only one dairy serving per day. More recently, Devaraj *et al.* (97) noted that the first quartile of serum  $25\text{(OH)D}$  level, compared with the fourth quartile, was associated with an adjusted odds ratio (OR) of prediabetes (defined as a 2-h glucose concentration of 140–199 mg/dl, a fasting glucose concentration of 110–125 mg/dl, or a glycosylated hemoglobin value of 5.7–6.4%) of 1.47 [95% confidence interval (CI), 1.16–1.85]. In that study,  $25\text{(OH)D}$  levels were significantly and inversely correlated with fast-

ing glucose ( $r = -0.29$ ;  $P = 0.04$ ) and homeostasis model of assessment ( $r = -0.34$ ;  $P = 0.04$ ) in North American adults with the metabolic syndrome (97). A population-based study from Norway showed a similarly strong inverse association between elevated BMI and serum 25(OH)D (116).

In children and adolescents, the association seems more consistent and prominent. More than 50% of Norwegian children and adolescents with excess body weight had a low 25(OH)D status, and 19% had vitamin D deficiency (117). In obese African-American adolescents, low 25(OH)D levels correlated with low adiponectin levels, obesity, and insulin resistance (118). The association with increased adiposity was demonstrated in another study of both black and Caucasian youth (119). Analogous results (association of low vitamin D status with BMI and adiposity) were demonstrated in children in tropical environments such as Malaysia and Columbia and in adults in the Mediterranean region, such as Spain and France (105, 120–122). Meta-analysis of observational studies confirms the association of low 25(OH)D with incident diabetes (OR, 0.82; 95% CI, 0.72–0.93) (106).

Nevertheless, these studies remain observational and only document an association without causality, despite attempts to control for known confounders. For example, in one study the investigators adjusted for age, sex, race/ethnicity, season, geographic region, smoking, alcohol intake, BMI, outdoor physical activity, milk consumption, dietary vitamin D, blood pressure, serum cholesterol, C-reactive protein, and glomerular filtration rate to identify an association (114). The inability of these studies to evaluate temporality (*i.e.*, which occurred first, the vitamin D deficiency or obesity) and confounding (*i.e.*, an unknown factor may have caused both conditions) precludes conclusions about causality and whether vitamin D replacement would actually resolve or mitigate the observed outcome (obesity or glucose intolerance).

### C. Randomized trials of vitamin D in obesity, type 2 diabetes mellitus

Until recently, there were no randomized trials testing the efficacy of vitamin D supplementation on the risk of developing type 2 diabetes mellitus. In 2008, de Boer *et al.* (107) evaluated the effect of calcium plus vitamin D supplementation and the risk of incident diabetes in the Women's Health Initiative (WHI) trial. Postmenopausal women received 1000 mg/d elemental calcium plus 400 IU/d of vitamin D<sub>3</sub> or placebo in a double-blind fashion. The 2291 women with newly diagnosed type 2 diabetes were followed a median of 7 yr (107). The hazard ratio (HR) for incident diabetes mellitus associated with calcium/vitamin D treatment was 1.01 (95% CI, 0.94–1.10) based on intention-to-treat principles.

This null result was robust in subgroup analyses, efficacy analyses accounting for nonadherence, and analyses examining change in laboratory measurements (107). However, the supplement contained only 400 IU/d of vitamin D, and many women in the WHI were already taking upwards of 400 IU/d in their diet and with supplements.

A systematic review and meta-analysis was commissioned by The Endocrine Society to support the development of the Society's guidelines on vitamin D (123). Other than the trial by DeBoer *et al.* (107), this systematic review did not identify any other randomized controlled trials that reported the incidence of diabetes. Furthermore, that systematic review demonstrated that vitamin D supplementation did not affect glycemia (eight trials; weighted mean difference,  $-0.10$  mg/dl; 95% CI,  $-0.31$ ,  $0.12$ ;  $P = 0.38$ ;  $I^2 = 82\%$ ) (123).

However, other surrogate end points have been examined, and subgroup analyses have been performed in randomized controlled trials of vitamin D. Von Hurst *et al.* (124) supplemented the diets of nondiabetic overweight South Asian women with 4000 IU/d vitamin D<sub>3</sub> for 6 months and found a significant improvement in insulin sensitivity compared with a placebo group. Notably, it was the women with the lowest 25(OH)D levels at study initiation who achieved levels greater than 80 nmol/liter who had the greatest response with respect to glucose tolerance. In a subgroup analysis of the RECORD trial in which calcium (1000 mg/d), vitamin D (800 IU/d), both, or neither was randomly assigned to elderly people in Scotland, there was no difference in the incidence of self-reported development of type 2 diabetes among groups (125). Finally, Jorde *et al.* (116) performed a 1-yr, randomized, placebo-controlled trial in Norway of 438 obese women [ages 21–70 yr with a baseline 25(OH)D of 58 nmol/liter] using 40,000, 20,000, or 0 IU/wk of vitamin D<sub>3</sub> and found no differences in glucose tolerance among any of the groups despite an increase in serum 25(OH)D to 140 nmol/liter in the highest-dose vitamin D group.

### D. Conclusions

At both the cellular and physiological level, the precise relationship between vitamin D and adiposity is not certain, although it remains an area of intense investigation. The ever-expanding obesity epidemic has been associated with a rising prevalence of vitamin D deficiency, but a cause-and-effect relationship has not been established; neither has a direct relationship been proven between low 25(OH)D levels and the pathogenesis of type 2 diabetes mellitus. Most of the evidence to date is correlational (*i.e.*, noninterventional) and derived from observational and longitudinal cohort studies of various populations. There remains a paucity of randomized controlled trials of vitamin D for the prevention of diabetes; hence, few conclu-

sions can be firmly established. At present, strong evidence does not exist to support the tenet that vitamin D supplementation reduces the risk of type 2 diabetes or the metabolic syndrome.

## V. Vitamin D for the Prevention of Falls and Improvement in Quality of Life

### A. Introduction

Rickets in children and osteomalacia in adults are characterized by undermineralized osteoid, resulting in “soft” bones (95, 126). Clinically, osteomalacia is associated with very low bone mass, bone pain, fractures, and muscle weakness. The myopathy associated with hypovitaminosis D includes type II muscle fiber atrophy and in some cases fatty infiltration of the muscles. However, these changes are nonspecific and can be found in other types of myopathy. Serum 25(OH)D levels are usually very low, which makes that measurement an extremely sensitive but not specific predictor of disease status (127). However, very low calcium intake in the face of normal vitamin D stores can also lead to osteomalacia. Conversely, in some forms of osteomalacia, calcium levels may be low normal, but with very low serum phosphorus, there is undermineralized osteoid and severe proximal muscle weakness. Supplementation with high doses of vitamin D rescues the phenotypic manifestations of osteomalacia due to dietary deficiency, including correction of low serum calcium and phosphorus, albeit only when serum 25(OH)D levels are restored to normal ranges. However, improvement in symptoms of muscle weakness and pain with osteomalacia can take up to 18 months after initiation of therapy, and these changes do not significantly correlate with the rise in serum 25(OH)D (126).

Several lines of evidence support the concept that there is a strong and direct effect of vitamin D on muscle function. First, the syndrome of vitamin D deficiency [*i.e.*, serum 25(OH)D levels <10 ng/ml or 25 nmol/liter] is frequently accompanied by profound muscle weakness that responds to vitamin D treatment, although as noted the myopathy is nonspecific (102, 126, 127). In children, the proximal muscle weakness is readily reversible with cholecalciferol supplementation. With the adult syndrome, there is relatively strong evidence that elders living in an institutional setting are more prone to vitamin D deficiency due to reduced solar and dietary exposure to vitamin D. These men and women often exhibit signs of muscle weakness, bone pain, frailty, and fractures that also respond to vitamin D replacement, although it is unclear whether this is direct or indirect, due to the effect of vitamin D on calcium entry into skeletal muscle cells and the

marked reduction in phosphate stores (128). Second, this phenotype is recapitulated in the heritable conditions of vitamin D resistance and impaired receptor function where muscle weakness, bone pain, and poor skeletal mineralization are quite common (73, 127, 129). Third, the VDR is widely expressed in many tissues, and genetic deletion of this receptor can lead to poor muscle function in mice (130). Some, but not all, studies have demonstrated by immunohistochemistry with several different antibodies that the VDR is expressed in adult muscle tissue, although this recently has come into question (131). Finally, there is biochemical evidence that activation of the VDR by 1,25-(OH)<sub>2</sub>D in skeletal muscle induces fast, nontranscriptional responses involving stimulation of the transmembrane second messenger systems, including adenylyl cyclase/cAMP/PKA, PLC/DAG+ IP(3)/PKC/Ca(2+), and MAPK cascades. Short treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> also induces reverse translocation of the VDR from the nucleus to plasma membranes (132). Hence, there is some support for a direct relationship between vitamin D and muscle function.

There is also experimental evidence to suggest that the effects of vitamin D on muscle function may be indirect. First and foremost, Wang and DeLuca (131), using a highly specific antibody to VDR, recently reported that they could not identify strong VDR positivity by immunohistochemistry in adult muscle from either mouse or man. Second, genetic deletion of the VDR in intestinal tissue results in a phenocopy of the VDR null mouse with dramatic musculoskeletal and skin changes. Furthermore, high-dose supplementation with calcium and phosphorus rescues the skeletal phenotype in VDR<sup>−/−</sup> mice. And a knock-in of the VDR in the intestine of VDR<sup>−/−</sup> mice rescues the musculoskeletal phenotype (133). Third, some individuals with very low serum 25(OH)D levels (*i.e.*, <10 ng/ml) do not exhibit signs of osteomalacia either clinically or histologically, most likely because they have adequate calcium intake (134). These observations, plus data from both mice and humans that high doses of calcium alone can reverse the clinical syndrome of osteomalacia, suggest that the effects of vitamin D on muscle function may be mediated in part through changes in calcium absorption rather than directly via a putative muscle VDR. In summary, there is significant controversy about the role of vitamin D in adult muscle function. It remains to be determined whether the effects are mediated directly by VDR activation of second messengers in stem cells or skeletal muscle cells or through changes in calcium absorption that affect PTH secretion and ultimately determine intracellular calcium levels.

Conflicting data about the effects of vitamin D on muscle function translate into heterogeneous results from clin-

ical trials and produce significantly different conclusions from meta-analyses of vitamin D for fall prevention (see *Section V.C.*). Attempts to define an absolute threshold level of 25(OH)D above which muscle function is optimal or falls can be prevented are fraught with significant issues. First, as noted, although serum 25(OH)D is a sensitive indicator of osteomalacia, it is not specific likely because of variability in calcium intake. In a recent autopsy series from Priemel *et al.* (134) of more than 600 individuals, nobody had histological evidence of osteomalacia with levels greater than 30 ng/ml, but many subjects with values less than 15 ng/ml had no evidence of osteomalacia on bone biopsy. Notably, daily calcium intakes were not available in this study, making it difficult to discern the reason why so many individuals with low vitamin D levels had no histological evidence of osteomalacia. Second, according to the recent IOM report, serum 25(OH)D is only a measure of exposure to vitamin D, not a biomarker of a disease state (135). This is also clearly illustrated in the Priemel *et al.* study (134), as well as in clinical observations in which calcium insufficiency alone with normal levels of 25(OH)D can rarely cause osteomalacia. Third, there is some evidence to suggest that the protective effects of calcium plus vitamin D in respect to hip fracture risk reduction are more apparent in individuals with baseline low vitamin D levels (136, 137). Finally, the etiology of falls is multifactorial and involves numerous confounding determinants, including neurological factors, gait speed, mental status, and medications. As such, the complex interaction between calcium and vitamin D makes it difficult to define an absolute threshold for serum 25(OH)D below which muscle function is impaired or, conversely, above which, falls are prevented. Notwithstanding, it is noteworthy that in virtually every observational study, individuals with the lowest levels of serum 25(OH)D are at the greatest risk of falls and fractures (3).

### B. Observational studies of vitamin D and falls

Several observational studies have pointed to an association between serum 25(OH)D levels and falls and/or frailty. However, analysis in the most recent Agency for Healthcare Research and Quality (AHRQ) systematic review identified a significant inconsistency across studies (138). In part this relates to defining a threshold value for serum 25(OH)D that would prevent falls and improve muscle function. For example, in the Longitudinal Aging Study in Amsterdam, a prospective cohort study, the investigators found that serum levels less than 25 nmol/liter were associated with the greatest risk of falling in subjects who had experienced multiple falls (139). On the other hand, in National Health and Nutrition Examination Survey (NHANES) III, higher serum 25(OH)D concentra-

tions among 4100 older adults were associated with better lower extremity function, with the greatest effect occurring in those individuals with serum levels between 20 and 40 nmol/liter (137). In a Dutch study of men and women more than age 65 yr, serum levels below 20 nmol/liter were associated with a significant decline in physical function over a 3-yr period (140). More recently, Ensrud *et al.* (141) examined indices of frailty both cross-sectionally and after 6 yr in the large Study of Osteoporotic Fractures (SOF) cohort of elderly women and found increased frailty indices for women with levels below 20 ng/ml and a plateau for risk of frailty between 20 and 30 ng/ml. Additionally, in the Osteoporotic Fractures in Men Study (MrOS), a prospective cohort study in older men, serum levels of 25(OH)D below 20 ng/ml were independently associated with greater evidence of frailty at baseline, but unlike the Dutch study, did not predict greater frailty status at 4.6 yr (142).

In sum, observational data from cross-sectional and cohort studies suggest that serum 25(OH)D levels that are often considered deficient (*i.e.*, <20 ng/ml) are associated with greater frailty indices and likely increased the risk of falls among elderly individuals. However, as noted, there is considerable heterogeneity among the subjects, their calcium intake, the assay used for measuring serum 25(OH)D, and the primary outcome that was measured. In respect to the serum measurement of 25(OH)D, several distinct assays (*e.g.*, RIA, ELISA, liquid chromatography-mass spectrometry) have been used in large observational studies, and each has its own strengths and limitations. Notwithstanding, it is apparent that comparisons across studies to define a single 25(OH)D threshold level for falls are likely to be confounded by significant variations in the measurement tool.

### C. Randomized trials of vitamin D on falls

There have been several randomized controlled trials of vitamin D or vitamin D plus calcium to prevent falls and improve frailty indices, although the quality of the evidence has been rated as “fair” by two AHRQ reviews (138). Surprisingly, more meta-analyses of vitamin D and falls have been published recently, such that the ratio of randomized clinical trial (RCT) publications to meta-analyses is now a mere 1.9:1. Thus, with fewer clinical trials and more meta-analyses, the results become less clear-cut. For example, outcome measurements (*i.e.*, falls *vs.* fallers), population heterogeneity, and serum measurements of 25(OH)D all complicate interpretation. More importantly, selection of the most appropriate studies for analysis based on *a priori* criteria is essential because the number of well-executed randomized trials is limited.

The most recent systematic review and meta-analysis by Murad *et al.* (143), also commissioned by The Endocrine Society to support the development of clinical practice guidelines, found a statistically significant reduction in the risk of falls in 26 randomized trials of vitamin D supplementation (OR = 0.85; 95% CI, 0.77–0.95;  $I^2$  = 60%). This effect was more prominent in patients who were vitamin D deficient at baseline, a finding consistent with previous observational studies ( $P < 0.05$ ) (143). Interestingly, the effect of vitamin D on fall reduction was only noted in studies that used both calcium and vitamin D supplementation. Not surprisingly, the evidence supporting a reduction in falls with supplementation among individuals with very low levels of 25(OH)D has become more robust and relatively consistent in systematic reviews, including the recent report from the U.S. Public Health Services Task Force (137, 143–145).

The optimal dose and timing of supplementation with vitamin D has not been settled, in part due to issues related to compliance. Two recent well-designed, randomized, placebo-controlled trials in older individuals using high-dose intermittent cholecalciferol have been reported. Saunders *et al.* (146) administered 500,000 U vitamin D once yearly for 3 yr or placebo to older postmenopausal women (mean age, 76 yr) at high risk for falling (one third had also suffered previous fractures). The authors found that vitamin D raised serum levels of 25(OH)D from 53 nmol/liter (approximately 21 ng/ml) at baseline to 120 nmol/liter at 1 month, 90 nmol/liter at 3 months, and 75 nmol/liter (28 ng/ml) at 1 yr, but was not associated with fewer fractures or falls compared with placebo (146). Glendenning *et al.* (147) performed a 9-month, randomized, placebo-controlled trial in older Australian postmenopausal women (mean age, 76 yr) using 150,000 U cholecalciferol every 3 months and also found no reduction in falls among the vitamin D group despite an increase in serum 25(OH)D from 65 to 74 nmol/liter at 3 months. In both of these studies, the risk of falling was greater in the vitamin D-treated group than placebo, although the difference was only significant in the former trial (HR, 1.16;  $P = 0.003$ ). However, caution must be exercised in extrapolating serum 25(OH)D levels from these studies to adverse events because peak levels were never ascertained in either of the two “high-dose” trials.

#### D. Effects of vitamin D supplementation on pain and quality of life

The effect of vitamin D supplementation on other functional outcomes such as pain and quality of life is less clear. Six studies assessed the effect of vitamin D on patients' quality of life using standardized instruments (SF-36, SF-12, and the Medical Outcome Survey Short Form-8)

(148–153). Meta-analysis demonstrated no significant change in the physical component score (standardized mean difference, 0.07; 95% CI, –0.03 to 0.16;  $I^2$  = 54%) or the mental component score (standardized mean difference, 0.02; 95% CI, –0.05 to 0.09;  $I^2$  = 29%). The individual domains of quality-of-life surveys were reported in three studies and did not significantly differ at the end of follow-up (150, 153, 154). This includes follow-up of the WHI, which is fairly large and better powered to demonstrate a difference (154). Lastly, vitamin D administration in elderly patients with congestive heart failure resulted in no significant benefit in terms of physical performance using a timed up-and-go test, subjective measures of function, or daily activity. Quality of life measured by a disease-specific tool (the Minnesota Living with Heart Failure questionnaire), worsened by a small, but significant amount in the treatment group (148).

The effect of vitamin D on pain was reported in five studies, but the results were too heterogeneous to be pooled in a meta-analysis (150, 153, 155–157). Three of these studies showed a possible beneficial effect. Arvold *et al.* (158) randomized patients with mild-to-moderate vitamin D deficiency (level, 10–25 ng/dl) to vitamin D<sub>3</sub> 50,000 U/wk for 8 wk or placebo. The study measured scores on the Fibromyalgia Impact Questionnaire and reported that those with mild-to-moderate deficiency had more fatigue and joint and muscle aches at baseline than placebo but no impairments in terms of the activities of daily living. Supplementation led to statistically significant improvements in fatigue symptoms compared with placebo. A third arm of severe deficiency (not randomized) had more severe baseline symptoms and marked improvements with supplementation. Brohult and Jonson (157) reported decreased pain and analgesic use by rheumatoid arthritis patients after using a large dose of vitamin D for 1 yr (67% of patients in the vitamin D group improved *vs.* 36% of control patients;  $P < 0.05$ ). Grove and Halver (159) showed similar results in postmenopausal women with osteoporotic fractures who took vitamin D, calcium, and fluoride compared with placebo (83% of patients in the vitamin D group improved *vs.* 31% of control patients;  $P = 0.05$ ).

The other studies did not demonstrate a benefit of vitamin D on pain scores in the elderly at risk of falls (as a component of SF-36), in postmenopausal osteoporotic women with vertebral fractures, or in patients with diffuse musculoskeletal pain and osteoarthritis who have 25(OH)D levels no greater than 20 ng/ml (150, 153, 155, 156). In summary, conclusions from studies that evaluated the effects of vitamin D on pain and quality of life are quite limited due to their heterogeneous nature in terms of population, cohort size, outcome definition, and imprecision.

## E. Conclusions

In a somewhat distinct vein from the IOM report, we believe vitamin D supplementation is likely to reduce the risk of falls, particularly in those individuals who have low baseline levels (<20 ng/ml) and are supplemented with calcium as well (3). However, the absolute threshold level of 25(OH)D needed to prevent falls in an elderly population is not known in part because of the lack of true dose-ranging studies. Importantly, recommendations for vitamin D intake in a given individual must also be considered within the context of optimal calcium intake. Notwithstanding, the effect of vitamin D supplementation on falls could have important public health implications considering the morbidity associated with falls, particularly in the frail elderly. Selecting patients at risk for falls and defining the appropriate dose remain as areas in need of further research.

## VI. Vitamin D and Cancer

### A. Introduction

Vitamin D has received widespread attention in the medical literature and popular press for its potential role in cancer prevention. Thus, it surprised many in the biomedical community that this research did not have a prominent role in establishing new Dietary Reference Intakes for vitamin D by the IOM (3, 135). After a comprehensive and rigorous review of the scientific research, the IOM Committee concluded that the evidence that vitamin D prevented cancer was inconsistent and did not meet criteria for establishing a cause-effect relationship. A systematic review conducted by the AHRQ in 2009 (160) and in 2011 (161), as well as other recent reviews summarized in the report (3, 162), have reached similar conclusions. Importantly, no previous large-scale RCT of vitamin D had been completed with cancer as the primary prespecified outcome (3, 163). Most of the available evidence on vitamin D and cancer was derived from laboratory studies, ecological correlations, and observational investigations of serum 25(OH)D levels in association with cancer outcomes. Although measures of serum 25(OH)D concentrations were considered to be a useful marker of current vitamin D exposure, the committee was concerned about the limitations of association studies. Specifically, low serum 25(OH)D levels may be linked with numerous confounding factors that are known to relate to higher cancer risk, including obesity (due to vitamin D sequestration in adipose tissue), lack of physical activity (correlated with less time outdoors and less incidental solar exposure), race/dark skin pigmentation (less skin synthesis of vitamin D in response to sun), and diet or supplement-taking prac-

tices (3, 135, 163). Reverse causation bias is also a concern if poor health reduces outdoor activities and sun exposure or adversely affects diet, thereby resulting in lower serum 25(OH)D levels. Because of these potential biases and limitations, association cannot prove causation (163, 164).

There is strong biological plausibility for a role of vitamin D in cancer prevention. The VDR is expressed in most tissues. Studies of *in vitro* cell culture and *in vivo* experimental models suggest that 1,25-(OH)<sub>2</sub>D promotes cell differentiation, inhibits cancer cell proliferation, and exhibits antiinflammatory, proapoptotic, and antiangiogenic properties (163, 165, 166). Through binding to the VDR, 1,25-(OH)<sub>2</sub>D has been shown in laboratory studies to inhibit the growth of cancer cells by regulating several genes responsible for cell proliferation—*e.g.*, activating cyclin-dependent kinase inhibitors such as p21 and p27; repressing growth factors such as IGF-I and epidermal growth factor receptor; and activating growth regulatory genes such as TGF- $\beta$ . 1,25-(OH)<sub>2</sub>D-VDR transcriptional signaling may also exert antiinflammatory effects on cancer cells by down-regulating the prostaglandin pathway and cyclooxygenase-2, leading to growth inhibition. In addition, 1,25-(OH)<sub>2</sub>D exhibits proapoptotic effects in cancer cells by repressing several prosurvival proteins such as BCL2 and telomerase reverse transcriptase and by activating proapoptotic proteins such as BAK. Recent *in vivo* and *in vitro* studies have further suggested that vitamin D signaling is particularly relevant for advanced-stage or high-grade tumors because of its inhibitory effects on angiogenesis, invasion, and metastatic potential. Treatment of cancer cells with 1,25-(OH)<sub>2</sub>D may inhibit cell tube formation and tumor growth by repressing vascular endothelial growth factor and IL-8. Although the mechanistic studies are promising, they cannot provide conclusive evidence that vitamin D prevents the development of cancer in humans or slows its progression to invasive and metastatic forms.

### B. Total cancer and cancer mortality: research findings

Although several observational studies have linked low serum levels of 25(OH)D with increased cancer incidence and mortality, no previous randomized trial has assessed cancer as a primary prespecified outcome (3, 135, 163). Three previous trials of vitamin D have assessed incident cancer or cancer mortality as secondary outcomes, but the results were null (167–169) (Table 1). For example, in a British trial of 2686 men and women aged 65–85, in which 100,000 IU of vitamin D<sub>3</sub> every 4 months (average intake, ~833 mg/d) was compared with placebo, the relative risk (RR) for cancer incidence over 5 yr was 1.09 (95% CI, 0.86–1.36) (167). In a 4-yr trial among 1179 postmenopausal women (mean age, 67 yr) in Nebraska, women

**TABLE 1.** RCT of vitamin D supplementation and total cancer incidence

Site of study	Cohort	Intervention dose	No. of subjects (treated/control)	RR (95% CI)
Oxford, United Kingdom	2,686 men and women, ages 65–85 yr	Vitamin D <sub>3</sub> 100,000 IU every 4 months (~833 IU/d) vs. placebo	188/173	1.09 (0.86–1.36)
Nebraska, United States	1,179 postmenopausal women, mean age 67 yr	Vitamin D <sub>3</sub> 1,100 IU/d + calcium vs. calcium alone	13/17	0.76 (0.38–1.55)
WHI, United States	36,282 postmenopausal women, ages 50–79 yr	Vitamin D <sub>3</sub> 400 IU/d + calcium vs. placebo	1634/1655	0.98 (0.91–1.05)

Data are from Refs. 160 and 163.

receiving calcium plus vitamin D (1000 IU/d) had a lower rate of malignancies than those receiving placebo but did not have a significantly lower risk of cancer than those receiving calcium alone (13 vs. 17 cases; RR = 0.76; 95% CI, 0.38–1.55) (168). Interestingly, calcium alone tended to reduce cancer incidence vs. placebo, although this was not statistically significant. In that trial, assignment to vitamin D raised mean serum 25(OH)D by 24 nmol/liter, from 72 nmol/liter at baseline to 96 nmol/liter after 1 yr of treatment. Among 36,000 postmenopausal women aged 50–79 in the WHI trial of calcium (1000 mg/d) plus low-dose vitamin D<sub>3</sub> (400 IU/d), the 7-yr intervention did not reduce the incidence of total cancer (RR = 0.98; 95% CI, 0.91–1.05) or cancer mortality (RR = 0.89; 95% CI, 0.77–1.03) (169, 170).

## C. Vitamin D and the risk of site-specific cancers

### 1. Breast cancer

**a. Overview.** The influence of 1,25-(OH)<sub>2</sub>D on breast cancer cells *in vitro* includes anticancer effects such as cell cycle inhibition, reduced proliferation, enhanced sensitivity to apoptosis, and induction of differentiation markers (171). These responses appear to be mediated by the VDR, which is expressed on nearly all established breast cancer cell lines (172). Although target genes regulated by vitamin D show variability in different model systems, some common features include inducing cellular differentiation, remodeling of the extracellular matrix, and enhancing innate immunity (172). Many of the genes identified show a consensus VDRE in their promoter elements, suggesting that they are specific targets of the VDR complex (172, 173).

**b. Observational studies.** The 2009 AHRQ report did not find any qualified systematic reviews that evaluated associations between vitamin D intake or serum 25(OH)D and risk for breast cancer (160). Three observational studies of sufficient methodological quality were identified that assessed the association between 25(OH)D levels and breast cancer risk. A prospective cohort study within a subgroup of NHANES III reported that women with higher

25(OH)D levels were at significantly lower risk for breast cancer. In this study, however, only eight women were in the higher 25(OH)D category, and a linear trend analysis was nonsignificant (174). In a nested case-control study using data from the Nurses' Health Study, no significant relationship between higher plasma 25(OH)D concentrations and lower risk for breast cancer was observed overall, but a significant trend was seen for women above age 60 (175). Another nested case-control cohort study of postmenopausal women participating in the Prostate, Lung, Colorectal, and Ovarian Cancer (PLCO) Screening Trial found no evidence that higher plasma 25(OH)D concentrations were associated with reduced risk of breast cancer (176). More recently, a large case-control study in Italy of women aged 20–74 yr found an inverse association between vitamin D intake and risk for breast cancer, with an apparent threshold at intakes of 188 IU/d or greater (177). In contrast, a study of Canadian women found no association between dietary intake of vitamin D or calcium with breast cancer risk, except for a reduced risk associated with vitamin D supplements greater than 400 IU/d (178). In the WHI, an inverse association between baseline 25(OH)D levels and incident breast cancer disappeared after adjustment for BMI and physical activity levels (170).

**c. Randomized controlled trials.** Only one randomized trial (the WHI calcium/vitamin D trial of 1000 mg calcium combined with 400 IU/d of vitamin D<sub>3</sub>) was large enough to assess breast cancer as a separate, although secondary, outcome (170). Overall, the WHI showed no significant effect of the intervention on breast cancer incidence (HR, 0.96; 95% CI, 0.86–1.07) or breast cancer mortality (HR, 0.99) over 7 yr. When the study population was stratified by baseline vitamin D intake (diet plus supplements), evidence for effect modification was seen. Women who had the lowest baseline intakes of vitamin D had a reduced risk of breast cancer with the intervention (HR, 0.79; 95% CI, 0.65–0.97), whereas women with the highest baseline intakes (≥600 IU/d) had significantly increased breast cancer risk (HR, 1.34; 95% CI, 1.01–1.78) (*P* for interac-

tion = 0.003). Thus, randomized trial data on vitamin D and breast cancer suggest possible benefits from supplementation among women with low baseline intake but raise the possibility of harm at higher (*e.g.*, above recommended dietary allowance) levels of intake.

**d. Conclusions.** Vitamin D and breast cancer. Although experimental laboratory studies are suggestive of a role for vitamin D in breast biology, available observational research is inconsistent, and randomized trial evidence is limited and not supportive of benefit.

## 2. Colorectal cancer

**a. Overview.** The VDR and the enzyme  $1\alpha$ -hydroxylase, which converts 25(OH)D to  $1,25\text{-(OH)}_2\text{D}$ , are expressed in colorectal tissue (179, 180).  $1,25\text{-(OH)}_2\text{D}$  and its analogs have been shown to regulate cell proliferation and differentiation in human colon cancer cell lines (181, 182). Injection of colon cancer cells into vitamin D-sufficient and vitamin D-deficient mice led to significantly greater tumor growth in the vitamin D-deficient animals (183).  $1,25\text{-(OH)}_2\text{D}$  may also affect the development and progression of colon cancer by acting directly on calcium homeostasis, increasing intracellular calcium flux (182, 184).

**b. Observational studies.** Observational studies of serum 25(OH)D levels in relation to colorectal cancer incidence generally support an inverse association (3, 135, 162). In a meta-analysis of prospective data from five studies with a total of 535 cases (including the large-scale Nurses' Health Study and WHI), those with serum 25(OH)D of at least 33 ng/ml ( $\geq 83$  nmol/liter) had about half the risk for colorectal cancer of those with levels of 12 ng/ml or less ( $\leq 30$  nmol/liter) (185). The large-scale European Prospective Investigation into Cancer and Nutrition study reported a similarly strong inverse association (162, 186). The Japan Public Health Centre-based Prospective Study did not find an inverse relation between plasma 25(OH)D and colon cancer in either men or women, although an inverse association with rectal cancer was apparent (187). A 2008 meta-analysis by the International Agency for Research on Cancer (IARC) found a significant inverse association between serum 25(OH)D and colorectal cancer risk, although there was significant between-study heterogeneity (162). Similarly, a recent systematic review by AHRQ (161) found a 6% (95% CI, 3 to 9%) reduction in colorectal cancer risk for each 10-nmol/liter increase in 25(OH)D concentrations in observational studies but concluded that the evidence was not sufficiently robust to draw conclusions regarding a cause-effect relationship.

**c. Randomized controlled trials.** Randomized trial evidence for vitamin D in the prevention of colorectal cancer is limited. In a 5-yr British trial, in which 2686 older men and women were randomized to 100,000 IU of vitamin D<sub>3</sub> or placebo every 4 months ( $\sim 833$  IU/d), the intervention was not associated with a reduction in colorectal cancer incidence, a secondary outcome (28 colorectal cancers in the vitamin D group *vs.* 27 in the placebo group; RR = 1.02; 95% CI, 0.60–1.74) (167). Similarly, among 36,000 women in the WHI calcium-vitamin D trial, a combination of calcium (1000 mg/d) plus low-dose vitamin D<sub>3</sub> (400 IU/d) for a mean of 7 yr did not reduce colorectal cancer incidence (168 *vs.* 154 cases; RR = 1.08; 95% CI, 0.86–1.34) or deaths from colorectal cancer (34 *vs.* 41 cases; RR = 0.82; 95% CI, 0.52–1.29; *P* = 0.39) (169).

**d. Conclusions: Vitamin D and colorectal cancers.** The experimental laboratory and observational research on vitamin D is more compelling for colorectal cancer than for other cancers. However, randomized trial evidence remains limited and has not demonstrated benefits to date. Whether randomized trials testing higher doses of vitamin D and providing longer duration of treatment will demonstrate efficacy in colorectal cancer prevention remains unknown.

## 3. Prostate cancer

**a. Overview.** Studies *in vitro* demonstrate that prostate cancer and epithelial cells in culture respond to  $1,25\text{-(OH)}_2\text{D}$  with antiproliferative effects and increased cell differentiation (3, 188). Like epithelial cells of other tissue origins, these effects appear to be mediated by the VDR expressed in prostate cells (3). Gene expression array studies suggest that  $1,25\text{-(OH)}_2\text{D}$  inhibits growth factor signaling and cell cycle progression, promotes differentiation, and has antiinflammatory and antiangiogenic effects (3, 189, 190).

**b. Observational studies.** Although ecological studies suggest that prostate cancer mortality is inversely related to sun exposure, observational analytic studies of serum 25(OH)D and prostate cancer have been inconsistent. Eight of 12 nested case-control studies found no association between baseline serum 25(OH)D and risk for prostate cancer, whereas one reported a significant inverse association [for baseline serum 25(OH)D levels  $< 30$  compared with levels  $> 55$  nmol/liter] (163, 191). A more recent case-control analysis of data from the  $\alpha$ -Tocopherol,  $\beta$ -Carotene Prevention Study found no association between serum 25(OH)D levels and incidence of prostate cancer (192). Moreover, a meta-analysis of 45 observational studies of dairy and milk intake in relation to risk of

prostate cancer showed no significant association with dietary intake of vitamin D (193).

**c. Randomized controlled trials.** No relevant RCT of vitamin D supplementation and risk of prostate cancer were identified.

**d. Conclusions: Vitamin D and prostate cancer.** Although laboratory data suggest a role for vitamin D in inhibiting prostate carcinogenesis, observational studies of serum 25(OH)D and risk of prostate cancer have provided mixed results, and data from randomized controlled clinical trials are lacking.

#### D. Other site-specific cancers

The large-scale Cohort Consortium Vitamin D Pooling Project of Rarer Cancers found no evidence linking higher serum concentrations of 25(OH)D to reduced risk of less common cancers, including endometrial, esophageal, gastric, kidney, pancreatic, and ovarian cancers, and non-Hodgkin lymphoma (194). In aggregate, these cancers represent approximately half of all cancers worldwide. Moreover, the report provided suggestive evidence for a significantly increased risk of pancreatic cancer at high levels [ $\geq 40$  ng/ml ( $\geq 100$  nmol/liter)] of 25(OH)D (194). An increased risk of esophageal cancer at higher levels of 25(OH)D also has been reported (3, 195).

#### E. Conclusions

Despite biological plausibility for a role of vitamin D in cancer prevention, most recent systematic reviews and meta-analyses, as well as a comprehensive review by the IOM Committee, have found that the evidence that vitamin D reduces cancer incidence and/or mortality is inconsistent and inconclusive as to causality. Importantly, no large-scale randomized trials have been completed with cancer as the primary prespecified outcome, and trials with cancer as a secondary outcome have been sparse and generally unresponsive. Observational evidence is strongest for colorectal cancer but is weak or inconsistent for breast, prostate, other cancer sites, and total cancer. Moreover, concerns about potential increased risk for selected cancers with high levels of 25(OH)D have been raised. New trials assessing the role of moderate- to high-dose vitamin D supplementation in cancer prevention, including the large-scale VITamin D and Omega-3 Trial (196), are in progress and should provide additional information within 5–6 yr. It is worth noting that many micronutrients that seemed promising in observational studies (*e.g.*,  $\beta$ -carotene, vitamins C and E, folic acid, and selenium) were not found to reduce the risk of cancer in RCT and some were found to cause harm at high levels of

supplementation (135, 163). Although future research may demonstrate clear benefits for vitamin D in relation to cancer and possibly support higher intake requirements for this purpose, the existing evidence has not reached that threshold.

## VII. Vitamin D and Cardiovascular Disease

### A. Introduction

For CV diseases (CVD), as with other outcomes, the assessment of 25(OH)D levels, intakes and supplementation is somewhat more complex due to a number of issues, including the relationship of sun exposure to serum 25(OH)D, the seasonal fluctuation in serum levels, the lack of information about the relationship between vitamin D dietary and supplement intake to serum levels, and, perhaps most importantly, the possibility that the benefits or risks of vitamin D supplementation may depend on initial levels of 25(OH)D. The assessment of vitamin D is further complicated by the fact that CVD is a heterogeneous category, and it is possible that vitamin D has a different relationship to individual types of clinical endpoints [*e.g.*, stroke *vs.* myocardial infarction (MI) *vs.* hypertension]. Notwithstanding, there is biological plausibility to the concept that vitamin D could impact CV events such as MI or hypertension, either directly through actions of the VDR in smooth muscle cells of the vasculature or cardiac muscle in the heart, or indirectly by promoting calcium absorption at the expense of lipid absorption or lipid excretion in the gut (197). Several basic mechanisms have been proposed, including endothelial dysfunction from lack of adequate vitamin D, vascular compliance impairment due to smooth muscle changes, enhanced inflammation or effects related to high levels of PTH, or the renin-angiotensin system. Although the VDR null mouse has been studied with respect to CVD outcomes and evidence has suggested that these mice might be at higher risk of vascular disease, several animal studies have not shown a relationship of vitamin D supplementation to development of CVD, and one animal study found increased thrombogenicity associated with vitamin D supplementation (198). On the other hand, vitamin D supplementation could lower vascular risk by improving glucose tolerance and/or inhibiting inflammatory components in the metabolic syndrome. However, vitamin D supplementation in animals with impaired renal function may actually worsen vascular responsiveness. And a recent meta-analysis of calcium use alone has suggested the possibility that enhanced calcium absorption (either from calcium supplements or possibly through increased vitamin D) may increase the risk of CV events (199).

## B. Studies of hypertension and lipids

Ecological studies have suggested that rates of CVD and hypertension may increase with increasing distance from the equator (200), which suggests the possibility that lower vitamin D levels are associated with higher risk of CVD. Pittas *et al.* (115) reviewed prospective observational studies of vitamin D levels in relationship to incident hypertension and identified three cohort studies for this question. In a meta-analysis of these three, they found a significant association between lowest levels of 25(OH)D (<37 to 51 nmol/liter) and incidence of hypertension over 7 to 8 yr. A recently performed systematic review of randomized trials that studied vitamin D and its impact on mean blood pressure and lipids (total cholesterol, triglycerides, low-density lipoproteins, and high-density lipoproteins) (123) included between 11 and 14 relevant studies (depending on the endpoint). The authors found no significant effect of vitamin D on any of these endpoints (the significance levels varied from 0.27 to 0.91), although they saw significant heterogeneity of meta-analytic estimates of low-density lipoprotein and high-density lipoprotein analyses. Thus, whereas current data do not support an effect of vitamin D on blood pressure and lipids, further studies of the effect of vitamin D on lipids are warranted.

## C. Studies of other CVD endpoints

### 1. Observational studies

There are a number of prospective observational studies that examined vitamin D status and risk of CVD using other endpoints for CVD. The Pittas *et al.* review (115) identified a total of seven studies, which included nine different analyses in six different cohorts. The outcomes used in these studies have varied and included MI, combined CVD, stroke, and CV mortality. The primary predictor in all of these studies was serum 25(OH)D concentration. These cohorts together have included more than 43,500 people with a mean follow-up ranging from 5 to 27 yr. Pittas *et al.* (115) judged five of the seven to be of good and two of poor quality.

Of the nine studies, five found that low vitamin D levels were associated with a high risk of CVD. The Framingham Offspring Study included 1739 participants without prior CVD (201). Over an average follow-up of 5 yr, the adjusted HR for overall incident CV events was 1.62 (95% CI, 1.11 to 2.36;  $P = 0.01$ ) in the 28% of the cohort with low 25(OH)D levels (<15 ng/ml) *vs.* the remainder. A secondary analysis suggested that this association may have been significant only in those with initial hypertension. Similarly, the Health Professional Follow-up Study using a nested case-control study in 18,225 men found an

increased risk of MI in those with 25(OH)D levels below 15 ng/ml compared with those with levels above 30 ng/ml (RR = 2.42; 95% CI, 1.53–3.84) (202). In a cohort study of 3258 patients undergoing coronary angiography, after 8 yr, those at the lowest 25(OH)D levels [<8 ng/ml (20 nmol/liter)] had significantly higher CV mortality compared with those with higher levels [>28 ng/ml (69 nmol/liter)] (203). An analysis of 13,331 women and men over 8.7 yr from NHANES III found only a trend toward an increased risk (RR = 1.2; nonsignificant) in the lowest (<17.8 ng/ml) compared with the highest 25(OH)D levels but lower risk (nonsignificant) in the intermediate quartiles (204). However, they found that overall mortality was significantly higher (RR = 1.26;  $P < 0.001$ ) in the lowest *vs.* highest quartiles (201). Marniemi *et al.* (205) found no significant relationship of serum 25(OH)D levels (lowest tertile) to MI but did find a relationship to stroke incidence. In a prospective cohort study of 1490 men over age 65 yr, followed for an average of 7.5 yr in the MrOs study (206) published since the Pittas review, there was no relationship of 25(OH)D level to CV mortality (HR = 1.01; 95% CI, 0.89–1.14) across its entire range. However, there was a trend (nonsignificant) toward a higher risk in those at the lowest level (<20 ng/ml) *vs.* those above 30 ng/ml (HR = 1.51; 95% CI, 0.82–2.76). Pittas *et al.* (115) did not perform a meta-analysis of observational cohort studies due to heterogeneity of outcomes.

Although most cohort studies have focused on risk of CVD among those with the lowest levels of serum 25(OH)D, several analyses allowed an examination of the higher levels and have suggested that risk does not continue to decrease at levels above 30 ng/ml. This includes the Framingham Osteoporosis Study for all CV events (201). The NHANES study found a higher risk for CVD mortality in those with 25(OH)D levels above 30 ng/ml overall, although risk began to increase with levels above 30 ng/ml and then declined with levels above 40 ng/ml (207). There are also a number of observational studies suggesting that overall mortality does not decrease further and may increase in those with higher levels of 25(OH)D. The IOM report suggested the possibility of increased risk of CV risk and mortality at the highest levels of 25(OH)D and that this possibility should be studied further (135).

Grandi *et al.* (208) pooled data from prospective observational studies and demonstrated an overall association of 25(OH)D baseline levels in the lowest compared with the highest 25(OH)D categories defined in each study. There was a significant relationship for incident composite CV events (pooled HR = 1.54; 95% CI, 1.22–1.95) and for CV mortality (HR = 1.83; 95% CI, 1.19–2.80). There was, however, significant heterogeneity and an indication for a possible publication bias in some of

these analyses (particularly for mortality), making the reported HR less reliable. The IOM report summarized the observational data as showing overall positive evidence for a relationship of low 25(OH)D levels to CVD but does not support the view that higher levels of 25(OH)D are associated with further lowering of risk (3). In sum, there is some evidence from observational data to suggest that low levels of 25(OH)D are associated with a greater risk of CVD. On the other hand, modestly higher levels of 25(OH)D may be associated with better health indices such as nutrition, sunlight exposure, and physical activity, which in turn could lead to a lower risk of CVD. Thus, confounding factors, even when controlled for, make it difficult to draw conclusions from these observational studies.

## 2. Randomized trials

There is a limited amount of evidence from randomized trials of vitamin D alone (*i.e.*, not in combination with calcium) *vs.* placebo to address the relationship to CVD. The Pittas *et al.* (115) review lists two such trials. Trivedi *et al.* (167) performed a randomized trial of vitamin D<sub>3</sub> (100,000 U every 4 months for 5 yr) *vs.* placebo. The primary endpoints were fractures and cause-specific mortality. They found a nonsignificant trend (RR = 0.84; 95% CI, 0.055–1.10) toward a reduction in CV deaths. Another RCT studied added vitamin D to ongoing calcium supplementation in 302 women; the primary endpoint was risk of falls (209). They reported as adverse events ischemic heart disease event rates of 1.3% in those on vitamin D *vs.* 2.0% for placebo (two *vs.* three events). Combining these two trials into a meta-analysis, Wang *et al.* report a pooled RR of 0.90 (0.77–1.05, not statistically significant) (210).

The Pittas *et al.* (115) review reports on two other randomized trials using vitamin D in combination with calcium *vs.* double placebo. In the largest of these, the WHI, more than 36,000 women were randomized to receive both 400 IU of vitamin D<sub>3</sub>/d and 1000 mg of calcium/d or placebo (211, 212). After a 7-yr follow-up, no significant effect was reported on any of three CV outcomes (MI, coronary heart disease death, or stroke). The investigators also reported a nearly significant increased risk (RR = 1.09; 95% CI, 0.99 to 1.19) for a combined endpoint of nonfatal MI, coronary heart disease death, or revascularization. One other small trial (n = 192) of vitamin D (800 IU) in combination with calcium reported 11 CV events, which did not differ by treatment (213).

A recent meta-analysis by Elamin *et al.* (123) examined randomized trials of vitamin D (with or without calcium) for the endpoints of MI and stroke, as well as all-cause mortality. The authors performed a comprehensive literature search and found six studies (the four above plus two

others) that have reported on MI and/or stroke (including trials with nonfatal events as well as those with only fatal events). For MI, the pooled RR was 1.02 (95% CI, 0.93–1.13), and for stroke it was 1.05 (95% CI, 0.88–1.25). It is worth noting that the WHI accounted for approximately 90% of the participants in the pooled studies. Based on the result for MI and stroke, as well as the null results for overall mortality, the authors concluded that current trial evidence was not consistent with recommending vitamin D to patients to reduce CV risk.

## 3. Conclusions

The results from many, although not all, prospective observational studies of the relationship between CVD and 25(OH)D levels suggest that low levels of serum 25(OH)D are associated with future increased risk of CV outcomes. However, the interpretation of these findings is limited by the different outcomes assessed in the studies. More importantly, whereas the observational studies might suggest an association between low levels of 25(OH)D and future CVD, this association may not be causal, and therefore it cannot be assumed that increasing 25(OH)D levels through supplementation will reduce CV risk. As has been shown with several antioxidant vitamins and supplements (*e.g.*, vitamin E and selenium), well-conducted randomized trials can yield results that are inconsistent with previous positive observational evidence (214). Therefore, caution is required in generalizing observational evidence directly into clinical practice.

The randomized trial evidence is currently inadequate to define the relationship between vitamin D and reduction in CV events. The two trials of vitamin D alone discussed in *Section VII.C.* suggest a trend but not a significant reduction in CV events. The Trivedi *et al.* (167) trial used 100,000 IU four times per year, and therefore results may not be generalizable to more frequent/lower dose vitamin D supplementation. Although the WHI did not find a relationship between vitamin D supplementation and CVD, it used a low dose of vitamin D (400 IU) in combination with calcium. A recent meta-analysis raises the possibility that calcium supplementation might increase CV risk (199). Therefore, it is conceivable that vitamin D, by facilitating calcium delivery, might increase CV risk. Furthermore, the possibility that calcium increases CV risk complicates the interpretation of trials of vitamin D in combination with calcium with respect to CVD and suggests greater reliance on the limited evidence from trials that used vitamin D alone *vs.* placebo.

In conclusion, whereas there is a possibility that vitamin D supplementation may lower CVD risk, the limitations of applying observational data to clinical practice

and the insufficiency of the evidence from clinical trials do not support recommending vitamin D supplementation for lowering CVD risk at this time. This is consistent with the conclusions from the recent trial meta-analysis as well as the recent IOM report (3, 123). Additional research, particularly from randomized trials, is needed—particularly research examining whether there is a dose-response relationship of vitamin D supplementation to CV outcomes or whether high levels of supplementation might increase CVD.

## VIII. Vitamin D and Immune Function

### A. Introduction

The human immune response to invading microbes and cells is composed of two basic elements, the innate and adaptive immune response. “Innate” derives from the Latin word “innātus” meaning inborn or not conditioned by an acquired event. The human innate immune response is modulated principally by two cell types: the monocyte/macrophage and the dendritic cell. These two cell types are purposed to recognize, inactivate, or kill invaders as well as to draft cells of the adaptive immune response to protect the host from that invader. The so-called adaptive arm of the human immune response is composed of T and B lymphocytes. Through directed cell-to-cell contact or by the elaboration of cell-targeted, specific lymphokines T (Th<sub>1</sub>, Th<sub>17</sub>) and B Ig-producing lymphocytes act to promote destruction of the offending invader. On the other hand, Treg and Th<sub>2</sub> lymphocytes act to quell what might otherwise be an overzealous immune response to the invader that could illicit off-target damage to the host.

The first hint that cells of the innate and adaptive immune response in humans might be potential targets for 1,25-(OH)<sub>2</sub>D-directed gene expression and the subject of functional consequences regulated by vitamin D balance in the host came from observations of human immune cell populations *in vitro*: 1) that human lymphocytes and monocyte-macrophage cells expressed the VDR when exposed to mitogens or specific antigens (215–219); 2) that the major effect on lymphocytes and monocytes was to limit their proliferation in response to mitogen and antigen stimulation (218, 219); 3) that disease-activated macrophages harvested from the lungs of patients with active granuloma-forming diseases, such as sarcoidosis and tuberculosis, were extremely efficient at synthesizing 1,25-(OH)<sub>2</sub>D when incubated with substrate 25(OH)D (220); and 4) that 1,25-(OH)<sub>2</sub>D production by the macrophage was driven by the T-cell cytokine interferon- $\gamma$  (IFN- $\gamma$ ) (221). *In vivo* clinical correlates of these findings were: 1) the utility of 1-hydroxylated vitamin D metabolites or an-

alogs as therapeutic agents to control hyperproliferative disorders such as psoriasis (222); 2) the course of patients (lacking a renal source for the vitamin D hormone) with widespread, active granuloma-forming diseases can be complicated clinically by hypercalciuria or frank hypercalcemia if macrophage-produced 1,25-(OH)<sub>2</sub>D escapes the local inflammatory microenvironment to the general circulation (223, 224); 3) going back to the preantibiotic era in the late 1800s and the early 1900s, observations that direct sunlight exposure at high altitudes was beneficial in the management of cutaneous and pulmonary tuberculosis (225); and 4) colocalization and concentration of the 25(OH)D-CYP27B1-hydroxylase stimulating Th1 cytokine IFN- $\gamma$  and product 1,25-(OH)<sub>2</sub>D at sites of inflammation in the human host with active granuloma-forming disease (226, 227).

What was not clearly recognized until recently was the molecular means by which macrophages could be activated to express the VDR and CYP27B1-hydroxylase in the presence of a disease-causing agent. For insight in this direction, lessons learned from the human macrophage exposed to the human pathogen *Mycobacterium tuberculosis* (mTB) will be the focus of much of the subsequent discussion. Liu *et al.* (67) investigated the consequence of the interaction of human macrophages with a pathogen-associated membrane pattern molecule from mTB recognized to activate a pair of pattern recognition receptors (PRR), the TLR 2/1 dimer pair (67, 228); PRR such as the TLR are unique in that they are activated not by specific antigens but by nonspecific products of microbes and other cells (229). The TLR, like other PRR in the human macrophage, are known to engage an intracellular adaptor proteins (e.g., myD88), which signals through the cell's kinase systems to activate and translocate nuclear signaling molecules (e.g., nuclear factor  $\kappa$ B) to transcribe monokine gene products that are then used to regulate (either up or down) the innate immune response in that cell or to direct the adaptive immune response (230). Working from previous studies that showed that the cathelicidin gene and its endogenous antibiotic-like gene product LL37 were under stimulatory control of a VDRE in the promoter of the cathelicidin gene, Liu *et al.* (67, 231) showed that inhibition of the CYP27B1-hydroxylating activity, blockade of the VDR with a competitive nonacting analog of 1,25-(OH)<sub>2</sub>D, and small interfering RNA-directed knock-down of the LL37 mRNA translation all resulted in failure to synthesize LL37. These and more recent data suggest that: 1) the synthesis of 1,25-(OH)<sub>2</sub>D and the VDR inside the human macrophage represents a self-contained, intracrine-acting system capable of killing mTB inside that cell by a combination of antimicrobial gene expression and co-opting the cell's autophagy pathway (232–234); 2) this

antimicrobial action is amplified under the influence of IFN- $\gamma$ -driven, autocrine IL-15 and IL-1 $\beta$  production (234, 235); and 3) this is dependent on extracellular availability of free 25(OH)D to the macrophage (236–238).

### B. Clinical observations and trials

To date, clinical observations imputing a role for the vitamin D synthetic-metabolic system as a *bona fide* regulator of the human immune response *in vivo* has been largely confined to cross-sectional studies, many very large, in which a disease is associated with low 25(OH)D levels (239). For the most part, these diseases are ones in which TLR have been implicated in pathogenesis of the particular disorder and for which VDR and CYP27B1 expression lie downstream in the innate immune response elicited by that disease. For example, atherosclerosis, an inflammatory disease of the vasculature in which the TLR are implicated in its pathogenesis, has been shown to be significantly associated with 25(OH)D less than 30 ng/ml (240). In fact, an increase in all-cause mortality in the U.S. population has been linked to low population serum 25(OH)D levels (204). Most of this mortality is attributed to the consequences of atherosclerotic vascular disease, the number one killer in the United States. The occurrence of cancer, particularly colon cancer, in which the TLR2 and TLR4 signaling pathways are activated, has been associated with vitamin D deficiency (241). Infectious mycobacterial (TLR2/1 and TLR4) (233, 235), bacterial (TLR4 and TLR6) (242, 243), and viral diseases (TLR 7) (244), as well as autoimmune diseases (TLR7) (245) have all been significantly associated with low serum 25(OH)D levels in cross-sectional studies.

The IOM found inadequate data from clinical trials to support the utility of vitamin D supplementation in the treatment and prevention of infectious, inflammatory, hyperproliferative, and autoimmune disorders (135). One area of recent investigation that is at the intersection of immunology and metabolism has been the association of type 1 diabetes mellitus with vitamin D status. Although there are few high-quality randomized trials of vitamin D supplementation for the prevention of this type of diabetes, theoretically, changes in immune status (*i.e.*, self-recognition) could prevent or forestall the onset of  $\beta$ -cell dysfunction (246). Alternatively, vitamin D supplementation could alter the innate immune response to latent viruses, thereby impacting the disease course in a different manner (247). Intuitively, boosting the endogenous innate response with vitamin D and/or 25(OH)D supplementation in subjects susceptible to chronic diseases should be a safe and relatively inexpensive intervention. The 20,000-subject VITAL RCT (163) will likely provide important insights as to whether supplemental vitamin D will lower

the risk for infections, inflammatory disease, autoimmune disease, musculoskeletal deficiency, type 2 diabetes, and hypertension (secondary outcomes). Barring larger preliminary studies in type 1 diabetes mellitus, it is unlikely that a vitamin D intervention trial will be performed in younger individuals at high risk for developing type 1 diabetes mellitus.

### C. Conclusions

There is a large body of evidence being generated *in vitro* and *ex vivo* to implicate the substrate-dependent, intracellular conversion of 25(OH)D to 1,25-(OH) $_2$ D with subsequent modulation in the bioactions of activated, VDR-expressing monocytes, macrophages, dendritic cells, and lymphocytes to control both the innate and adaptive immune response in man (Fig. 3). With the possible exception of psoriasis, in which the topical administration of 1-hydroxylated vitamin D metabolites and analogs has been shown to be both efficacious and safe, delivery of such vitamin D metabolites and analogs parenterally or orally to achieve an immunomodulatory effect in the host has been thwarted by off-target activation of the VDR in tissues that contribute an influx of calcium into the general circulation, resulting in hypercalciuria and hypercalcemia. These disappointing results and general failure to discover a nonhypercalcemic analog with immunomodulatory potential have led others to begin to employ the use of vitamin D and 25(OH)D supplements to boost the availability of 25(OH)D to the disease (TLR)-activated monocyte-macrophage. This approach would permit the host macrophage to generate 1,25-(OH) $_2$ D in a regulated fashion, which may in turn engage the VDR and turn on the host immune innate immune response. The expectation would be that this macrophage will be enhanced in its ability to: 1) neutralize invading microbes and foreign cells; 2) instruct the adaptive immune response in promoting this neutralization response at same time; and 3) prevent what might turn into an overzealous adaptive immune response that would prove detrimental, not beneficial, to the health of the host.

## IX. Vitamin D, the Placenta, and Maternal/Fetal Health

### A. Introduction

The placenta forms a physical and functional barrier between the maternal and fetal circulations. Within it, 1,25-(OH) $_2$ D could conceivably play autocrine, paracrine, or endocrine roles in regulating host defenses, trophoblast invasion, nutrient and gas exchange, hematopoiesis, hormone production, and fetal growth

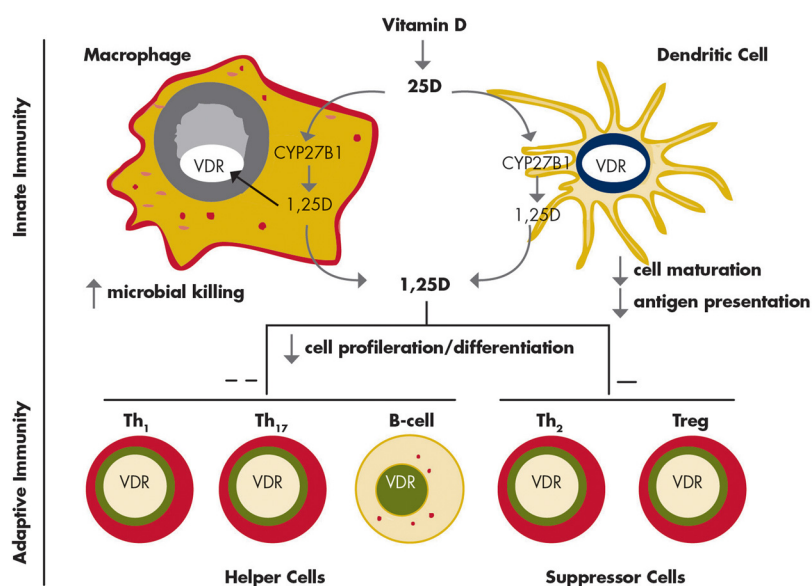
**Figure 3.**

Figure 3. Impact of vitamin D on the human innate (*upper panel*) and adaptive immune response (*lower panel*). When activated by mitogen or specific antigen, macrophages, dendritic cells, and lymphocytes express the VDR, thereby becoming targets for the active vitamin D metabolite, 1,25-(OH)<sub>2</sub>D. Macrophages and dendritic cells can also express the CYP27B1-hydroxylase that synthesizes 1,25-(OH)<sub>2</sub>D from substrate 25(OH)D, the major circulating metabolite of vitamin D and acknowledged best indicator of the amount of vitamin D entering the host via cutaneous synthesis or that ingested in the diet. Operating in an intracrine mode, 1,25-(OH)<sub>2</sub>D promotes microbial killing in the macrophage, whereas it inhibits maturation and the antigen-presenting capacity of the dendritic cell. If 1,25-(OH)<sub>2</sub>D escapes the confines of the macrophage or dendritic cell in sufficient amount, it can act on VDR-expressing lymphocytes recruited to the local inflammatory microenvironment. The major bioaction of 1,25-(OH)<sub>2</sub>D acting through the VDR in lymphocytes is to inhibit their proliferation and differentiation to maturity; this antiproliferative effect is more profound on the classes of helper than suppressor cells, leading to generalized suppression of the adaptive immune response. 1,25D, 1,25-dihydroxyvitamin D; 25D, 25-hydroxyvitamin D.

and development. Since the 1970s, we have known that trophoblasts and maternal decidua convert 25(OH)D to 1,25-(OH)<sub>2</sub>D, whereas more recent studies have confirmed that this is due to expression of CYP27B1 (248–252). In 1983, placental expression of VDR was inferred by binding of radiolabeled calcitriol to rat trophoblasts (253), and subsequent studies confirmed that trophoblasts, yolk sac, and decidua of humans, sheep, mouse, and rat express VDR (254–256). CYP24A1 is also expressed by trophoblasts, yolk sac, and decidua, where it converts 25(OH)D and 1,25-(OH)<sub>2</sub>D into inactive forms (251, 257, 258).

One study of human placentas found high levels of VDR and CYP27B1 mRNA, but low levels of CYP24A1 mRNA, as compared with adjacent decidua (259). When these findings are taken together with evidence that CYP24A1 is methylated in human placenta (260), some investigators have concluded that trophoblast synthesis of 1,25-(OH)<sub>2</sub>D is

unopposed or “unfettered” by degradation to 24-hydroxylated forms (260, 261). However, this conclusion is not supported by the burden of functional evidence. Human and rodent placentas preferentially metabolize 25(OH)D to 24,25-dihydroxyvitamin D over calcitriol (262–264), resulting in fetal levels of 24-hydroxylated forms that are up to 40-fold higher than 1,25-(OH)<sub>2</sub>D in humans, rats, and sheep (263–267). The placenta controls passage of vitamin D metabolites such that 25(OH)D freely crosses hemochorial placentas whereas 1,25-(OH)<sub>2</sub>D is blocked (263, 268). This explains why cord blood 25(OH)D levels are typically about 75–100% of maternal values at term whereas 1,25-(OH)<sub>2</sub>D is 25–40% of maternal levels (269). Maternal nephrectomy did not alter fetal 24,25-dihydroxyvitamin D or 1,25-(OH)<sub>2</sub>D levels, confirming that these metabolites are independently synthesized in the fetal-placental unit (264). Placenta and fetal kidneys both contribute to the low level of 1,25-(OH)<sub>2</sub>D in the fetal circulation (270). On the other hand, low vitamin D binding protein in the fetal circulation means that the free 1,25-(OH)<sub>2</sub>D level may be normal or even increased (271).

Maternal levels of 1,25-(OH)<sub>2</sub>D double or triple during pregnancy, whereas free levels do not increase until the third trimester (269, 271.) Placental production of 1,25-(OH)<sub>2</sub>D has often been assumed to explain the higher maternal levels, but this is incorrect. The rat placenta contributes a small amount of 1,25-(OH)<sub>2</sub>D to the maternal circulation, whereas only one sixth remnant of maternal kidney is sufficient to enable the normal pregnancy-induced increase in 1,25-(OH)<sub>2</sub>D (248, 272, 273). 1 $\alpha$ -Hydroxylase null pigs have very low levels of 1,25-(OH)<sub>2</sub>D, with no increase during pregnancy despite bearing heterozygous placentas (274). The most compelling human data come from a pregnant anephric woman who had very low 1,25-(OH)<sub>2</sub>D levels that did not change significantly during pregnancy (275). Overall, in pregnant mammals it appears that most or all of the 1,25-(OH)<sub>2</sub>D comes from a 2- to 5-fold up-regulation in CYP27B1 within maternal kidneys (276, 277). 1,25-(OH)<sub>2</sub>D that is produced by the fetal-placental unit likely doesn’t affect the mother.

## B. Biological plausibility

The placenta has immunoregulatory functions that enable successful implantation, block most maternal anti-

bodies and blood cells, and defend against microbial organisms. The importance of this role is underscored by the realization that intrauterine infections explain much of the risk of preterm birth and intrauterine growth retardation (278, 279). The TLR are essential components of the innate response to pathogen-associated microbial products, and trophoblasts express at least 10 TLR to some degree (280). Trophoblasts also express cathelicidin and other antimicrobial proteins, including bactericidal/permeability-increasing protein, secretory leukocyte protease inhibitor, human  $\beta$ -defensin 2, and acyloxyacyl hydrolase (281). Placenta and adjacent decidua also contain abundant immune cells (macrophages, dendritic cells, lymphocytes) that, as mentioned earlier, synthesize and respond to 1,25-(OH)<sub>2</sub>D.

There is biological plausibility for 1,25-(OH)<sub>2</sub>D to play a role in regulating placental defenses against infection. Injection of normal pregnant mice with lipopolysaccharide (LPS), a TLR ligand, caused marked elevation in placental expression of 1 $\alpha$ -hydroxylase and VDR (282). *Vdr* null and *Cyp27b1* null trophoblasts cultured *in vitro* had dysregulated inflammatory markers at baseline (increased IFN- $\gamma$ , decreased IL-10) and in response to treatment with LPS (increased TLR2, IFN- $\gamma$ , and IL-6) (282). Treatment of a trophoblast cell line with 25(OH)D before challenge with *Escherichia coli* protected against trophoblast cell death and led to fewer bacterial colony-forming units being formed (283). TLR2 was also increased in placentas obtained from pregnancies with documented preterm infection (284). In contrast to what occurs in macrophages, cathelicidin and other antimicrobial proteins were not induced by LPS or other TLR ligands (281, 283), and this was thought to be caused by absence of TLR4 in trophoblasts (281).

Because preeclampsia is considered a disease caused by dysfunctional trophoblasts, there is biological plausibility that altered vitamin D metabolism could locally predispose to preeclampsia. Several TLR (TLR-2, TLR-3, TLR-4, and TLR-9) were up-regulated in placentas from preeclamptic *vs.* normal women (285). Other investigators sought a causative role for altered placental expression of 1 $\alpha$ -hydroxylase but found conflicting results of decreased (286) and increased expression of 1 $\alpha$ -hydroxylase in preeclamptic *vs.* normal placentas (287).

These and other data support the hypothesis that 1,25-(OH)<sub>2</sub>D may act in a paracrine or autocrine manner to influence trophoblast growth and responses to infection and inflammation. In turn, loss of such actions might predispose to preeclampsia, placental infections and insufficiency, preterm birth, and certain immune-related disorders (*e.g.*, type 1 diabetes). None of these studies address what intake of vitamin D or circulating level of 25(OH)D

is required to achieve the postulated effects of 1,25-(OH)<sub>2</sub>D on trophoblasts *in vivo*.

### C. Animal data

Pregnancy and fetal development have been examined in severely vitamin D-deficient rats (288–290), 1 $\alpha$ -hydroxylase null pigs (291), and *Vdr* null mice (292, 293). 1 $\alpha$ -Hydroxylase null (*Cyp27b1* null) mice are infertile. Fetal-placental mineral and skeletal homeostasis appears unaffected in all of these models, and these data are reviewed elsewhere (269, 294). Local production of 1,25-(OH)<sub>2</sub>D by maternal decidua, immune cells, and invading trophoblasts has been proposed to critically regulate implantation and growth at the maternal-fetal interface (261, 295). At first glance, this theory appears to be supported by the findings that severely vitamin D-deficient rats and *Vdr* null mice conceive less often and bear fewer pups than normal and that *Cyp27b1* null mice were reported to be infertile (73, 289, 296, 298). However, the conception rate and litter sizes of *Vdr* null mice are normalized simply with a higher calcium diet (292, 293, 299, 301), whereas *Cyp27b1* null mice may have more fundamental problems of hypoplastic uteri and failure to ovulate (73). Although the size and weight of pups born of severely vitamin D-deficient rats are normal (289, 296, 298), pups born of *Vdr* null mothers are globally smaller than normal (293), which is consistent with a role for VDR in fetal growth or nutrition. Gestational length is normal in all mouse, rat, and pig models, with no reported evidence of infections, preeclampsia, or preterm births. Severe vitamin D deficiency beginning during gestation does not increase the risk of type 1 diabetes in otherwise normal rodents, but it causes diabetes to emerge earlier in nonobese diabetic (NOD) mice (302). Supraphysiological doses of vitamin D given *in utero* did not prevent diabetes in NOD mice (303). In contrast, *Vdr* null mice are not predisposed to develop type 1 diabetes, and *Vdr*-NOD double-mutants have the same risk of diabetes as NOD mice (304). These studies suggest that severe vitamin D deficiency increases the risk of type 1 diabetes only in genetically predisposed NOD mice and that the effect is not mediated by VDR. Vitamin D deficiency during rat gestation has been shown to cause subtle changes in brain development that may lead to impaired dopaminergic and cognitive function as adults (305–307).

### D. Observational and association studies

Available observational data come from vitamin D-deficient to -sufficient pregnant women who participated in observational studies and in the placebo arms of clinical trials of vitamin D supplementation (269). In none of these reports were increased adverse obstetrical/neonatal out-

comes reported, but low numbers of subjects meant that few adverse events occurred. Similarly, women with genetic absence of 1 $\alpha$ -hydroxylase or VDR (vitamin D-dependent rickets types I and II, respectively) have been reported to have normal fertility and uneventful pregnancies (269). Numerous associations have been examined between single measurements of maternal 25(OH)D or estimates of vitamin D intake and various obstetrical/neonatal outcomes. At best, these studies indirectly implicate altered fetal-placental vitamin D physiology as causing the outcome of interest. Some studies suggest that low maternal 25(OH)D predicts increased risk of preterm birth (308), threatened preterm delivery (308), and cesarean section (309), whereas other studies found no significant associations with these outcomes (310–313). These studies had low power, differed in their methods to measure 25(OH)D, and differed in the cut-points used to define vitamin D deficiency. They were also confounded by factors that predict low 25(OH)D and the outcomes of interest, including race/ethnicity, maternal overweight/obesity, lower socioeconomic status, poor nutrition, *etc.* For example, overweight/obesity predisposes to preeclampsia, gestational diabetes, vaginal infections, macrosomia, and other obstetrical complications (314).

Preeclampsia is associated with normal maternal ionized and albumin-corrected serum calcium, but lower calcium intake, hypocalciuria, lower 1,25-(OH)<sub>2</sub>D levels, and reduced creatinine clearance (294). 1,25-(OH)<sub>2</sub>D may be reduced to the level of healthy nonpregnant women. Low maternal 25(OH)D and low estimated vitamin D intake have also been associated with preeclampsia (315–318), although an equal number of studies refuted this association (310, 319–321). Rather than causing preeclampsia, the low 1,25-(OH)<sub>2</sub>D and hypocalciuria may result from the renal damage that occurs with the condition. Consistent with this, preeclamptic women had normal 1,25-(OH)<sub>2</sub>D levels earlier in pregnancy and low calcitriol only after developing hypertension and proteinuria (322). Moreover, because serum levels of fat-soluble vitamins A, D, and E were all lower in preeclamptic women *vs.* normotensive pregnant and nonpregnant controls (323), a confounding factor such as overweight/obesity or nutrition may explain why the fat-soluble vitamins were reduced.

Other associational studies have explored “fetal programming,” the concept that low 25(OH)D stores during gestation program the emergence of disorders in the child or adult. (Studies that used season of birth to implicate vitamin D during late gestation are not specific enough to warrant consideration in this review.) For type 1 diabetes, a higher maternal dietary intake of vitamin D during pregnancy was associated with decreased prevalence of islet

cell antibodies and diabetes in the children; curiously, maternal use of vitamin D supplements had no effect (324). In other studies, recalled use of vitamin D supplements during pregnancy was associated with lower childhood incidence of type 1 diabetes (325, 326). But another study found no association between maternal 25(OH)D during pregnancy and type 1 diabetes in the offspring (327). For childhood asthma and allergy, some studies found that higher maternal intake of vitamin D during pregnancy decreased the risk (328, 329), whereas other studies found that higher maternal 25(OH)D levels during pregnancy increased the risk as much as 5-fold (330, 331). Investigators who found effects of fetal vitamin D deficiency on rat brain neurodevelopment looked at banked human serum and found no association between maternal 25(OH)D levels during late pregnancy and risk of schizophrenia in the offspring (307).

Associational and ecological studies are hypothesis-generating but do not prove causality. The studies described above provide inconsistent and conflicting evidence, and no proof that higher intakes of vitamin D during pregnancy will prevent any adverse nonskeletal outcomes.

## E. Randomized interventional trials

The finding that preeclampsia is associated with low calcium intake and hypocalciuria prompted numerous randomized, placebo-controlled clinical trials of calcium supplementation in women at risk of preeclampsia. A recent meta-analysis found that 1 g of supplemental calcium significantly reduced the risk of preeclampsia in women who had low dietary calcium intake at baseline (RR = 0.36), or a high baseline risk of preeclampsia (RR = 0.22). There was no benefit when dietary calcium intake was adequate (332). In the same meta-analysis, preterm birth was also significantly decreased by the use of calcium supplements (RR = 0.76) (332). Multiple clinical trials have tested whether vitamin D supplementation improves maternal or fetal outcomes of pregnancy (269). The consistent finding is that supplemental vitamin D increases maternal and cord blood 25(OH)D levels. No study demonstrated any other obstetrical benefit, including those that specifically reported preeclampsia (333, 335–337) or preterm birth and low birth weight (333, 335, 338). The most extreme example raised the cord blood 25(OH)D level from 10 to 138 nmol/liter without obvious obstetrical benefits (334). However, none of these studies was sufficiently powered to detect a difference in many of the outcomes.

Two recent studies by Hollis *et al.* (333) warrant additional consideration. The first study randomized 494 women at 12–16 wk to receive 400, 2000, or 4000 IU of

vitamin D<sub>3</sub>/d; 350 subjects (70.9%) completed the trial. Achieved mean maternal 25(OH)D was  $78.9 \pm 36.5$ ,  $98.3 \pm 34.2$ , and  $111.0 \pm 43.0$  nmol/liter, respectively (333). The second study enrolled 257 women at 12–16 wk to receive 2000 or 4000 IU of vitamin D<sub>3</sub>/d; 160 subjects (62.3%) completed it. The first study has been published, whereas results of both studies have been presented at conferences and on YouTube (333, 335). In the intention-to-treat analysis for each study, no effect was seen on birth weight, gestational length, preterm birth, early preterm birth (<32 wk), preeclampsia, infections, cesarean sections, gestational diabetes, or other obstetrical outcomes (333, 335). Consequently, because no maternal or fetal/neonatal benefit was found, these two studies do not provide any evidence to justify a particular level of 25(OH)D or intake of vitamin D during pregnancy.

## F. Conclusions

1,25-(OH)<sub>2</sub>D and its receptor are well poised in the placenta to influence obstetrical and neonatal outcomes, but whether they truly play a significant role remains unproven. Consequently, there is insufficient evidence to recommend a particular maternal intake of vitamin D or 25(OH)D blood level during pregnancy to achieve any purported nonskeletal benefit of vitamin D. On the other hand, the biological plausibility may be sufficient to justify clinical trials to test whether vitamin D supplementation during pregnancy will prevent type 1 diabetes in the offspring.

## X. Summary and Future Direction

Vitamin D is a pleiotropic hormone that affects classical and nonclassical tissues principally through the VDR. Its primary sites of action are still considered to be the intestine, bone, and kidneys. In regard to the former, changes in intestinal calcium absorption may play a major role in modulating cardiac and skeletal muscle function, although it is not absolutely clear whether in certain muscle cells, vitamin D may directly regulate function through the VDR. Nevertheless, it is likely that deficiencies in vitamin D may contribute to a modest risk for falls, particularly in older individuals. On the other hand, there is emerging evidence that vitamin D may directly regulate immune function, both innate and adaptive. However, it will require large well-designed clinical trials to prove that vitamin D supplementation could enhance innate immunity or reduce the severity of autoimmunity.

The role of vitamin D in the CV system is complex and will require further trials to define whether outcomes such as hypertension, MI, and stroke are directly related to

vitamin D supplementation. The *in vitro* antiproliferative effects of vitamin D on neoplastic tissue are well recognized, but the clinical evidence has been relatively modest due to the short duration of follow-up and the relatively small number of subjects in previous trials. The 20,000-person VITAL trial, which has just been initiated, may help determine what effect vitamin D supplementation has on both neoplastic and CV outcomes. Although all the elements of the vitamin D regulatory system are present in skin, randomized trials have not demonstrated that this hormone prevents skin cancer or is better than other agents for the treatment of proliferative skin disorders. In respect to the relationship between vitamin D and placenta and maternal/fetal health, the observational studies provide somewhat conflicting evidence, and the randomized trials for preeclampsia and neonatal outcome do not show clear benefits for mother or child.

In summary, not surprisingly there remains a persistent need for large randomized controlled trials and dose-response data to test the effects of vitamin D on chronic disease outcomes including autoimmunity, obesity, diabetes mellitus, hypertension, and heart disease. The VITAL trial, as noted above, could help determine whether higher doses of vitamin D (*i.e.*, 2000 IU/d) will reduce the risk of osteoporosis, cancer, and CVD. Similarly, a very large, placebo-controlled, randomized trial of vitamin D, 4000 IU/d, to prevent the onset of type 2 diabetes mellitus in prediabetics is currently in the planning stage. Any potential benefit of high-dose vitamin D supplementation on maternal or fetal outcomes will also await larger trials. Notwithstanding, large-scale clinical trials of a single nutrient may not fully answer the many questions inherent in vitamin D actions. Thus, the role of vitamin D supplementation in the prevention and treatment of chronic nonskeletal diseases remains to be determined.

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