nutrients

A Commemorative Issue in Honor of Centennial of the Discovery of Vitamin D

The Central Role of Vitamin D in Physiology

Edited by
Carsten Carlberg

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A Commemorative Issue in Honor of Centennial of the Discovery of Vitamin D-The Central Role of Vitamin D in Physiology

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About the Editor

Carsten Carlberg

Carsten Carlberg graduated in 1989 with a PhD in biochemistry from the Free University Berlin (Germany). After holding positions as a postdoc at Roche (Basel, Switzerland), a group leader at the University of Geneva (Switzerland), and a docent at the University of Düsseldorf (Germany) he is, since 2000, a full professor of biochemistry at the University of Eastern Finland in Kuopio (Finland) and, since 2022, ERA chair for nutrigenomics at the Polish Academy of Sciences in Olsztyn (Poland). His work focuses on mechanisms of gene regulation by nuclear hormones, in particular on vitamin D. At present, Prof. Carlberg focuses projects on the epigenome-wide effects of vitamin D on the human immune system in the context of cancer.

Preface to "A Commemorative Issue in Honor of Centennial of the Discovery of Vitamin D-The Central Role of Vitamin D in Physiology"

In 2022, we celebrated the 100 year anniversary of the naming of vitamin D by Dr. McCollum. At the beginning of the last century, small molecules were found to cure several diseases caused by nutritional deficiencies and were, therefore, termed vitamins. Vitamin D received the index "D" because it was the fourth vitamin named after vitamins called A, B, and C. The specific purpose of vitamin D was described as the protection of bone growth and the prevention of rickets. In the last 100 years, tremendous advances in the understanding of the physiological role and mechanisms of action have been achieved. Vitamin D2 was identified in 1932 and vitamin D3 was identified in 1937, but it took until 1978 to prove that vitamin D3 is synthesized in human skin after UV-B irradiation. At the beginning of the 1960s, 1,25(OH)2D3 (calcitriol) was found to be a biologically active form of vitamin D. The vitamin D receptor (VDR) was isolated in 1969, and its gene was cloned in 1987. This was the basis for understanding vitamin D as a direct regulator of hundreds of genes in various tissues. VDR's ligand-binding domain was crystalized in 2000, and since 2010, we know VDR's genome-wide binding pattern in various cellular models. In parallel, 1,25(OH)2D3 and its synthetic analogues have been known since the early 1980s as regulators of monocyte differentiation during hematopoiesis as well as inhibitors of cellular proliferation in various in vitro and in vivo models. Today, it is obvious that an appropriate vitamin D status is essential for maintaining the health and well-being of everyone. Thus, vitamin D3 does not only regulate our calcium level in order to allow proper bone formation, but it also modulates our immune system and, in this way, indirectly protects us from cancer.

This vitamin D centenary issue provides a fantastic opportunity to present recent developments and the latest research in the fascinating broad range of today's vitamin D biology, from evolution to systems biology. In 21 articles, leading authors in the field presented original work and review articles to the whole biomedical community.

Carsten Carlberg
Editor

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Editorial

A Pleiotropic Nuclear Hormone Labelled Hundred Years Ago Vitamin D

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This year we are celebrating 100 years of the naming of vitamin D, but the molecule is, in fact, more than one billion years old [1]. *Nutrition Bulletin* and *Endocrine Connections* are also honoring the centenary of vitamin D's discovery, but with 21 original publications and review articles written by experts in the field, *Nutrients* represents the largest collection [1–21].

At the beginning of the last century, small molecules were found to cure several diseases caused by nutritional deficiencies and were, therefore, termed vitamins. These diseases are xerophthalmia (a clinical spectrum ranging from night blindness to complete blindness), anemias, and neurological disorders, such as beriberi and scurvy (a disability affecting the repair of bone, skin, and connective tissue), which can be healed and prevented using the supplementation of vitamins A, B and C, respectively. Thus, when McCollum and colleagues demonstrated in 1922 that experimentally induced rickets in rats could be cured by a factor isolated from cod liver oil, they followed the nomenclature termed and named it vitamin D [22].

The term "vitamin" implies that these molecules should be regularly supplied as part of our diet or as pills. However, some vitamins can be endogenously produced by our bodies, such as vitamin D_3 in UV-B-exposed skin [23]. Thus, changes in our lifestyle, such as spending more time indoors, wearing clothes outdoors, and living at latitudes with seasonal variations of UV-B intensity, made vitamin D a vitamin. The insufficient production or supplementation of vitamin D_3 causes a low vitamin D status, which is determined by the serum concentration of the most stable vitamin D_3 metabolite, 25-hydroxyvitamin D_3 ($25(OH)D_3$). Severe vitamin D deficiency, defined as $25(OH)D_3$ serum levels below 30 nM (12 ng/mL), can lead to bone malformations, such as rickets in children and osteomalacia in adults [24], and at all ages, lead to a malfunctioning immune system [25]. The latter may increase the risk for severe consequences of infectious diseases, such as COVID-19 (coronavirus disease) [26] or tuberculosis [27], as well as for the onset and progression of autoimmune diseases, such as type 1 diabetes [28] and multiple sclerosis [29].

Vitamin D and vitamin A differ from other vitamins due to the interesting property that a few metabolic steps can convert them into nuclear hormones that bind with high affinity to members of the nuclear receptor superfamily [17]. In the case of vitamin D_3, this is the metabolite 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) activating the transcription factor VDR (vitamin D receptor) [4]. In fact, $1,25(OH)_2D_3$ binds to VDR with a K_D of 0.1 nM, which is a significantly higher affinity than that of other nuclear receptors for their specific ligands. This suggests that the genomic signaling of $1,25(OH)_2D_3$ via VDR is the predominant means of vitamin D's mechanism of action [30]. However, there are also indications for non-genomic vitamin D signaling [19] as well as the biological activity of vitamin D metabolites other than $1,25(OH)_2D_3$ [16].

In genomic signaling, in all VDR-expressing tissues and cell types, vitamin D controls the transcription of hundreds of target genes [4]. The Genotype-Tissue Expression (GTEx) project provides gene expression data from 54 tissues obtained from 948 post-mortem

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donors [31]. The selected tissues are representative of more than 400 tissues and cell types that constitute our body. At present, the publicly available dataset (https://gtexportal.org) (accessed on 12 December 2022) is the gold standard for comparing tissue-specific gene expression. The expression of the *VDR* gene is highest in the skin, intestines, and colon and lowest in different regions of the brain (Figure 1). In other tissues, such as the blood and kidneys, the *VDR* gene shows intermediate levels of expression. The shortcut interpretation of this gene expression panel is that vitamin D, via the activation of VDR, primarily impacts the tissue of its synthesis and that its main action is in the gut, while in the brain, it may have no direct function. However, one has to distinguish the role of vitamin D as a controller of calcium transport in the gut [7] from its regulatory function, e.g., in the immune system [3]. Therefore, immune cells may not need as many VDR proteins as intestinal cells.

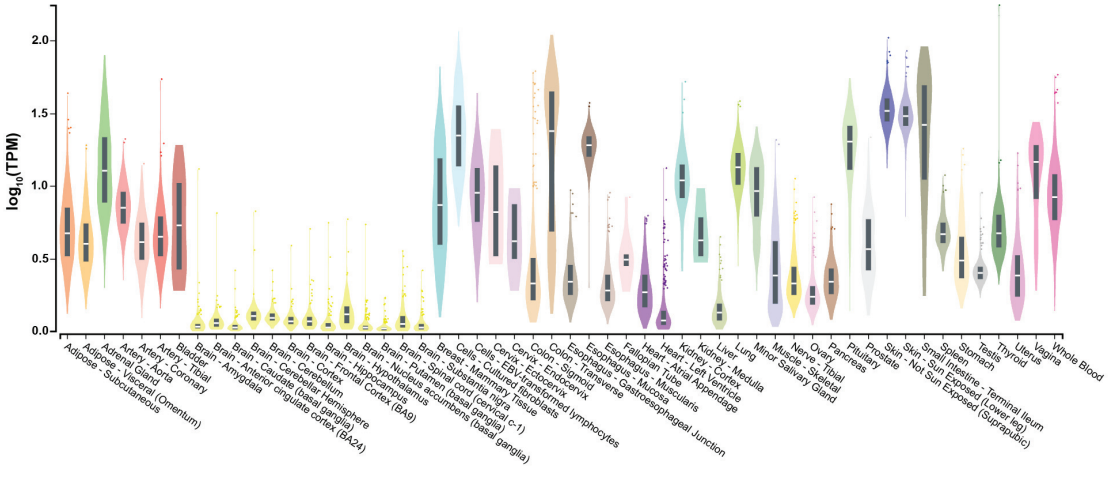

Figure 1. Expression of the *VDR* gene in 54 different human tissues. Normalized RNA sequencing (RNA-seq) data are shown in TPM (transcripts per million), where all isoforms were collapsed into a single gene. Box plots display the median as well as 25th and 75th percentiles. Points indicate outliers that are 1.5 times above or below interquartile range. Data are based on GTEx analysis release V8 (dbGaP Accession phs000424.v8.p2) [31].

From an evolutionary perspective, the calcium-absorbing and bone-remodeling function of vitamin D (Figure 2) was obtained less than 400 million years ago, when species left the ocean and needed a stable skeleton [1]. Thus, the best-known role of vitamin D was not why evolution created vitamin D endocrinology. However, regarding this physiological function, VDR and its ligand became dominant regulators [7,20]. In this context, vitamin D learned to control the expression of parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) [11]. The peptide hormones are expressed in the parathyroid gland and osteocytes, respectively, and up- and down-regulate the production of $1,25(OH)_2D_3$ (Figure 2).

Most likely, the first function of VDR was the regulation of energy metabolism [32]. Energy is essential for basically all biological processes, but particularly for the function of innate and adaptive immunity [33]. Vitamin D and its receptor obtained via the control of immunometabolism a modulatory role on immunity [34] (Figure 2).

Taken together, despite the story of its naming, vitamin D is not only a vitamin that prevents bone malformations. In contrast, via $1,25(OH)_2D_3$, vitamin D is a direct regulator of gene expression, resulting in pleiotropic physiological functions.

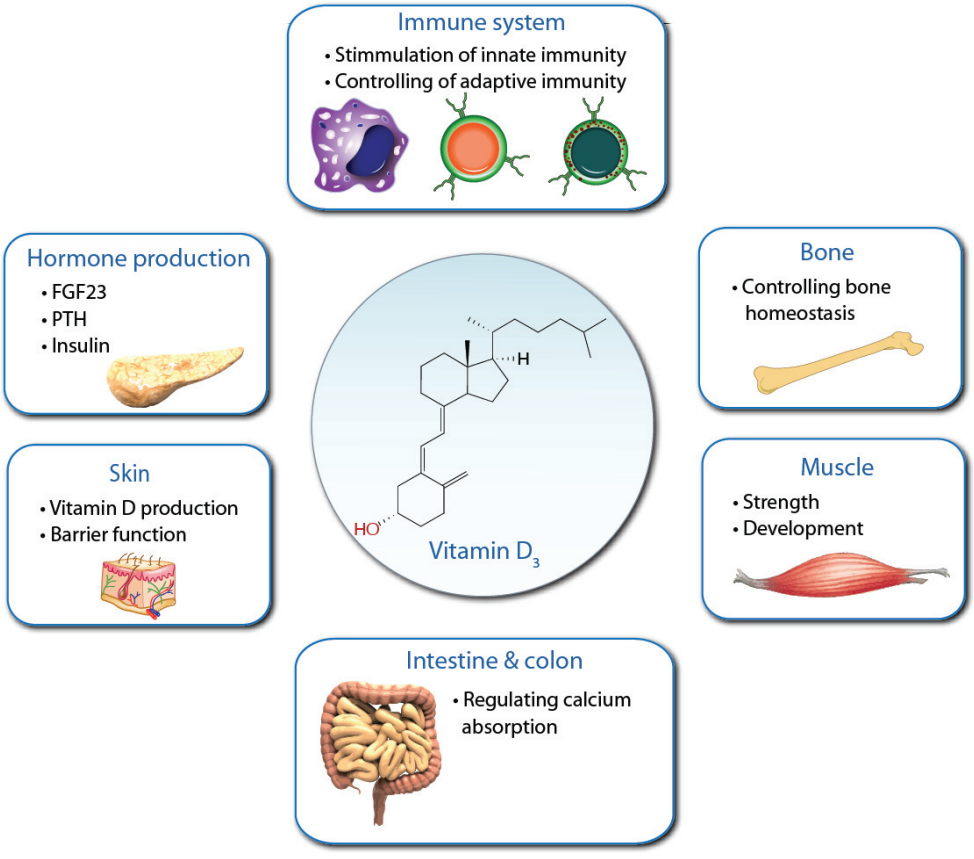

Figure 2. Pleiotropic physiological functions of vitamin D. Vitamin D_3 (center) can be produced endogenously in UV-B exposed skin. Via its metabolite $1,25(OH)_2D_3$, vitamin D_3 activates VDR in various tissues and cell types, where it regulates the indicated major physiological functions.

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Review

The One-Hundred-Year Anniversary of the Discovery of the Sunshine Vitamin D_3: Historical, Personal Experience and Evidence-Based Perspectives

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Abstract: The discovery of a fat-soluble nutrient that had antirachitic activity and no vitamin A activity by McCollum has had far reaching health benefits for children and adults. He named this nutrient vitamin D. The goal of this review and personal experiences is to give the reader a broad perspective almost from the beginning of time for how vitamin D evolved to became intimately involved in the evolution of land vertebrates. It was the deficiency of sunlight causing the devastating skeletal disease known as English disease and rickets that provided the first insight as to the relationship of sunlight and the cutaneous production of vitamin D_3. The initial appreciation that vitamin D could be obtained from ultraviolet exposure of ergosterol in yeast to produce vitamin D_2 resulted in the fortification of foods with vitamin D_2 and the eradication of rickets. Vitamin D_3 and vitamin D_2 (represented as D) are equally effective in humans. They undergo sequential metabolism to produce the active form of vitamin D, 1,25-dihydroxyvitamin D. It is now also recognized that essentially every tissue and cell in the body not only has a vitamin D receptor but can produce 1,25-dihydroxyvitamin D. This could explain why vitamin D deficiency has now been related to many acute and chronic illnesses, including COVID-19.

Keywords: vitamin D; McCollum; sunlight; rickets; 25-hydroxyvitamin D; 1,25-dihydroxyvitamin D; food fortification; vitamin D receptor; vitamin D_2; vitamin D_3; COVID-19

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1. Prequel

The sunshine vitamin D is likely to be the oldest hormone to be produced almost from the beginning of time when eukaryotes began to evolve in the oceans more than 1–1/2 billion years ago. The first evidence for this was the observation that a phytoplankton species *Emiliania huxlei*, which has existed for more than 750 million years unchanged in the Sargasso Sea, contained ergosterol (provitamin D_2; 1 mcg/g dry weight), which was converted to previtamin D_2 when exposed to simulated sunlight. Why these early eukaryotic organisms would contain such a large amount of ergosterol and produce vitamin D_2 is unknown. It has been speculated that early photosynthetic eukaryotes that required sun exposure for their energy source were also at risk of being exposed to ultraviolet radiation (UV) that could damage their precious DNA, RNA and proteins. The UV absorption spectra for provitamin D_2, previtamin D_2 and vitamin D_2 have high-extinction coefficients that completely overlap the UV-absorption spectra for these UV-sensitive macromolecules, thereby protecting them from solar ultraviolet radiation damage. It was also suggested that ergosterol in the plasma membrane not only served as an ideal sunscreen but also, upon exposure to ultraviolet radiation, was converted to previtamin D_2. Previtamin D_2, which is a planar structure embedded into the membrane, is thermally unstable and isomerizes into a non-planar vitamin D_2 structure. This process would have caused a significant structural change in the bilipid layer of the plasma membrane, resulting in vitamin D_2 being released into the environment. This process would have opened a tiny pore in the membrane that could have permitted the entrance of calcium and other cations into the

cell. This could help explain why sunlight, vitamin D and calcium are so intimately related. When vertebrate life forms left the oceans for terra firma, they were leaving a calcium-rich environment into a poor calcium environment. It is possible that the association of the production of vitamin D_2 in the plasma membrane of a phytoplankton, permitting the entrance of calcium into the cell, may have been taken advantage of in land vertebrates. During exposure to sunlight, land vertebrates would have produced vitamin D_3 in their skin, which would have entered their circulation and increased the efficiency of calcium absorption. Although it is unknown when vitamin D_3 was first metabolized and activated, it is likely that this occurred as marine vertebrates began to leave the oceans and explore and ultimately dominate terra firma. Most vertebrates require sunlight for their vitamin D requirement to maintain a healthy skeleton. Some species such as cats, other heavily furred vertebrates, and mole rats obtain their vitamin D from dietary sources. Fish-eating bats and vampire bats easily obtain an adequate amount of vitamin D from their diet, and recently, it was reported that insects, including mosquitoes exposed to sunlight, produce vitamin D_3 and are excellent dietary sources for insect-eating bats [1–4].

2. The Discovery of the Sunshine Vitamin D

By the 16th century, there was a migration of the population into crowded and air-polluted cities (Figure 1). Simultaneous with this migration was the appearance of a devastating crippling bone-deforming disorder that affected most of the children.

Figure 1. Photograph from Glasgow, Great Britain, in about 1870 showing that the buildings are built near each other. Ref. [5] Holick, copyright 1994. Reproduced with permission.

In 1650, Glisson, DeBoot and Whistler published their observations and concluded that this was a distinct disease that caused deformities of the skeleton, including enlargement of the joints of the long bones and rib cage, curvature of the spine and thighs, short stature, generalized muscle weakness and enlargement of the head (Figure 2). This disease, commonly known as rickets or English disease, continued to spread throughout European cities, and when the industrial revolution arrived in the 19th century, rickets became endemic in the northeastern United States. By the 20th century, it was estimated based on autopsy studies performed in Boston and Leiden that between 80–90% of children living in industrialized cities in Europe and the United States were suffering from this catastrophic bone-softening skeletal disorder. Besides causing bone deformities and growth retardation, vitamin D deficiency also gave rise to a flattened deformed pelvis with a small pelvic outlet in females of childbearing age. As a result, young women had a difficult time with child birthing, often experiencing serious complications including death of both the mom and newborn. In 1889, Murdock Cameron, a Scottish surgeon, began routinely

performing C-sections to save both the mother and infant from a prolonged, difficult, and life-threatening childbirth [6–8].

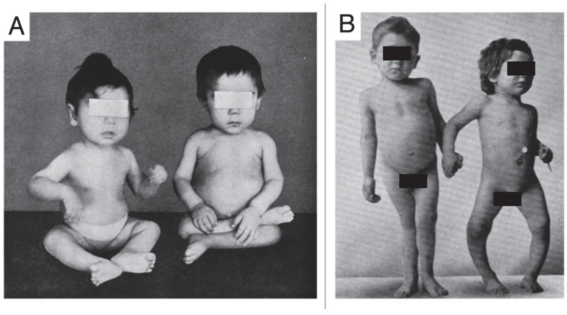


Figure 2. Skeletal deformities observed in rickets. (**A**) Photograph from the 1930s of a sister (left) and brother (right), aged 10 mo and 2.5 y, respectively, showing enlargement of the ends of the bones at the wrist, carpopedal spasm, and a typical "Taylorwise" posture of rickets. (**B**) The same brother and sister 4 y later, with classic knock knees (genu valgum) and bowed legs (genu varum), growth retardation, and other skeletal deformities. Ref. [7] Holick, copyright 2006. Reproduced with permission.

The first association relating inadequate sunlight exposure as a cause for rickets was made by the Polish physician scientist Sniadecki in 1822 [9]. He connected the dots and realized that children living in industrialized Warsaw who were not exposed to direct sunlight were plagued with rickets, whereas children who lived in rural farm areas and were outdoors and exposed to sunlight had no evidence of this bone-deforming disease. This was followed by Palm who in 1890 recognized that the lack of sunlight was a common denominator that could be associated with the high incidence of rickets in children living in the inner cities in Great Britain when compared to children living in underdeveloped countries [10]. He encouraged systematic sunbathing as a means for preventing and curing rickets, as did Sniadecki [9].

It was common folklore in the 19th century in fishing communities on the coast of England to give their children cod liver oil as a way of preventing rickets. Bretonneau in 1827 treated a 15-month-old child with severe rickets with cod liver oil and noted the incredible speed at which the patient was cured. His student Trousseau further demonstrated that oils from aquatic mammals including seals and whales as well as oily fish including herring were also effective in treating rickets [7,8]. These observations suggested that rickets was caused by a nutritional deficiency. Finally, in 1918, Mellanby reported that he could produce rickets in beagles by placing them on an oatmeal diet and then reversing the disease by giving them cod liver oil [11]. This observation convinced the scientific community that rickets was caused by a nutritional deficiency and the hunt was on to determine what nutrient was deficient. It was originally concluded that vitamin A in cod liver oil was responsible for its antirachitic activity. However, Elmer McCollum, who had discovered vitamin B, was not convinced that the antirachitic factor was vitamin A. It was known, based on the observation by Hopkins, that vitamin A was very sensitive to heat and could easily be destroyed by heating and aerating it [8]. McCollum used this information and conducted a study where he aerated and heated cod liver oil and demonstrated that it no longer had vitamin A activity but was still capable of healing rickets. This eureka discovery resulted in McCollum naming this new nutrient vitamin D [12].

At the same time, sunlight was beginning to be appreciated for its health-promoting properties. For his observations that direct sunlight exposure was effective in treating cutaneous tuberculosis and other skin disorders, Finsen received the Nobel Prize in 1903 [8]. In 1919, Huldschinski demonstrated that exposing children to radiation from a sun quartz lamp (mercury arc lamp) or carbon arc lamp resulted in marked increases in the mineralization of the long bones in the children's X-rays. He evaluated a similar group of children

not exposed to his lamps and reported no significant change in their X-rays. (Figure 3) He concluded that exposure to ultraviolet (UV) radiation was an infallible remedy against all forms of rickets in children. He also exposed one arm of some children to one of his lamps and demonstrated that the improvements in the mineralization in the X-rays of the children were identical in both arms. He concluded that the UV exposure to the skin was causing some type of photochemical reaction, producing a substance that entered the circulation and had a systemic effect on the skeleton [13,14]. These observations prompted Alfred Hess to expose seven rachitic children in New York City to varying periods of sunshine, and he reported marked improvement in rickets of each child as evidenced by calcification of their epiphyses (growth plates at the end of the long bones). He also concluded that children of color were at higher risk for developing rickets and required longer exposure times to sunlight to have the same effect that was observed in white children [15] (Figure 4).

Figure 3. Photographs of researchers who made crucial contributions to vitamin D and rickets research. (**A**) Jędrzej Sniadecki, (**B**) Kurt Huldschinsky, (**C**) Alfred Hess, (**D**) Harry Steenbock, and (**E**) Elmer McCullum. Ref. [8] Holick, copyright 2013. Reproduced with permission.

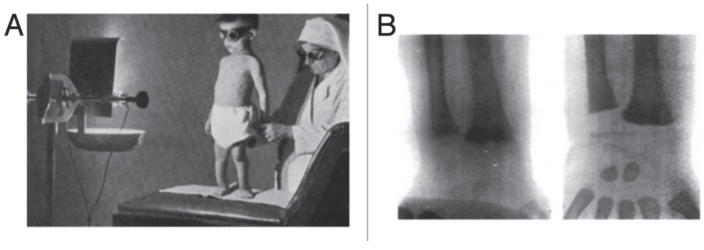


Figure 4. UV radiation therapy for rickets. (**A**) Photograph from the 1920s of a child with rickets being exposed to UV radiation. (**B**) Radiographs demonstrating florid rickets of the hand and wrist (**left**) and the same wrist and hand taken after treatment with 1 h UV radiation 2 times per week for 8 weeks. Note mineralization of the carpal bones and epiphyseal plates (**right**). Ref. [7] Holick, copyright 2006. Reproduced with permission.

These two disparate observations for the cure of rickets caused confusion. In 1921, Powers et al. fed a rachitic diet to rats and demonstrated that exposure to ultraviolet radiation was effective in preventing rickets, similar to dietary vitamin D [16]. However, the true appreciation for the health benefits of preventing rickets by exposing foods to UV radiation did not occur until 1924. Steenbock and Hess independently reported that exposure of foods, cotton seed and corn oil and especially the sterol fraction of foods with UV radiation produced antirachitic activity in rodents and chickens. Whereas Hess commented that "the question of the therapeutic value of this procedure is of secondary importance", Steenbock patented the process for the UV irradiation of foods and other substances as a means of increasing their antirachitic properties. These included yeasts, cereals, grain, oils, fats, butter and milk. The dairy industry appreciated the importance of providing milk to children with antirachitic activity and initiated the addition of ergosterol, obtained from yeast, to milk, followed by UV irradiation. Later, UV-irradiated ergosterol

was added to milk to provide antirachitic activity [17,18]. By the early 1930s, essentially all milk in the United States and most European countries including Great Britain encouraged the fortification of milk with vitamin D_2. At the same time, the United States Department of Labor sent out a brochure encouraging parents to give their children a coat of tan from sun exposure to prevent rickets and to improve bone health (Figure 5). Within a few years, these interventions resulted in the essential eradication of rickets in the industrialized countries. Vitamin D fortification was so popular worldwide that beer, soda pop, hot dogs, custard and even soap and shaving cream were fortified with vitamin D (Figure 6). Parents were also able to purchase a mercury arc lamp from their local pharmacy and expose their children at home to ultraviolet radiation to prevent rickets (Figure 5).

Figure 5. (**A**). Brochure from the US Department of Labor promoting sensible sun exposure for children in 1931. (**B**). Mercury arc lamp turned on for the use of preventing rickets in children in the 1930s. (**C**). The Sperti lamp available in the 1940s was used to expose children to ultraviolet radiation to promote bone health and to prevent rickets. Holick copyright 2023. Reproduced with permission.

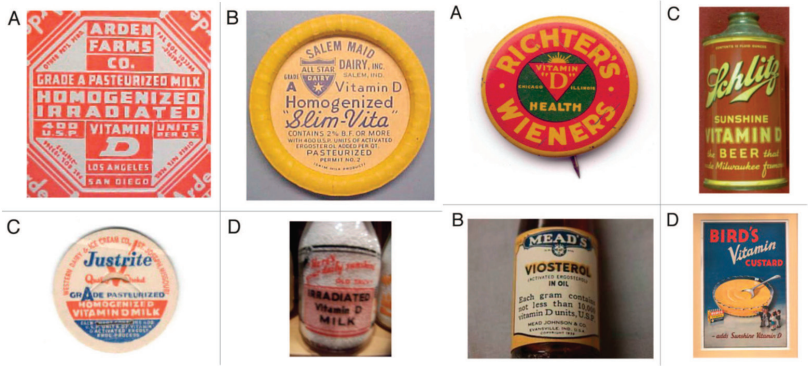


Figure 6. (**Left panel**). (**A**) Seal of a milk bottle that denoted that the milk was irradiated with UV radiation and contained vitamin D. (**B**) Cap of a milk bottle stating that activated ergosterol had been added to the milk. (**C**) Cap of milk bottle stating that the milk had been fortified with vitamin D. (**D**) Seal of a bottle of milk that denoted that the milk had been irradiated and contained vitamin D. Holick, copyright 2013. Reproduced with permission. (**Right panel**) (**A**) Seal denoting that this product was fortified with vitamin D. (**B**) Bottle of oil denoting that it contained irradiated ergosterol. (**C**) Beer can denoting that it was fortified with vitamin D. (**D**) Advertisement denoting that Bird's custard contained vitamin D. Ref. [8] Holick, copyright 2013. Reproduced with permission.

The vitamin D craze abruptly came to a halt when, in the early 1950s, an outbreak of infants with elfin facies, mild mental retardation, cardiac problems, and hypercalcemia was reported. The Royal College of physicians initiated an investigation and concluded that this outbreak in children was due to ingestion of excessive amounts of vitamin D in milk. This was based on a published observation that pregnant rats that received high doses of vitamin D delivered pups with some of the same clinical presentations including altered facial appearance, supravalvular aortic stenosis and hypercalcemia. Since it was difficult to monitor the content of vitamin D in milk, as a precautionary measure and based on the recommendation from the Royal College of Physicians, legislation quickly followed banning the fortification of any food or personal use product with vitamin D [19,20]. This ban quickly spread across Europe, Asia, India, and South America. Most of these countries still ban the fortification of foods with vitamin D with a few exceptions. Finland and Sweden now permit milk, margarine, and some cereals to be fortified with vitamin D. India recently introduced in 2017 a vitamin D_2 fortification program for milk and cooking oil. In retrospect, it is more likely that the children in Great Britain were suffering from the rare genetic disorder, Williams syndrome, which is associated with elfin facies, supravalvular aortic stenosis, mild mental retardation, and a hypersensitivity to vitamin D that causes hypercalcemia [21]. Unfortunately, this ban on vitamin D fortification persists in many countries throughout the world. It is responsible for the increased risk of rickets in children and vitamin D deficiency and insufficiency in children and adults.

3. Structural Identification of Vitamin D_2 and Vitamin D_3

When the oily sterol extract from yeast, containing ergosterol, was exposed to UV radiation, it was assumed that this resulted in the production of the antirachitic factor, vitamin D. However, it was quickly realized that there were other photoproducts present in the irradiated oil, and therefore, the UV-exposed sterol oil was called visterol. (Figure 6, right panel B). When animal oily sterols that mainly contained cholesterol were exposed to UV radiation, it resulted in the oils processing antirachitic properties. It was presumed that there was the contaminant, ergosterol, in the cholesterol oil that was transformed after UV irradiation into vitamin D. In 1932, Windaus in Germany and Bourdillon in England isolated pure crystalline vitamin D from irradiated ergosterol. Windaus and his colleagues

named the compound vitamin D_2, whereas the British investigators called their crystalline compound ergocalciferol [22,23]. Originally irradiated ergosterol was given the name vitamin D_1. Once it was realized that the irradiated ergosterol contained vitamin D and lumisterol, the term was no longer used.

It was originally believed that vertebrate animals such as pigs and humans produced vitamin D_2 in their skin when exposed to sunlight. Waddell, in 1934, found that irradiated ergosterol was less effective as an antirachitic factor in chickens when compared to the same amount given to rachitic rodents. He concluded that there was a contaminant in cholesterol, and it was not ergosterol, that was responsible for the antirachitic activity in cholesterol oil exposed to UV radiation [24]. This conundrum was finally solved when Windaus and colleagues synthesized 7-dehydrocholesterol, and after its irradiation, the isolated vitamin D had a high antirachitic activity in chickens, whereas vitamin D_2 had only 1–3% the activity in chickens that it had in rats. Further investigations revealed that the vitamin D isolated from halibut and blue fin tuna oil was identical to the vitamin D generated from 7-dehydrocholesterol. Windaus named his vitamin D as vitamin D_3. He received the Nobel Prize in 1928 for his work on the constitution of sterols, including the identification of vitamins associated with them. Schenck, in 1937, finally determined that Windaus's vitamin D in his irradiated sterols was vitamin D_3 based on its successful crystallization [25]. The structures for vitamin D_2 and vitamin D_3 can be found in Figure 7.

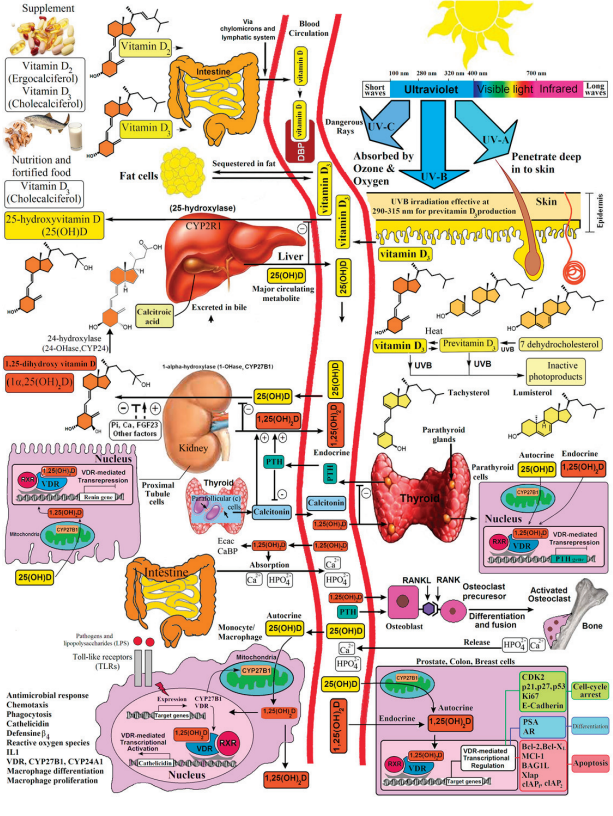


Figure 7. Schematic representation of the synthesis and metabolism of vitamin D for skeletal and extra-skeletal function. During exposure to sunlight, 7-dehydrocholesterol in the skin is converted to previtamin D_3. Previtamin D_3 immediately converts by a heat-dependent process to vitamin D_3. Excessive exposure to sunlight degrades previtamin D_3 and vitamin D_3 into inactive photoproducts.

Vitamin D_2 and vitamin D_3 from dietary sources are incorporated into chylomicrons and are transported by the lymphatic system into the venous circulation. Vitamin D (D represents D_2 or D_3) made in the skin or ingested in the diet can be stored in and then released from fat cells. Vitamin D in the circulation is bound to the vitamin D-binding protein (DBP), which transports it to the liver, where vitamin D is converted by the vitamin D-25-hydroxylase to 25-hydroxyvitamin D [25(OH)D]. This is the major circulating form of vitamin D that is used by clinicians to measure vitamin D status (although most reference laboratories report the normal range to be 20–100 ng/mL, the preferred healthful range is 30–60 ng/mL). It is biologically inactive and must be converted in the kidneys by the 25-hydroxyvitamin D-1a-hydroxylase (1-OHase) to its biologically active form 1,25-dihydroxyvitamin D [$1,25(OH)_2D$]. $1,25(OH)_2D_3$ is then taken up by target cells and targeted to intracellular D-binding proteins (IDBP) to mitochondrial 24-hydroxylase or to the vitamin D receptor (VDR). The $1,25(OH)_2D_3$-VDR complex heterodimerizes with the retinoic acid receptor (RXR) and binds to specific sequences in the promoter regions of the target gene. The DNA-bound heterodimer attracts components of the RNA polymerase II complex and nuclear transcription regulators. Serum phosphorus, calcium fibroblast growth factors (FGF-23), and other factors can either increase or decrease the renal production of 1,25(OH)2D. $1,25(OH)_2D$ feedback regulates its own synthesis and decreases the synthesis and secretion of parathyroid hormone (PTH) in the parathyroid glands. $1,25(OH)_2D$ increases the expression of the 25-hydroxyvitamin D-24-hydroxylase (24-OHase) to catabolize $1,25(OH)_2D$ to the water-soluble, biologically inactive calcitroic acid, which is excreted in the bile. $1,25(OH)_2D$ enhances intestinal calcium absorption in the small intestine by stimulating the expression of the epithelial calcium channel (ECaC) and calbindin 9K (calcium-binding protein, CaBP). $1,25(OH)_2D$ is recognized by its receptor in osteoblasts, causing an increase in the expression of the receptor activator of the NF-kB ligand (RANKL). Its receptor RANK on the preosteoclast binds RANKL, which induces the preosteoclast to become a mature osteoclast. The mature osteoclast removes calcium and phosphorus from the bone to maintain blood calcium and phosphorus levels. Adequate calcium and phosphorus levels promote mineralization of the skeleton. Autocrine/paracrine metabolism of 25(OH)D occurs when a macrophage or monocyte is stimulated through its toll-like receptor 2/1 (TLR2/1) by an infectious agent such as Mycobacterium tuberculosis or its lipopolysaccharide the signal upregulates the expression of VDR and 1-OHase. A 25(OH)D level of 30 ng/mL or higher provides adequate substrate levels for 1-OHase to convert 25(OH)D to $1,25(OH)_2D$ in mitochondria. $1,25(OH)_2D$ travels to the nucleus, where it increases the expression of cathelicidin, a peptide capable of promoting innate immunity and inducing the destruction of infectious agents such as M. tuberculosis. It is also likely that the $1,25(OH)_2D$ produced in monocytes or macrophages is released to act locally on activated T lymphocytes, which regulate cytokine synthesis, and activated B lymphocytes, which regulate immunoglobulin synthesis. When the 25(OH)D level is approximately 30 ng/mL, the risk of many common cancers is reduced. It is believed that the local production of $1,25(OH)_2D$ in the breast, colon, prostate, and other tissues regulates a variety of genes that control proliferation, including p21. Ref. [26] Holick, copyright 2013. Reproduced with permission.

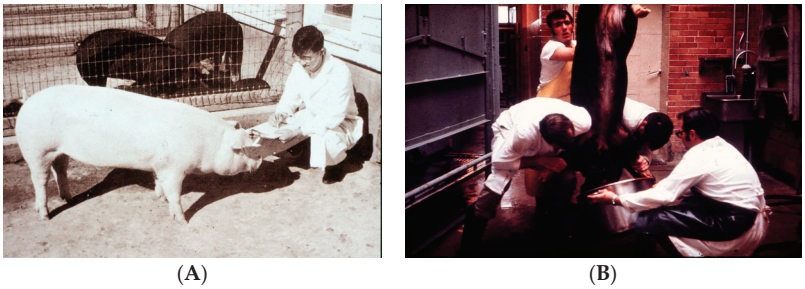
4. Understanding How Vitamin D Works to Promote Bone Health

By 1940, it was acknowledged that rickets could be prevented and cured in two ways: by irradiation of the skin to solar or artificial UV radiation or by the ingestion of vitamin D (D represents D_2 or D_3). It was recognized that rachitic children had low blood concentrations of phosphate and calcium and that treatment with vitamin D corrected them to normal. It was unclear however how vitamin D affected this. In 1923, Orr et al. observed decreased fecal excretion of calcium in rats exposed to UV radiation. They concluded that this was likely due to vitamin D increasing intestinal calcium absorption [27]. This was confirmed in 1953 by Nicolaysen et al. who established that the important function of vitamin D was for stimulating intestinal calcium absorption [28]. It was also generally believed that vitamin D was playing a fundamental role in bone mineralization. However, studies by Carlsson demonstrated that vitamin D, rather than stimulating the direct deposition of calcium into bone, stimulated the mobilization of calcium from the

skeleton [29]. We now recognized that the fundamental function of vitamin D is to maintain the serum calcium and phosphate in the normal range for maintaining most metabolic functions, including neuromuscular and cardiac activity [7,30]. Vitamin D also maintains an adequate circulating and extracellular calcium–phosphate product, resulting in the passive deposition of calcium hydroxyapatite, i.e., mineral into the skeleton [7,30]. There is also evidence that vitamin D enhances intestinal phosphate absorption, which is also critically important for energy and muscle function through the action of ATP and is a major component of calcium hydroxyapatite in the skeleton (Figure 7) [30].

5. The Saga for the Quest to Identify the Active Form of Vitamin D_3

In the early 1950s, using radiolabeled calcium, it was determined that when a vitamin-D-deficient rat or chicken received vitamin D, intestinal calcium absorption began to increase, with maximum absorption occurring at around 24 h. Kodicek and coworkers reasoned that to understand the mode and sites of action of vitamin D, investigations were needed to understand its storage, distribution and metabolism. They produced ^{14}C-beled vitamin D_2 with a low specific activity of 0.46 mCi/mmol and administered pharmacologic doses to rats. They concluded, based on determination of radioactivity in the tissues and excreta by reverse chromatography, that vitamin D_2 was likely the biologically active form of the vitamin [31]. In the early 1960s, Norman and DeLuca prepared randomly labeled ^{3}H-vitamin D_3 and ^{3}H-vitamin D_2 with much higher specific activities of 7.3 and 3.25 mCi/mmol, respectively, and gave them to vitamin-D-deficient rats. The chromatography results from their study revealed that there was accumulation of radioactivity mainly in the liver and to a lesser degree in the ileum and kidneys and much less in the adrenal glands. This resulted in the investigation of the metabolism of tritiated vitamin D_3 in rat kidney and intestinal mucosa. At least three lipid-soluble radioactive compounds other than ^{3}H-vitamin D_3 were detected, and all showed partial vitamin D activity [32]. The DeLuca group then made [1, 2-^{3}H] vitamin D_3 with very high specific activity and found by silicic acid chromatography a polar metabolite labeled as metabolite IV circulating in rats. They found that this metabolite had at least the same antirachitic potency as vitamin D_3. Furthermore, they discovered that the amount of this metabolite continued to increase with increasing doses of vitamin D_3. Utilizing this fact, Blunt et al. gave four hogs 6.25 mg of vitamin D_3 daily for 26 days and then collected their blood. They separately gave a hog [1, 2-^{3}H] vitamin D_3 and collected its blood. Organic extractions were made of both blood pools and were then combined. The lipid extract underwent a series of straight-phase and reverse-phase chromatographies, resulting in the recovery of 1.2 mg of metabolite labeled as peak IV. The vitamin D_3 metabolite was identified as 25-hydroxyvitamin D_3 [33]. Suda et al. performed a similar procedure, only this time using pharmacologic amounts of vitamin D_2 and tritiated vitamin D_2 and identified the polar metabolite as 25-hydroxyvitamin D_2 (Figure 8) [34]. These observations in pigs were interesting, but it was unknown whether this metabolism also occurred in humans.



(**A**) (**B**)

Figure 8. (**A**). This hog was treated with 6.25 mg vitamin D_2 by Dr. Suda; (**B**) the blood was then collected. After purification of the blood lipid extract, the vitamin D_2 metabolite was identified as 25-hydroxyvitamin D_2. Holick copyright 2023. Reproduced with permission.

6. A Personal Experience and Perspective for the Identification of the Major Circulating Form of Vitamin D_3 in Human Blood

Clearly, a different strategy would be needed to determine if 25-hydroxylated vitamin D was present in human circulation. I arrived at the University of Wisconsin Madison in 1968 in the Department of Medical Microbiology. This was a time when there was great excitement about the discovery of DNA and RNA. Molecular biology was in its infancy, and my goal was to achieve a Ph.D. degree by investigating the molecular biology of microorganisms. However, no one in the department had any interest in this area, and I, after completing the year, went to the Biochemistry Department and explained to them that I had been accepted into the University's graduate program and that they should accept me into the graduate program in the Biochemistry Department. Much to my amazement, I was accepted. I quickly realized, however, that I had a big problem to overcome. I was considered to have completed my first year of graduate work, and to proceed into the second year, I needed to take the biochemistry preliminary exam. Although I had a chemistry degree with a special interest in organic chemistry and had biochemistry laboratory experience, I had never taken a biochemistry course. As a result, I barely passed the exam. I wanted to find a research mentor who had a special interest in molecular biology. However, the professors, after seeing my poor performance on the preliminary exam, informed me that there were no openings but that I should see Professor DeLuca, who was making important observations about vitamin D. I explained to various professors that I had no interest in vitamin D since I considered it a boring subject. I was informed that this was my only option. When I met Dr. DeLuca in June 1969, he was pleased that I had significant biochemistry research experience and a publication while I was in the chemistry program at Seton Hall University. However, after seeing my preliminary exam results, he informed me that I needed to go into a softer science program such as physiology or pharmacology and that biochemistry would be too difficult for me. I reassured him that it was my goal to receive a Ph.D. degree in biochemistry and that I would do whatever was necessary to accomplish this. He said that he did not want to discourage me but to be realistic, I needed to first receive a master's degree that would likely take 2 years followed by being quizzed by the biochemistry faculty to determine if I could proceed into the Ph.D. program. He said that the Ph.D. program would take at least 4–5 years. Thus, he said it could take up to 7 years before I would receive a Ph.D. degree. I accepted the challenge, and he accepted me into his research program in July 1969. As the newest graduate student, I was given a desk against the wall where the entrance was located with no laboratory space. In July of 1969, Dr. DeLuca received a phone call from Dr. Avioli, at Washington University in St. Louis, informing him that he has been treating hypoparathyroid patients with pharmacologic doses of vitamin D_3 (1.5 mg daily) and had been collecting their heparinized blood plasma. He asked Hector if he would like the blood plasma to see if his laboratory could identify 25-hydroxyvitamin D_3 in the blood plasma. Since all his graduate students and postdoctoral fellows had ongoing research projects and I had none, I was informed by Dr. DeLuca that this would be my project. He instructed me that all I needed to do was to follow the exact chromatography procedures that were successfully used to identify 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in hog blood. With great enthusiasm, I began by obtaining ^{3}H-tracer from the blood of vitamin-D-deficient rats that were injected with [1, 2-^{3}H] vitamin D_3. I performed a lipid extraction on 600 mL of human blood plasma and rat serum and combined the lipid extracts. I proceeded to follow the exact chromatography methods used to successfully obtain purified 25-hydroxyvitamin D from hog blood. I conducted all the chromatographies that were previously reported. After exhausting all chromatographic procedures known at the time, I realized at the end of November 1969 that there was a lipid contaminant in human blood that was not present in hog blood and that the contaminant could not be separated from the vitamin D metabolite for its identification. This was Thanksgiving weekend. Since I could not afford to travel home to New Jersey to be with my family, I went to the laboratory. I unlocked the door early in the morning and walked in distressed because of my predicament of failing to purify the

vitamin D metabolite. As I was walking to my desk, I noticed on one of the shelves on the research bench that was next to my desk a bottle containing Sephadex LH-20. I remembered from my college days that Sephadex, which is a glucose polymer, can separate proteins based on size. I reasoned that maybe I could use Sephadex LH-20 to separate the vitamin D metabolite from other contaminants based on size. I made a slurry of Sephadex LH-20 with methanol and poured the slurry into a column. After the Sephadex LH-20 settled, I placed my human plasma blood lipid extract, which had undergone multiple chromatographies, on the column and collected fractions. The vitamin D metabolite peak was identified by determining the location of the tritiated peak IV. The peak was recovered and the metabolite was now pure. I recovered 25 mcg that was identified as 25-hydroxyvitamin D_3 (25(OH)D_3). This metabolite is the major circulating form of vitamin D_3 that is clinically measured to determine a person's vitamin D status. What I did not realize at the time was that I had developed a new chromatography method known as liquid gel chromatography that became the standard chromatography technique in most vitamin D laboratories and was instrumental for purifying the active form of vitamin D_3 [35,36]. I had completed my master's research in 3 months, and for these discoveries, I was awarded a master's degree in January 1970.

7. The Hunt to Identity the Active Form of Vitamin D_3

It was found that 25(OH)D_3 was biologically more potent than vitamin D_3, and it was concluded that it was the active form [33]. However, it soon became evident that ^{3}H-25(OH)D_3 was rapidly metabolized to a more polar metabolite that quickly appeared in the intestine. Haussler et al. [37] reported a more polar metabolite of 25(OH)D_3 in nuclear fractions of the intestine in chickens that received ^{3}H-vitamin D_3. At the same time, Lawson et.al. [38] reported that during the formation of this more polar metabolite, the 1^{-3}H on their [4-^{14}C,1α-^{3}H] vitamin D_3 was lost, suggesting that this polar metabolite had some alteration on carbon 1. The realization that this more polar metabolite not only had all the biologic actions that vitamin D_3 and 25(OH)D_3 possessed but that it acted more quickly in stimulating intestinal calcium absorption. It became clear that 25(OH)D_3 was not the biologically active form, but rather, it was a more polar metabolite that needed to be identified.

This realization initiated a frenzy of activity in the Kodicek, Norman and DeLuca laboratories. Various strategies were undertaken, the first being in the DeLuca lab where they gave hogs pharmacologic doses of vitamin D_3 and then collected their intestines. Dr. Robert Cousins, who was a postdoctoral fellow in DeLuca's laboratory, was in charge. It was quickly realized that it made little sense to be giving pharmacologic doses of vitamin D_3 and expecting marked increases in the concentrations of the active form in the intestine. As a result, in the summer of 1970, it was decided that what was needed was many intestines from chickens that were receiving presumably physiologic amounts of vitamin D_3 in their diet. Dr. Cousins, Dr. Suda and I traveled an hour north in the department of biochemistry's pickup truck to the BrakeBush facility where they processed 20,000 chickens per day (Figure 9A). We collected approximately 400 pounds of chicken intestines and returned to the University of Wisconsin with the intestines. We quickly realized that the intestines had begun to putrefy since we had not brought ice with us, and it was a hot steamy day. It was decided that the entire laboratory would go back to the BrakeBush facility to collect, clean, and process approximately 400 pounds of chicken intestines and freeze them immediately on dry ice (Figure 9B,C). The realization quickly set in upon arriving back at the University that we had no ability to process such a large amount of intestinal material. The biochemistry department had large fermentation stainless steel containers in the basement. Dr. Cousins, Dr. Suda and I began to process the intestines in a sausage meat grinder. The mashed intestines were then added to a large fermentation tank (Figure 9D). Several hundred gallons of chloroform, methanol and water were added and stirred. An additional amount of chloroform was then added, which caused a separation of the organic phase and which contained the lipids that were present in the intestines,

from the water phase, which contained the water-soluble compounds. The several hundred gallons of the chloroform phase was taken to dryness and then chromatographed on a huge Shepadex LH-20 column that I designed. It became obvious to me that it was going to be futile to chromatograph and purify the minute quantity of the active form of vitamin D_3 that was present in several hundred grams of intestinal lipid extract.

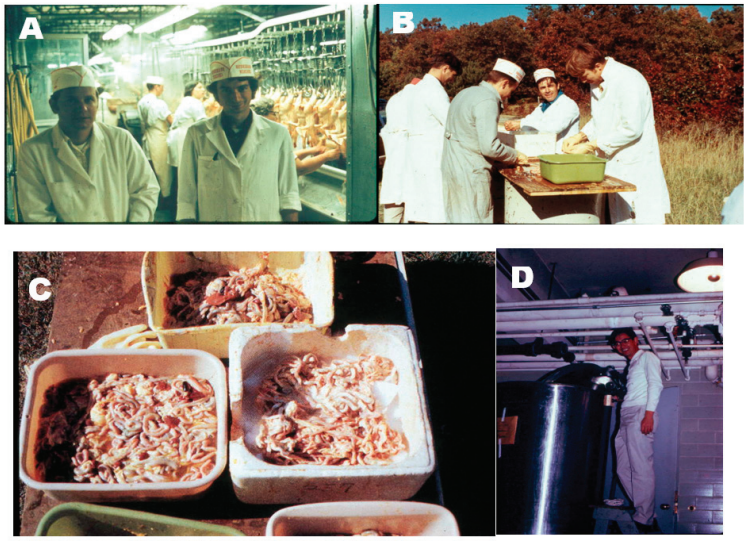


Figure 9. (**A**) The first attempt. Dr. Robert Cousins on the left and me collecting intestines from the trough behind us at the BrakeBush facility. (**B**) The second collection of intestines with several members of the DeLuca laboratory helping out. From left to right is Dr. Ian Boyle, Dr. Chuck Frolich, Dr. Jack Omdahl and Dr. Hector DeLuca. (**C**) Cleaned and processed intestines placed on dry ice. (**D**) Dr. Tatsuo Suda inspecting the huge stainless steel fermentation tank that contained approximately 400 pounds of mashed intestines and several hundred gallons of chloroform and methanol for the lipid extraction. Holick copyright 2023. Reproduced with permission.

Thus, while this process was underway, I went to Dr. DeLuca and suggested to him that we know based on multiple studies that there was approximately 1 ng of the active metabolite in 1 g of intestine. I suggested to him that a better way of isolating this intestinal metabolite was to give vitamin-D-deficient chickens intravenously 100 IUs of vitamin D_3 that contained [1, 2-^{3}H] vitamin D_3. Based on my calculation, I explained to him that I would need 1500 chicken intestines to obtain approximately 10 mcg of the active metabolite that could then be extracted, chromatographed, and ultimately purified for identification. He gave me the green light. I asked Dr. Jack Omdahl, who was a postdoctoral fellow in Dr. Deluca's laboratory, with extensive experience in working with vitamin-D-deficient chickens, for his assistance. Due to the capacity of the department of agriculture to house chickens, I was able to only order 500 chicks. I placed them on a vitamin-D-deficient diet that we produced in the biochemistry department. After 6 weeks on the vitamin-D-deficient diet, Jack gave an oral dose of 100 IUs (2.5 mcg) of vitamin D_3 containing trace amounts of [1, 2-^{3}H] vitamin D_3 in vegetable oil to all 500 chickens. The chickens were sacrificed 24 h later, and Jack and I removed and cleaned the small intestines and placed them in a freezer. At the same time, another 500 chickens were ordered and placed on the vitamin-D-deficient diet and underwent the same procedures. By November 1970, I had 1000 intestines stored in the freezer and was waiting for my last 450 chickens that were scheduled to be sacrificed in early December. However, at the end of November, late at night, we heard a news flash on BBC radio stating that the Kodicek laboratory

had made a major discovery related to vitamin D. We did not know what that discovery was. Dr. Deluca became extremely concerned that this meant that the Kodicek group had successfully identified the active form of vitamin D. I was asked to proceed immediately to process the 1000 chicken intestines I had in the freezer, with the intent of identifying the active form of vitamin D_3 as quickly as possible. My response was that I needed 1500 chicken intestines to be successful and that I did not want to proceed. Challenging my mentor was not easy but I initially stood my ground. I then relented, reiterating my concern that I would not be successful but that I would do as directed and begin the processing of the thousand chicken intestines the next morning. As I was beginning to collect the frozen intestines, early the next morning, Dr. DeLuca appeared and informed me that I had been right in the past with my research activities and that he would permit me to wait until I collected the additional 500 intestines. By the middle of December 1970, I had completed the lipid extraction of all 1450 chicken intestines. Based on the known specific activity of the [1, 2-^{3}H] vitamin D_3 that was given to the chickens, I estimated that I had approximately 11 mcg of the active metabolite in 22 g of oily lipid. I immediately began multiple chromatographies on my Sephadex columns (Figure 10A,B). It was now coming close to Christmas, and I realized that we were in stiff competition with the Kodicek and Norman laboratories. I flew home to New Jersey on Christmas Eve and wished my parents a Merry Christmas and flew back to Madison Wisconsin on Christmas day to continue to purify the active vitamin D_3 metabolite that was called peak V. When I returned, I learned that the Kodicek group published its newsworthy finding in the journal Nature. At this time, it was assumed that because this active metabolite appeared so quickly in the intestine that it was the intestine that was producing it. Therefore, it was obvious that to generate the active form of vitamin D_3 in sufficient quantities for its identification, all you would need to do is incubate intestinal homogenates with 25(OH)D_3. After numerous attempts by several laboratories including the DeLuca laboratory, incubation of intestinal homogenates with [^{3}H]-25(OH)D_3 did not yield any of the desired polar metabolite. The Kodicek group not only made homogenates of chicken intestines, they made homogenates of many of the other organs. They then incubated each of the homogenates with [4-^{14}C,1-^{3}H] 25(OH)D_3. What they observed was truly remarkable. They found that only kidney homogenates could produce the active metabolite [39]. For me, this revelation was very disconcerting, since it meant that an unlimited amount of active vitamin D_3 could be easily produced by incubating kidney homogenates with 25(OH)D_3. It was becoming increasingly more likely that the Kodicek group was close to purifying the active metabolite for its identification. By early January 1971, I realized after 17 chromatographies, I had approximately 8 mcg of active metabolite that was associated with a large amount, that I estimated to be about 2000 mcg, of lipid contamination (Figure 10C). There were no more chromatographies that I could do that would make any difference, and I concluded that I had failed. However, within a few hours, I reasoned that we knew that the active form of vitamin D had a secondary hydroxyl group on carbon 3 and a tertiary hydroxyl group on carbon 25. It was presumed that there was an additional secondary hydroxyl group on carbon 1 based on the observation by the Kodicek group and that this metabolite had lost its 1α−^{3}H on carbon 1. I considered that it was unlikely that the lipid contaminants in my preparation had the same number and type of hydroxyl groups. I reasoned that if I trimethylsilylated the active metabolite, and then selectively, by acid hydrolysis, removed the trimetylsilyl ether groups from the secondary hydroxyl groups, this would result in the 25-hydroxyl being protected with a trimethylsilyl ether. This modification would decrease the polarity of the vitamin D metabolite, thereby altering its chromatographic properties. I proceeded with this strategy and chromatographed the dihydroxyated active form on a Sephadex LH-20 column and recovered it in pure form. The purified monosilyl ether derivative was then hydrolyzed, and after purification, 2 micrograms of pure metabolite was recovered. The ultraviolet absorption spectrum showed a lambda max 265 nm and a lambda min 228 nm that was identical to the absorption spectrum for the 5,6-triene system for vitamin D_3. A small amount was immediately placed in the mass spectrometer, and it revealed a

mass spectrum with a molecular ion of 416 *m*/*e*. This was consistent with an additional hydroxyl group being present in the metabolite. The fragmentation pattern was consistent with the metabolite having the additional hydroxyl group in the A ring (Figure 11). Further chemistry on nanogram quantities of the purified active form of vitamin D_3, followed by the skillful mass spectroscopy by Dr. Heinrich Schnoes (Figure 10D), finally revealed its structure as 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) (Figures 7 and 11) [40].

Figure 10. (**A**) Lipid extraction of 1450 chicken intestines. The yellow chloroform organic phase that contained the active metabolite was being dried down under vacuum in preparation for its first chromatography on a Shephadex LH-20 column (**B**) The dried down lipid extract from the intestines was first chromatographed on a Shephadex LH-20 column and eluted with a mixture of 65% chloroform in hexane. (**C**) In one of the final chromatographies, the semi-purified active vitamin D_3 metabolite was dissolved in methanol and chromatographed on a Shephadex LH-20 column prepared in methanol. (**D**) Dr. Heinrich Schnoes in his office. Holick copyright 2023. Reproduced with permission.

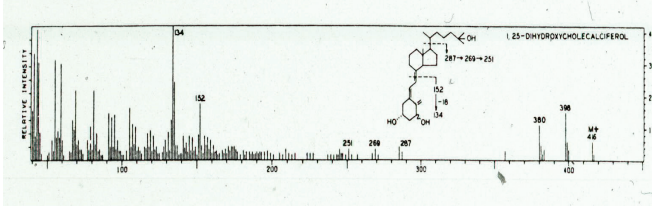


Figure 11. The mass spectrum of the purified metabolite demonstrated a molecular ion of 416 *m*/*e* consistent with an additional hydroxyl group on the metabolite. The fragments of 152 and 134 revealed that the hydroxyl group was somewhere in the A ring, most likely on carbon 1. Several chemical reactions were performed on nanogram quantities of the pure metabolite that provided evidence that the extra hydroxyl group was on carbon 1. Holick copyright 2023. Reproduced with permission.

During that same time, the Kodicek group isolated 60 mcg of the metabolite with approximately 30% purity, using Sephadex LH-20 chromatography, from chicken kidney

homogenates. They reported that the isolated metabolite had a UV absorption lambda maximum of 269 nm and a lambda minimum at 232 nm. The mass spectrum showed a molecular weight of 416, identical to what we had observed. Because they only had 30% purity, the only way they were able to conclude that the additional hydroxyl group was on carbon 1 was based on their observation that the 1-^{3}H on their [4-^{14}C,1α-^{3}H] vitamin D_3 was lost while it was being metabolized. They concluded that their metabolite was also 1,25-dihydroxyvitamin D_3 [41]. The Norman group also used the Kodicek kidney homogenate strategy and confirmed several months later that the structure of the active form of vitamin D was 1,25-dihydroxyvitamin D_3. [42] These three observations were submitted for publication on 12 February to PNAS, 19 February to Nature and 10 May 1971 to Science, respectively [40–42]. We had won the race in just one week. For my research accomplishments, I received my Ph.D. Degree in May 1971, a little less than 2 years after I joined the DeLuca laboratory.

The final evidence that the one hydroxyl group was on C-1 was confirmed by the 21- step synthesis of 1,25-dihydroxyvitamin D_3 by my roommate Eric Semmler and me [43]. We demonstrated that the synthetic metabolite migrated identically with the biologically produced active metabolite and had the same biologic activity on intestinal calcium absorption and bone calcium mobilization [43]. This was further confirmed several years later when my group made 1β, 25-dihydroxyvitamin D_3 and demonstrated that it was biologically inactive [44]. Soon after these discoveries, Dr. Glennville Jones, while a postdoctoral fellow in Dr. DeLuca's laboratory, began using the newly introduced high-performance liquid chromatography and identified 1,25-dihydroxyvitamin D_2 as the biologically active form of vitamin D_2. He and other investigators went on to identify numerous other vitamin D metabolites [45]. However, to date, it remains that 1α,25-dihydroxyvitamin D_3 is the biologically active form of vitamin D_3.

8. Clinical Uses for 1α,25-Dihydroxyvitamin D_3 and 1α-Hydroxyvitamin D_3

Once 1,25-dihydroxyvitamin D_3 was identified, there was a great interest in producing it chemically. It was decided that it would be worthwhile to see if the 1α-hydroxyl group could be introduced into the less expensive starting material, cholesterol. The synthesis of 1α-hydroxyvitamin D_3 was accomplished. The same methodology was then used to successfully produce 1,25-dihydroxyvitamin D_3 [46]. 1α-hydroxyvitamin D_3 was shown to be biologically active after it was metabolized in the liver on carbon 25 to form 1,25-dihydroxyvitamin D_3 [47].

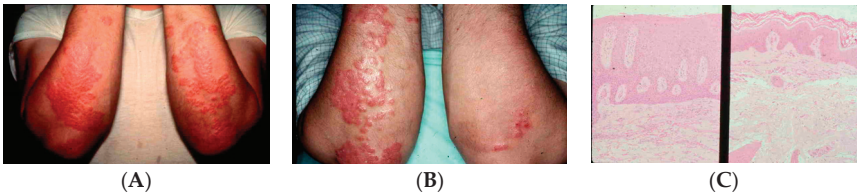
Several milligrams of both compounds were produced. They were immediately sent out to clinicians around the world who found that these active vitamin D compounds were extremely effective in treating metabolic calcium and bone diseases in children and adults with kidney failure [48,49]. It was known that kidney-failure patients had a resistance to the biologic actions of vitamin D that was difficult to understand. The revelation that the kidneys were responsible for metabolizing 25(OH)D_3 to 1,25-dihydroxyvitamin D_3 revealed the reason why. It was also observed that a rare form of hereditary rickets, known as vitamin-D-dependent rickets type I or pseudo-vitamin D deficiency rickets, was effectively treated with physiologic doses of 1,25-dihydroxyvitamin D_3 [50,51]. Both active vitamin D compounds were also effectively used to treat hypocalcemia in patients with hypoparathyroidism and pseudohypoparathyroidism [52,53]. These clinical successes prompted Dr. Milan Uskokovic at Hoffmann LaRoche to develop a streamlined method for producing the active metabolite, and it became commercially available as an FDA-approved pharmaceutical in the early 1970s.

9. Enter the Era of Noncalcemic Genomic Health Benefits of Vitamin D

In 1979, Stumpf et al. reported that ^{3}H-1,25$(OH)_2D_3$ was concentrated in the nuclei of most tissues in the body of a vitamin-D-deficient rat [54]. This provided evidence that the vitamin D receptor (VDR) not only existed in calcium-regulating tissues, but also was present in tissues that were not related to calcium and bone metabolism. The

physiologic significance of this revelation was not appreciated until it was observed that $1,25(OH)_2D_3$ inhibited the proliferation and induced terminal differentiation of HL-60 human myeloid leukemia cells [55]. Several laboratories in the early 1980s began to report that some cultured cancer cells possessed a VDR and that incubation of these cells with $1,25(OH)_2D_3$ resulted in decreased proliferative activity [56]. The observation that mice with M1 leukemia had prolonged survival when they were treated with $1\alpha(OH)D_3$ prompted great interest in seeing whether the same could be true for patients with preleukemia [57]. Eighteen patients with preleukemia were treated with $1,25(OH)_2D_3$. Although the patients performed quite well early in the treatment, they ultimately developed hypercalcemia, and all died in blastic phase [58]. As a result of these initial studies, a huge effort was made to develop $1,25(OH)_2D_3$ and its active analogs as a potential therapy for treating a variety of cancers, including prostate cancer. Unfortunately, these therapies were not found to be successful [59]. It appeared that the cancer cells cleverly designed mechanisms to bypass the antiproliferative and pro-differentiating properties of 1,25-dihydroxyvitamin D_3 and its active analogs. As a result, 40+ years later, there still has never been a vitamin D analog that has proven to be successful in treating any form of cancer.

At the same time, Bikle et al. [60] and our group [61] discovered that keratinocytes had a VDR, and when these cells were incubated with $1,25(OH)_2D_3$, they demonstrated a dramatic response. The keratinocytes decreased their proliferative activity and became fully differentiated. It had been reported that one osteoporotic patient being treated with 1α-hydroxyvitamin D_3 had improvement in their psoriasis. We had at the same-time initiated psoriasis clinical trials with both topical and oral $1,25(OH)_2D_3$, which demonstrated remarkable improvement in disease activity (Figure 12). Pharmaceutical companies began making analogs to see if they could reduce the calcemic action of $1,25(OH)_2D_3$ while maintaining the same or more potent anti-proliferative and pro-differentiating activity. Several analogs were developed, and they and $1,25(OH)_2D_3$ remain a first-line treatment for patients with minimal psoriasis [62–65].



(**A**) (**B**) (**C**)

Figure 12. (**A**) A young man with chronic psoriasis with no effective treatment. He was a participant in our clinical research trial evaluating the effectiveness of topical-applied 1,25-dihydroxyvitamin D3 that I formulated in Vaseline with a concentration of 50 mcg/gram. This was a double-blind placebo-controlled trial with one side receiving topical Vaseline and the other side receiving topical Vaseline containing 1,25-dihydroxyvitamin D3. (**B**) After 2 months, there was dramatic improvement on the right forearm that had received the active metabolite. (**C**) A skin biopsy was taken from each forearm and the histology of the skin biopsy from the right forearm shown on the right side of figure (**C**) revealed a dramatic improvement by marked decreases in the keratinocyte proliferation with induction of terminal differentiation to normalize the skin. Holick copyright 2023. Reproduced with permission.

Although it was dogma that only the kidneys could produce $1,25(OH)_2D_3$, many laboratories began reporting that a wide variety of tissues and cells not only had a vitamin D receptor but also expressed the 25-hydroxyvitamin D-1α-hydroxylase. Of particular interest was the observation that when macrophages were activated, toll-like receptors were turned on to induce the macrophage to express the 25-hydroxyvitamin D-1α -hydroxylase. This resulted in the ability of the macrophage to metabolize $25(OH)D_3$ to $1,25(OH)_2D_3$ (Figure 7). The reason for this activation was that once produced, $1,25(OH)_2D_3$ interacted with the macrophage VDR, resulting in expression and production of cathelicidin, a de-

fensen protein responsible for killing and lysing infectious agents, including bacteria and viruses [66]. It is also now recognized that $1,25(OH)_2D_3$ produced by immune cells is a major regulator of both innate and acquired immunity, as illustrated in Figure 13 [67]. When healthy vitamin-D-deficient/insufficient adults were given 600 IUs or 10,000 IUs daily for 6 months, an evaluation of their circulating immune cells before and after revealed that even on 600 IUs daily, 128 genes were being influenced, whereas 1289 genes were being influenced in those taking 10,000 IUs daily [68]. What was also observed was although all the healthy vitamin-D-deficient/insufficient participants substantially raised their circulating concentrations of 25(OH)D in the range of 60–90 ng/mL, only 50% had a robust response. This observation demonstrates that there were other factors such as VDR polymorphisms that could influence the responsiveness of vitamin D, promoting genomic effects on the immune system (Figure 14)

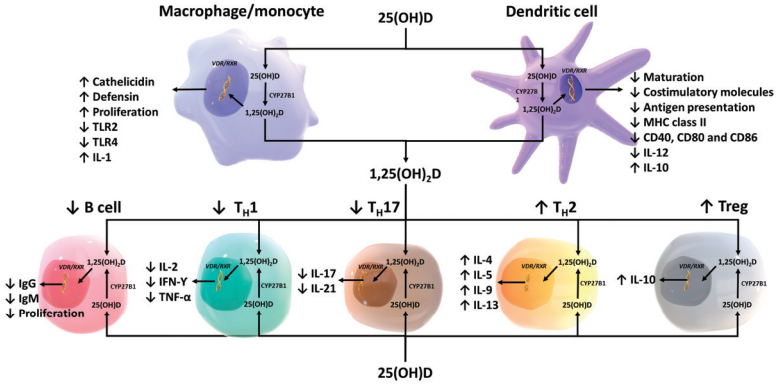


Figure 13. Schematic representation of paracrine and intracrine function of vitamin D and its metabolites and actions of 1,25-dihydroxyvitamin D on the innate and adaptive immune systems. Abbreviations: 1,25(OH)2D: 1,25-dihydroxyvitamin D; 25(OH)D: 25-hydroxyvitamin D, IFN-Y: interferon-Y; IL: interleukin; MHC: membrane histocompatibility complex, TH1: T helper 1; TH2: T helper 2; TH17: T helper 17; Treg: regulatory T cell, TNF-α: tumor necrosis factor-α; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4. Ref. [67] Holick MF, copyright 2020. Reproduced with permission.

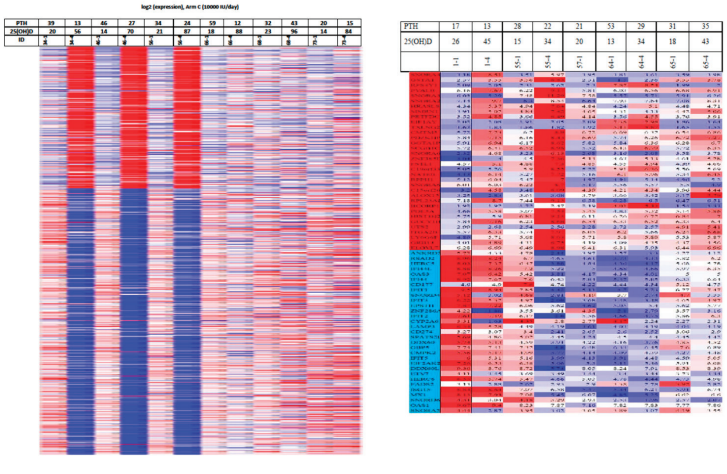


Figure 14. (**Left**) Heatmap of 1289 vitamin D-responsive genes whose expression response variation in 6 vitamin-D-deficient subjects taking 10,000 IUs per day of vitamin D_3 for 6 months, showing that

3 subjects had a robust response in gene expression compared to the other 3 subjects who had minimum to modest responses even though these subjects raised their blood levels of 25(OH)D in the same range of ~60–90 ng/mL. (**Rgiht**) Heatmap of only 128 vitamin D-responsive genes in 5 vitamin D deficient subjects taking 600 IUs per day for 6 months. Abbreviation: 0 m: 0 month; 6 m: 6 months; 25(OH)D: 25-hydroxyvitamin D; PTH: parathyroid hormone. Ref. [68] Holick MF, copyright 2019. Reproduced with permission.

10. Associating Vitamin D Deficiency with Acute and Chronic Illnesses

Most tissues and cells in the body have a VDR and can produce $1,25(OH)_2D$ in a wide variety of cells, including prostate, breast, colon, skin and the brain. The reason that the local production of $1,25(OH)_2D_3$ does not potentially cause hypercalcemia is that once it is produced and enters the nucleus, $1,25(OH)_2D$ immediately induces the 25-hydroxyvitamin D-24-hydroxylase. This enzyme begins to rapidly metabolize $1,25(OH)_2D_3$ to an inactive water-soluble carboxylic acid derivative (calcitroic acid). Simultaneously, $1,25(OH)_2D_3$ up- and downregulates more than 1000 genes responsible for cellular proliferation, differentiation, a variety of cellular metabolic activities, antiangiogenesis and apoptosis (Figure 7) [30,68].

Epidemiology studies have related vitamin D deficiency with a multitude of chronic illnesses, including the autoimmune disorders multiple sclerosis, type 1 diabetes and rheumatoid arthritis, cardiovascular disease, type 2 diabetes, neurocognitive dysfunction and infectious diseases, including COVID-19 (Figure 15) [69–71]. These observations will be discussed in more detail by other authors participating in this special edition.

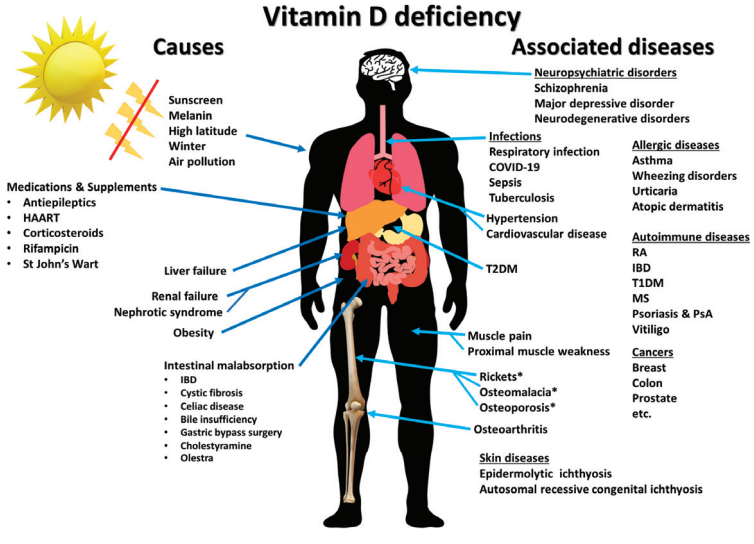


Figure 15. Summary of causes of vitamin D deficiency and diseases and disorders associated with vitamin D deficiency. Abbreviations: HARRT: highly active antiretroviral therapy; IBD: inflammatory bowel diseases; MS: multiple sclerosis; PsA: psoriatic arthritis; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; RA: rheumatoid arthritis. * denotes diseases that are direct consequences of vitamin D deficiency. Ref. [67] Holick MF, copyright 2020. Reproduced with permission.

11. The Future of Vitamin D for the Next 100 Years

The discovery of the antirachitic nutrient/hormone, vitamin D, by McCollum 100 years ago has had and continues to have far-ranging health benefits. This is due to the discovery that vitamin D must undergo sequential metabolism in the liver to 25(OH)D, which is then converted to its active form, $1,25(OH)_2D$, in the kidneys for regulating calcium and

phosphate metabolism. The new appreciation that essentially all organs and cells have a VDR, and that many cells can produce $1,25(OH)_2D$, opens a new chapter for vitamin D playing an important role in promoting good health and well-being by reducing the risk for chronic illnesses (Figures 7 and 15).

Vitamin D has by no means revealed all its biologic functions and clinical potential. It is hoped that there will be a resolution for recommendations for how much vitamin D and sensible sun exposure is necessary for maximum health. There continues to be debate as to what the definition of vitamin D deficiency is based on a circulating blood concentration of 25(OH)D. There is consensus that to achieve and maintain a healthy skeleton, the circulating concentration of 25(OH)D should be at least 20 ng/mL. To maintain maximum bone health and to prevent any evidence of vitamin D deficiency osteomalacia, the circulating concentration should be at least 30 ng/mL [72]. There is mounting evidence that when a blood concentration of 25(OH)D is at least 30 ng/mL, the noncalcemic health benefits of vitamin D become more apparent, based on association studies and randomized controlled trials. It is documented that Maasai herders and the Hazda maintain a circulating concentration of 25(OH)D in the range of 40–60 ng/mL. This concentration range has been associated with many of the noncalcemic health benefits including reduced risk for cardiovascular disease, neurocognitive dysfunction, several cancers and infectious diseases [73]. To achieve a circulating concentration of 25(OH)D in the range of 40–60 ng/mL requires a daily average intake of 2000–5000 IUs or the same amount of vitamin D_3 produced in the skin from sun exposure, as demonstrated in the Maasai and Hazda. An adult in a bathing suit exposed to enough sunlight to cause a minimal erythemal response results in the production of an amount of vitamin D equivalent to ingesting 15,000–20,000 IUs of vitamin D [72].

There is some evidence to suggest that vitamin D itself has its own health benefits. When screening for compounds that were most effective in stabilizing endothelial membranes, vitamin D_3 was found to be much more effective than its metabolites as well as a multitude of other compounds [74]. Extremely high doses, in the range of 20,000–50,000 IUs daily, have been effective in treating autoimmune disorders including psoriasis, multiple sclerosis, rheumatoid arthritis and vitiligo. The toxicity that is associated with ingesting these high doses of vitamin D is mitigated by having the patients on an extremely low calcium diet and maintaining good hydration [67,75].

It is likely in the future with the advent of the UVB-emitting LEDs that a variety of devices will become available for personalized in-home use for generating adequate amounts of vitamin D in the skin [76]. At this time, it is generally accepted that obtaining vitamin D from supplements and diet is the same as obtaining vitamin D_3 from sun exposure. There is evidence that during sun exposure, not only previtamin D_3 is produced in the skin, but there are other photoproducts of both previtamin D_3 and vitamin D_3 that have unique biologic activities not related to the classical nuclear function through the VDR [77] (Figure 7).

The reintroduction of $25(OH)D_3$ (calcifediol) for the treatment of vitamin D deficiency offers the advantage of its hydrophilic properties by being more bioavailable in patients with fat malabsorption syndromes and obesity. In the future, we may see a combination supplement containing $25(OH)D_3$ and vitamin D_3 [78].

The enthusiasm for the health benefits of vitamin D, which was initiated 100 years ago with its identification by McCollum, has not abated and continues to prosper.

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Review

Vitamin D in the Context of Evolution

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Abstract: For at least 1.2 billion years, eukaryotes have been able to synthesize sterols and, therefore, can produce vitamin D when exposed to UV-B. Vitamin D endocrinology was established some 550 million years ago in animals, when the high-affinity nuclear receptor VDR (vitamin D receptor), transport proteins and enzymes for vitamin D metabolism evolved. This enabled vitamin D to regulate, via its target genes, physiological process, the first of which were detoxification and energy metabolism. In this way, vitamin D was enabled to modulate the energy-consuming processes of the innate immune system in its fight against microbes. In the evolving adaptive immune system, vitamin D started to act as a negative regulator of growth, which prevents overboarding reactions of T cells in the context of autoimmune diseases. When, some 400 million years ago, species left the ocean and were exposed to gravitation, vitamin D endocrinology took over the additional role as a major regulator of calcium homeostasis, being important for a stable skeleton. *Homo sapiens* evolved approximately 300,000 years ago in East Africa and had adapted vitamin D endocrinology to the intensive exposure of the equatorial sun. However, when some 75,000 years ago, when anatomically modern humans started to populate all continents, they also reached regions with seasonally low or no UV-B, i.e., and under these conditions vitamin D became a vitamin.

Keywords: vitamin D; evolution; energy metabolism; immune system; calcium homeostasis; migration of *Homo sapiens*

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1. Introduction

Evolution is a dominant driver of the development of biological processes and their adaption to changes in the environment. The statement "Nothing in biology makes sense except in the light of evolution" [1] underlines that evolution is an essential component for understanding the mechanisms of these processes. Accordingly, this review will discuss vitamin D, its nuclear receptor (NR) VDR and their molecular action in the light of evolution.

It was exactly 100 years ago that vitamin D was named a vitamin, because it is able to cure experimentally induced rickets in dogs and rats [2]. Since rickets is a bone malformation disorder in children, this and many other studies linked vitamin D to calcium homeostasis and bone remodeling [3]. However, calcium homeostasis is only one of multiple biological processes being regulated by vitamin D, such as detoxification, energy metabolism as well as innate and adaptive immunity [4]. In fact, the relationship between vitamin D and bone remodeling developed as one of the most recent evolutionary functions of vitamin D.

Furthermore, the consequences of the migration of modern humans from equatorial East Africa to regions of higher latitude will be reflected in relation to vitamin D's possible role in skin lightening, particularly in European populations [5].

2. Sterols and Vitamin D Synthesis

Sterols are lipophilic molecules carrying a four-ring skeleton, a hydroxyl group at carbon (C) 3 and a flexible side chain at C17 (Figure 1). The use of sterols in cell membranes is a characteristic difference between eu- and prokaryotes [6]. Some 2.45 billion years ago,

atmospheric molecular oxygen (O_2) concentrations drastically rose in the so-called great oxidation event [7] (Figure 2). This stimulated the development of complex eukaryotes with the help of new enzymes and biochemical pathways [8]. A key example is the biosynthesis of sterols, where four different types of enzymes require O_2. Moreover, aerobic metabolism, such as oxidative phosphorylation, significantly increases the generation of energy from nutrients. In parallel, some of these new enzymes and pathways had the primary role of protecting from oxygen toxicity, which also may have been the primary role of sterols [9]. Thus, the occurrence of oxygen and sterols are tangled; without oxygen there is no sterol synthesis and many sterols protect from damage created by O_2 and reactive oxygen species. Furthermore, some sterols can be considered as oxygen sensors [10].

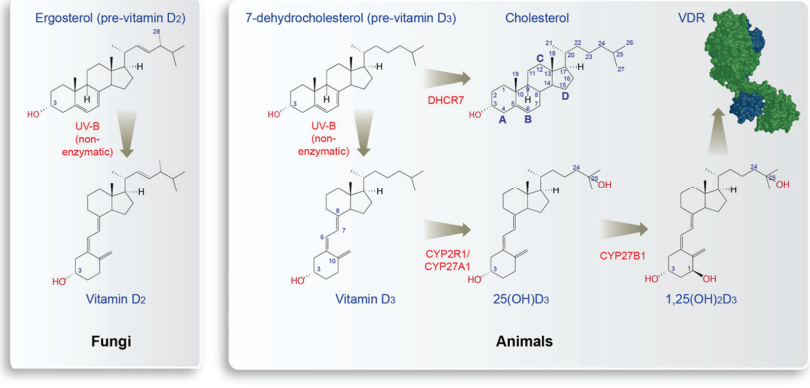

Figure 1. Principles of endogenous vitamin D_2 and vitamin D_3 production. In fungi, vitamin D_2 is produced non-enzymatically when the sterol ergosterol is exposed to UV-B radiation. In cholesterol synthesizing animals (as well as in some plants, such as phytoplankton), 7-dehydrocholesterol reacts to vitamin D_3 using the energy of UV-B. Animals express enzymes that convert vitamin D_3 first to 25(OH)D_3 and then to 1,25$(OH)_2D_3$. In animals (but not in fungi), vitamin D endocrinology developed and 1,25$(OH)_2D_3$ acts as a hormone binding with high affinity to the nuclear receptor VDR (green, the co-receptor RXR is shown in blue). In the example of cholesterol, the numbering of rings (A-D) and carbons (1-27) is indicated, while only key carbons are marked in the other molecules.

There is a large number of naturally occurring sterols and species can be phylogenetically distinguished by their sterol profile. Sterols are primarily distinguished by the modification at C24 in their side chain. In animals, 24-desmethylsterols such as cholesterol (no additional group, 27 carbon atoms in total) are typical, while fungi have 24-demethylsterols, such as ergosterol (28 carbon atoms) (Figure 1). In contrast, plants produce a wide range of more than 250 different sterols, the most common of which are sitosterol, campesterol and stigmasterol [11]. Interestingly, cholesterol represents 1–2% of the plant sterol content, i.e., cholesterol is not unique to animals but can also be produced at least by some plants, such as algae [12]. Cholesterol is not only critical for membrane fluidity but also an important precursor for bile acids and steroid hormones [13]. Most eukaryotic species, including humans, can synthesize sterols de novo, but some others, such as insects, depend on a supply of sterols via their diet [14].

When sterols like ergosterol (pre-vitamin D_2) in fungi or the direct cholesterol precursor 7-dehydrocholesterol (pre-vitamin D_3) in animals and phytoplankton are exposed to UV-B radiation of 280–315 nm, they transform non-enzymatically to vitamin D_2 and vitamin D_3, respectively (Figure 1). First, the double bond between C7 and C8 of both types of sterols absorbs the energy of the radiation, which creates thermodynamically unstable pre-vitamin D molecules, in which their B ring is opened between C9 and C10, creating secosteroids [15]. Then elevated temperature catalyzes the isomerization of pre-vitamin D

into vitamin D. Since both reactions do not require any enzyme, it is likely that vitamin D_2 and vitamin D_3 are evolutionary very old molecules that occurred as early as the ergosterol and cholesterol biosynthesis pathways evolved some 1.2 billion years ago (Figure 2). For example, for at least 750 million years, phytoplankton has produced vitamin D_3 [16]. However, in early evolution, vitamins D_2 and vitamin D_3 may have been primarily side products of sterol biosynthesis in UV-B exposure, i.e., they had no signaling function since a respective endocrine system (see Section 3) had not evolved. Interestingly, continuous UV-B exposure can convert pre-vitamin D_3 into lumisterol, tachysterol and other photoproducts [17], i.e., vitamin D_3 precursors and metabolites are able to perform UV scavenging by rearranging double bonds within the molecule [18]. Thus, the pre-endocrine function of vitamin D was and still is the protection of DNA and proteins from mutagenesis and degradation, respectively.

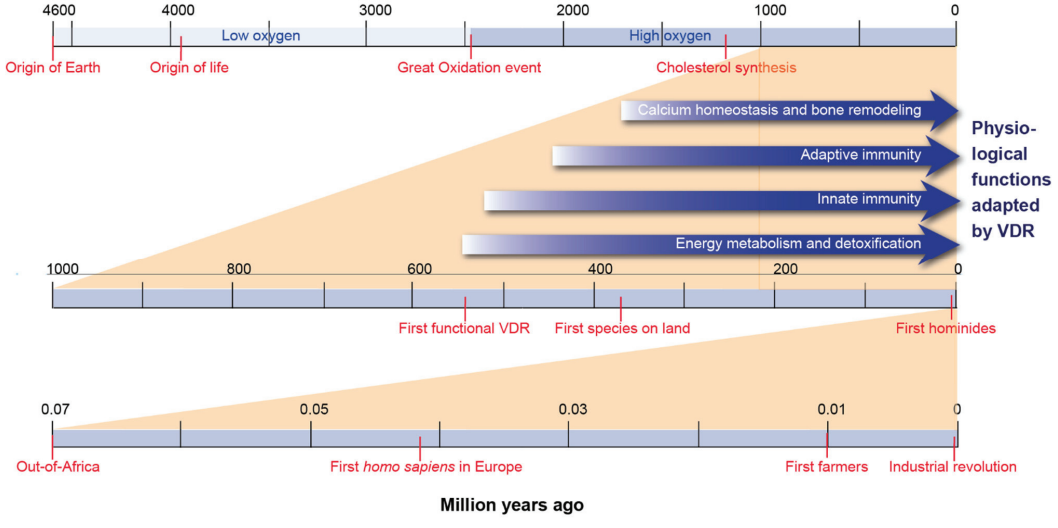

Figure 2. Timeline of evolution. The evolutionary history of the past 4.6 billion years (**top**), 1 billion years (**center**) and 70,000 years (**bottom**) are depicted. Important events discussed in this review are indicated and the time of their approximate occurrence is marked.

Interestingly, the accumulation of vitamin D_3 in the marine food chain [12,19] explains why salmon, as well as the liver of cod ("cod liver oil"), have a high content of the vitamins [20,21].

3. Evolution of Vitamin D Endocrinology

The core protein of an endocrine system is its receptor. A high-affinity receptor for vitamin D, the transcription factor VDR evolved some 550 million years ago [22]. However, contrary to its name, VDR is activated neither by vitamin D_2 nor by vitamin D_3 [23]. Carrying only one hydroxy group, both secosteroids are not polar enough to bind VDR. In fact, two hydroxylation reactions are required to form with 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) a vitamin D metabolite that offers three hydroxy groups for specific high-affinity binding to the ligand-binding domain (LBD) of VDR (Figure 1). This implies that the 25-hydroxylases cytochrome P450 (CYP) 2R1 and CYP27A1, as well as the 1α-hydroxylase CYP27B1, are key components of vitamin D endocrinology. They transform vitamin D_3 into 25-hydroxyvitamin D_3 ($25(OH)D_3$) and $25(OH)D_3$ into $1,25(OH)_2D_3$, respectively. Furthermore, as described for other hormones, the levels of $1,25(OH)_2D_3$ need to be tightly regulated. This happens via the 24-hydroxylase CYP24A1 that converts $1,25(OH)_2D_3$ to $1,24,25(OH)_3D_3$ and inactivates the VDR ligand in this way [24]. Despite their hydroxyl

groups, all vitamin D metabolites are lipophilic and need to be carried in hydrophilic serum and cellular liquids by transport proteins. Thus, for a functional endocrine system, specific receptor(s), metabolizing enzymes and transport proteins need to evolve [25].

VDR belongs to the transcription factor family of NRs, which in humans is formed by 48 genes [26]. Comparative genomics demonstrates that the closest relatives to VDR are the NR1I subfamily members pregnane X receptor (PXR) and constitutive androstane receptor (CAR), and the NR1H subfamily members liver X receptor (LXR) α and b, as well as the farnesoid X receptor (FXR) [27]. This indicates that VDR and its five relatives have a common ancestor and that the individual receptor genes developed by whole genome duplications in early vertebrate evolution [28]. Interestingly, the six NRs function as sensors for cholesterol derivatives, such as $1,25(OH)_2D_3$, oxysterols and bile acids [29]. Moreover, FXR, VDR, CAR and PXR detect toxic secondary bile acids, such as lithocholic acid, and get activated by them [30–33]. This suggests that the prime function of the common ancestor of NR1H- and NR1I-type NRs was to act as a bile acid sensor. Accordingly, one of the first functions of VDR and its relatives was the regulation of genes encoding for enzymes of marine biotoxin degradation [4,34].

Detoxification reactions represent a specialized form of metabolism that allows a response to environmental conditions, such as the rise in toxic compounds. However, the most dominant environmental challenge of species is their diet, which is primarily composed of macro- and micronutrients. This created an evolutionary pressure, with the push of which the sensing of the levels of nutritional molecules like fatty acids, cholesterol and vitamins became the main function of NRs, such as peroxisome proliferator-activated receptors (PPARs), LXRs, retinoid acid receptors (RARs) and VDR [35,36]. This function is closely linked to the control of energy metabolism, which was and still is a prime task of many NRs, including VDR [37]. Accordingly, a significant proportion of the hundreds of VDR targets are metabolic genes [38–41].

Archetypical NRs were orphan receptors, as some members of the NR superfamily still are [42]. Comparative genomics suggests that in a stepwise evolutionary adaption, orphan, NRs changed critical amino acids within their LBD, so that a ligand-binding pocket got accessible to potential small lipophilic ligands. The 40 or more amino acids forming this pocket are specifically adapted to the shape and polarity of the ligand. Some 550 million years ago, this evolutionary adaptation process resulted in the first known VDR that binds $1,25(OH)_2D_3$ at sub-nanomolar concentrations was found in the early jawless vertebrate sea lamprey (*Petromyzon marinus*) [22], meaning that VDR had evolved into a classical endocrine receptor, such as those for the steroid hormones estrogen, testosterone and progesterone. Crystal structure analysis of lamprey's VDR ligand-binding domain [43] confirmed similar binding of $1,25(OH)_2D_3$ as identified for human VDR [44]. In vertebrate evolution, amphibians, reptiles, bony fish, birds and mammals also learned to express functional VDR proteins [45]. Most species have only one *VDR* gene, but the genome of teleost fishes underwent a third whole genome duplication and contains even two *VDR* genes [46].

Since the levels of $1,25(OH)_2D_3$ in lamprey are similar to that in higher vertebrates, respective enzymes, such as CYP2R1 and CYP27B1, must have co-evolved with VDR [22]. Similar co-evolution also happened for the vitamin D transport protein vitamin D binding protein (encoded by the *GC* gene) [25]. This indicates that some 150 million years before the first species left the ocean and had the need for a stable skeleton, vitamin D endocrinology was already established. Thus, from an evolutionary perspective, the control of calcium homeostasis was rather a secondary than a primary goal for establishing the vitamin D endocrine system.

4. Evolution of the Physiological Functions of Vitamin D

Possible harming invaders created since the early times of life on Earth a strong evolutionary pressure for developing defense mechanisms, such as an immune system. The innate immune system is evolutionarily older and found already in many non-vertebrate

species, such as insects. It involves a number of barriers, such as skin and mucosa, and uses a limited set of pattern recognition receptors that detect only general features of possible pathogens. In contrast, the adaptive immune system developed some 500 million years ago in ectothermic cartilage fishes and uses antigen receptors, such as B and T cell receptors, that have a very high affinity and specificity to their antigens [47,48].

The growth of immune cells and their function in defense and tissue repair takes significant amounts of energy [49]. Key vitamin D target genes in this context are *PFKFB4* (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4) in dendritic cells [50] and *FBP1* (fructose-bisphosphatase 1) in monocytes [51]. Therefore, the regulatory function of vitamin D and its receptor on energy metabolism were essential during the development of the immune system (Figure 2). Moreover, vitamin D modulates innate immunity through further target genes, such as those encoding for the antimicrobial peptide CAMP (cathelicidin) [52] and the toll-like receptor 4 co-receptor CD14 [51] in monocytes. Furthermore, in dendritic cells, which present antigens to T cells of the adaptive immune system, many genes respond to vitamin D [53]. In this way, vitamin D was and still is involved in efficient responses to pathogens, such as the intracellular bacterium *Mycobacterium tuberculosis* [54]. Moreover, the cluster of *HLA* (human leukocyte antigen) genes on human chromosome 6, many of which are vitamin D targets [41], is a "hotspot" of vitamin D-induced chromatin accessibility [55]. Thus, most non-skeletal functions of vitamin D, like the modulation of the immune system, developed before its regulation of calcium homeostasis and bone remodeling had been established (Figure 2).

Some 385 million years ago, the next important step in vertebrate evolution happened: some species moved from the ocean onto land and had to develop a skeleton supporting locomotion under gravitational forces [25] (Figure 2). At earlier times, calcified cartilage and dermal bone had already been developed by cartilage fishes like sharks. Bone fishes even had replaced this cartilage with bone [56]. In the calcium-rich environment of water (approximately 10 mM), this transformation was not limited by calcium abundance. However, the calcium-poor conditions on land created an evolutionary pressure to tightly regulate the concentration of calcium in intra- and extracellular compartments of the body. Since the largest amounts of calcium are stored in bones, they serve as reservoirs to balance variations in the supply of the mineral by diet. In this process, vitamin D, as well as the peptide hormone PTH (parathyroid hormone), took the lead role. For example, the calcium channel TRPV6 (transient receptor potential cation channel subfamily V member 6), as well as the calcium-binding proteins CALB1 (calbindin 1) and CALB2 are encoded by vitamin D target genes [57].

Vitamin D regulates the activity of bone-resorbing osteoclasts by the cytokine RANKL, which is encoded by the vitamin D target gene *TNFSF11* (TNF superfamily member 11) [58]. Moreover, also bone mineralization is controlled by proteins encoded by vitamin D target genes, such as SPP1 (osteopontin) and BGLAP (bone gamma-carboxyglutamate protein, also called osteocalcin). Bone remodeling, i.e., the resorption of extracellular matrix by osteoclasts as well as bone formation by osteoblasts, requires, like immune functions, also a lot of energy. [59]. Thus, bone remodeling and immunity are connected via their dependency on energy metabolism [60]. Moreover, hematopoietic stem cells find in the interior of large bones, the bone marrow, a niche, i.e., a place where proliferating immune cells are effectively shielded from radiation and, in parallel, supported by calcium. Interestingly, already in bone fishes like zebrafish (*Danio rerio*) vitamin D regulates hematopoietic cell growth during embryogenesis [61]. Thus, the close connection of calcium homeostasis and bone remodeling to immunity, i.e., the co-evolution of both systems, illustrates why vitamin D shifted into this additional task.

Taken together, vitamin D evolved from one of the multiple factors controlling (energy) metabolism and immunity to a dominant regulator of calcium homeostasis and bone remodeling. This explains why bone malformations were observed as the first symptom of vitamin D deficiency.

5. How Does the Evolution of *Homo sapiens* Relate to Vitamin D?

The anatomically modern human (*Homo sapiens*) evolved just some 300,000 years ago in East Africa [62] and spread then over the whole continent. In order to protect from sunburn and skin cancer induced by the intensive equatorial sun, the skin of these humans was profoundly pigmented [63,64]. Despite dark pigmentation, there was and still is sufficient vitamin D_3 synthesis [65]. Just some 75,000 years ago, modern humans started to migrate to Asia and from there to Oceania, Europe and the Americas [66,67] (Figure 2). In Europe and in northern parts of Asia they experienced cold winter climates that let them cover their skin by clothes. In addition, the intensity of UV-B is at higher northern latitudes far lower, and in winter, for a few months, the radiation does not reach the surface [68]. Both clothing and northern latitude reduced the amount of vitamin D_3 produced in the skin and could cause vitamin D deficiency. The medical consequences of vitamin D deficiency, bone malformations and reduced potency of the immune system, may have created an evolutionary pressure that could have pushed for a reduced skin pigmentation [69], in order to explain today's North–South gradient in skin color [70].

Skin pigmentation depends on the load of keratinocytes with melanosomes [71], which are melanin-loaded organelles that origin from melanocytes [72] (Figure 3). The UV absorbing pigment melanin is produced via oxidation and polymerization of the aromatic amino acid tyrosine. The brown/black eumelanin is the most common form of melanin, while pheomelanin is yellow/red [73]. The difference in skin pigmentation of human populations and individuals (as well as that of their eyes and hair) primarily depends on SNPs (single nucleotide polymorphisms) in genes encoding for key proteins in melanogenesis [72]. The most relevant SNPs are those related to the genes *SLC24A5* (solute carrier family 24 member 5), *SLC45A2* and *OCA2* (OCA2 melanosomal transmembrane protein) [67,74] that encode for a potassium-dependent sodium/calcium exchanger, an ion transporter and a pH regulator in melanosomes, respectively [72]. Thus, the loss of function of these key proteins in melanin production leads to reduced skin pigmentation.

Modern humans arrived in Europe some 42,000 years ago [75,76] with dark skin like their African ancestors, but many of them had blue eyes due to variations of their *OCA2* gene [75,77] (Figure 3). By interbreeding, they outnumbered the ancestral Neanderthal hominins, which had lived in Europe already for some 400,000 years [77–79]. In net effect, today's Europeans have, on average, 2.3% Neanderthal DNA in their genomes [80]. These hunter–gatherers lived first in ice-free southwestern Europe [81] and started some 11–12,000 years ago to colonize also northern Europe [75]. Based on archeogenomic data the evolution and timing of trait changes within European populations had been discovered [82] (Figure 3). First, some 8400 to 6000 years ago, people from northwestern Anatolia spread over southern Europe. These Anatolian farmers started the Neolithic revolution in Europe by introducing the concept of agriculture, i.e., the domestication of animal and plant species, to the hunter-gatherers. Moreover, by interbreeding with the indigenous European population, the Anatolian farmers also brought them their *SLC24A5* gene variant for lighter skin. In a second wave, some 5000 years ago, Yamnaya pastoralists from the Eurasian steppe arrived in Europe and settled preferentially in the North (Figure 3). They introduced the horse, the wheel, their Indo-European languages as well as lighter skin due to SNPs in their *SLC45A2* and *SLC24A5* genes to the preexisting European populations [83–85]. Thus, the relative admixture of the hunter-gatherers, Anatolian farmers and Yamnaya pastoralists explains the variation in skin color (as well as many other traits) of present Europeans.

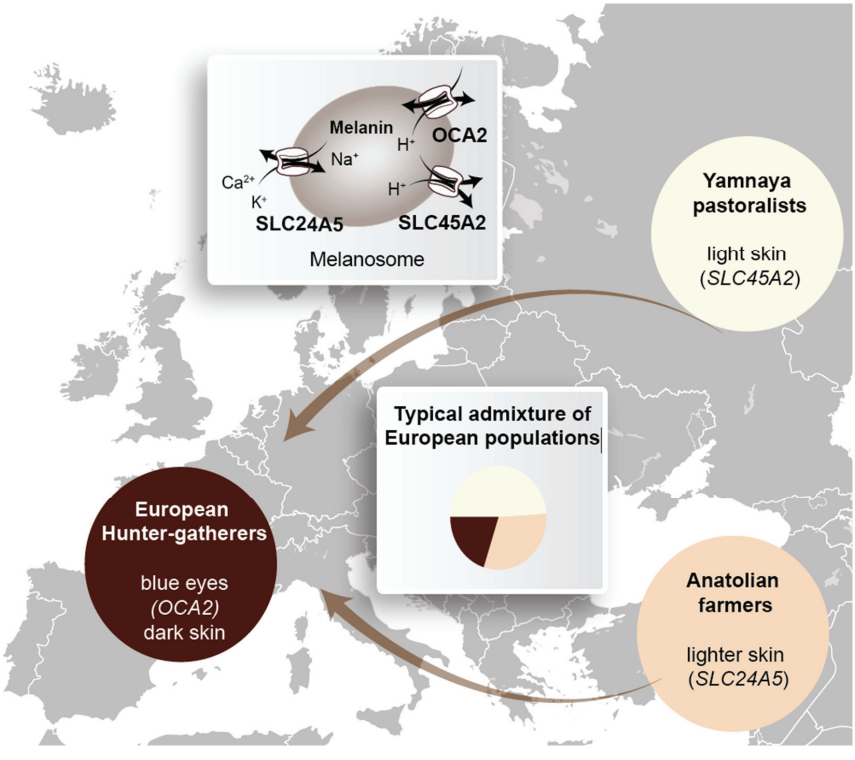

Figure 3. Schematic representation of the admixture of the European population. All European populations derived from European hunter–gatherers, Anatolian farmers and Yamnaya pastoralists [84]. A pie chart indicates the typical admixture of the founding populations (**center**). Melanin is produced in melanosomes under the control of the proteins OCA2, SLC24A5 and SLC45A2 (**top**).

Archeogenomic data demonstrated that *Homo sapiens* hunter-gatherer populations lived in Europe with dark skin for more than 30,000 years. Did they suffer from consequences of vitamin D deficiency? With the exception of people living an urban lifestyle already some 2000 years ago in the Roman empire [86], older bone samples do not show signs of malformation. Explanations could be a dominant outdoor lifestyle in southern Europe (the rest of Europe was covered by ice) or, in part, vitamin D_3 supplementation via a marine-based diet for populations living close to the coast. However, the most dominant effects were SNPs in the regulatory regions of the *DHCR7* (7-dehydrocholesterol reductase) gene, which reduced its expression, and by this, its enzymatic activity [82,87]. The resulting increased concentrations of 7-dehydrocholesterol in the skin led then to a more efficient synthesis of vitamin D_3 (Figure 1). Genome-wide association studies (GWAS) confirmed that the vitamin D status (as measured by 25(OH)D_3 serum levels) significantly depends on SNPs of the *DHCR7* gene [88]. Furthermore, other GWAS demonstrated the dependence of the vitamin D status on genes related to vitamin D endocrinology, such as *CYP2R1*, *CYP24A1* and *GC*, but not to skin color [89,90]. This suggests that at least in (western) Europe, skin lightening did not happen due to an evolutionary pressure caused by vitamin D deficiency but by interbreeding with populations from northwestern Anatolia and the northern Caucasus. Thus, the light skin color of today's Europeans is primarily based on the migration of populations from western Asia and the Near East to Europe [75]. Nevertheless, concerning their vitamin D status and their ability to populate also northern regions, the European populations benefitted from skin lightening.

6. How Vitamin D Became a Vitamin?

With the exception of highly developed societies, such as in the Roman empire, in the past, humans exposed larger percentages of their skin to the sun for a far longer part of the day than nowadays. In their evolutionary origin in East Africa, humans were every day around the year exposed to extensive UV-B radiation, which induced sufficient vitamin D_3 synthesis. Therefore, over a period of more than 200,000 years, they got used to a constantly high vitamin D status of 100 nM 25(OH)D_3 or more [65]. However, within the last 50–75,000 years, the migration toward regions with a latitude above 37 °N let them experience seasonal changes in sun exposure and periods of the year when vitamin D_3 cannot be produced endogenously. Furthermore, as a result of the industrial revolution, humans adapted to an urban lifestyle with predominant indoor work and activity. Both conditions, vitamin D winters and indoor preferences, often led to vitamin D deficiency in industrialized countries. For example, in England in the 19th century, rickets, also called the "English disease", was a very common disorder in children [91,92]. Moreover, also the severity of tuberculosis was and still is significantly increased in vitamin D deficient individuals [93]. Thus, not evolution but human migration and lifestyle changes made vitamin D_3 a vitamin.

7. Conclusions

Vitamin D_2 and vitamin D_3 started their "career" more than a billion years ago as side products of sterol biosynthesis, in fungi, some plants and animals that scavenge UV-B radiation. Just half a billion years later, the endocrinology of vitamin D evolved and the biologically active form of vitamin D_3, 1,25$(OH)_2D_3$, became a hormone. Via its high-affinity receptor VDR 1,25$(OH)_2D_3$ regulates genes that are involved in detoxification, energy metabolism, immunity and calcium homeostasis. With this pleiotropic functional profile vitamin D is an important contributor to organismal homeostasis and health. This explains why rather recently (from an evolutionary perspective) changes in human lifestyle caused a reduced endogenous vitamin D_3 production. Since, in parallel, the majority of human populations are not adapted to a marine-based diet [94], i.e., their average dietary intake of vitamin D is low, vitamin D deficiency became a common problem. Worldwide, vitamin D deficiency affects more than a billion people [95] and causes health problems, such as bone malformations and a decreased potency of the immune system.

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Review

Vitamin D and Systems Biology

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Abstract: The biological actions of the vitamin D receptor (VDR) have been investigated intensively for over 100 years and has led to the identification of significant insights into the repertoire of its biological actions. These were initially established to be centered on the regulation of calcium transport in the colon and deposition in bone. Beyond these well-known calcemic roles, other roles have emerged in the regulation of cell differentiation processes and have an impact on metabolism. The purpose of the current review is to consider where applying systems biology (SB) approaches may begin to generate a more precise understanding of where the VDR is, and is not, biologically impactful. Two SB approaches have been developed and begun to reveal insight into VDR biological functions. In a top-down SB approach genome-wide scale data are statistically analyzed, and from which a role for the VDR emerges in terms of being a hub in a biological network. Such approaches have confirmed significant roles, for example, in myeloid differentiation and the control of inflammation and innate immunity. In a bottom-up SB approach, current biological understanding is built into a kinetic model which is then applied to existing biological data to explain the function and identify unknown behavior. To date, this has not been applied to the VDR, but has to the related ERα and identified previously unknown mechanisms of control. One arena where applying top-down and bottom-up SB approaches may be informative is in the setting of prostate cancer health disparities.

Keywords: vitamin D receptor; systems biology; cell differentiation; prostate cancer

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1. Systems Biology and Biomedicine

1.1. The Opportunities of Applying Systems Biology Approaches in Biomedical Research

Much of the discovery in biomedicine has been centered on the classic paradigms of reductionist biology, in which phenotypes are interpreted as the interaction of either single or small groups of molecules. Across biomedical sciences, there are many remarkable examples of how this approach has led to the discovery of drivers of human disease, and equally remarkable examples of new therapies designed to target these drivers. For example, high-profile examples of this reductionist approach include the discovery of the oncogenic role of the BCR-ABL fusion gene in the etiology of chronic myeloid leukemia [1] and the development of a targeting kinase inhibitor such as Imatinib [2].

This is a striking example of the so-called bench-to-bedside research and represents one of the earliest examples of precision medicine. At times, however, this reductionist approach can appear limited both theoretically and clinically as the etiology of Imatinib-resistant phenotypes only too well demonstrates [3]. Across cancers and other disease phenotypes, a stumbling block can be identifying single strong disease driver mechanisms, which in turn accurately predict drug sensitivities and therapeutic effectiveness. Therefore,

delivering a fuller prediction of disease drivers and therapeutic vulnerabilities may require developing different methodologies. Ideally, any methodologies would also take advantage of the ever-increasing stream of high-dimensional biological data to inform diagnosis and prognosis. Perhaps these approaches (SB and reductionist) can actually be highly symbiotic.

Systems biology (SB) aims to apply mathematical approaches to build a predicative and quantitative model of biological systems, with the goal to use model predictions to define specific physiological or pathophysiological states and outcomes. The models derived from such SB approaches applied to experimentally derived biological data aim to identify the dynamic behavior of networks that are the center of cellular behaviors [4,5]. These approaches are readily scalable and not restricted by scope. SB approaches can be applied to discrete cell signaling systems, such as gene regulatory networks and signal transduction cascades, to cell–cell interactions, tissue organization, organismal behavior, and to complex multi-organism interactions as seen within, for example, the function of the gut and even complete ecosystems [6].

Furthermore, the models built by SB approaches aim to define the functioning of living organisms not solely by looking at the constituent molecules such as DNA, RNA, proteins, and metabolites but rather by the process-level biological systems they constitute, such as mitosis and metabolic control [7,8]. A key hallmark of these models is that they capture the states of the system in a predicative and quantitative manner and can be exploited to drive novel understanding. As these in silico models can be interrogated rapidly, multiple components of any given model can be dissected to reveal unintuitive findings [9–11].

A consequence of the integrated nature of biological signaling is the emergent complexity, which underpins human health. For example, the dexterous control of transcriptional networks is derived from a high ratio of transcription factors to regulated mRNA or miRNA targets. This ratio, combined with large regulatory regions, results in unparalleled plasticity over the choice, amplitude, and period of transcription [12,13]. More specifically, transcription factor modules recognize, interpret and sustain histone modifications and ultimately establish boundaries between transcriptionally rich euchromatin and transcriptionally restricted heterochromatin. These boundaries are cemented further by the regulation of CpG island methylation; these two processes are dynamically intertwined. Again, from an SB perspective, it is reasoned that modeling these transcriptional processes will help to explain the highly integrated nature and robustness of normal transcriptional control, whereby the processes have redundancies such that they do not radically alter in response to external signals and do not fail when a single component is altered. By contrast, transcriptional networks in cancer cells display a loss of transcriptional plasticity and do not display the full breadth of signaling capacities. The evolution of the malignant transcriptome is seen clearly in the nuclear receptor and MYC superfamilies (reviewed in [14]).

The concept of SB builds on so-called "holistic biology" developed in the 1960s and coupled with informatics theory and modeling approaches developed through the 20th century. However, the application of SB methodologies and the expansion of SB concepts in biomedicine has been boosted since the early 2000s by the technological advances in the development of high dimensional data approaches frequently derived from next-generation sequencing technologies coupled with bioinformatic approaches. This progression from the 1950s to the current state has been profoundly catalyzed by the human genome project, with its draft sequence published in 2001, and a final reference genome published in 2022 [15] alongside other reference genomes across the animal and plant kingdom. In this manner, the combination of high throughput experimental approaches in the wet lab coupled with complex statistical analyses and computational methods in the dry lab have led to a more comprehensive characterization of multiple diverse organisms, and a shift of focus from molecules to their interactions and the networks they form [16]. This sophistication and power of prediction are most likely set to increase when SB models also include a spatio-temporal characterization of cell behavior, including the dynamics of how molecules are exchanged between compartments and exported from the cell. Again, modeling these

aspects of cell behavior has been massively impacted by an explosion in single-cell and spatial technologies.

There are several well-justified advantages of applying SB approaches, which have the genuine potential to complement and extend the reductionist paradigm. Expressing the complete interactions within biological systems in mathematical terms reduces the impact of biases introduced by focusing on single proteins, and instead can reveal under-explored control points in the system. Furthermore, expressing biological systems in the context of processes, rather than well-understood individual components, has the potential to assimilate more readily new high dimensional data generated in biological experiments. Consequently, it is reasonably anticipated that significant, novel, and unpredicted strides will be made in understanding the control and responsiveness of biological events, which in turn can generate new insights into disease susceptibility and therapeutic opportunities [17,18].

1.2. Systems Biology Builds upon an Asymptotic Recursion between Wet Lab and Dry Lab

At its core, SB approaches have an asymptotic recursion between research activities in the wet lab and dry lab. Models are built in the dry lab that reflects the biological observations in the wet lab and are used to generate predictions for how the system can behave. Such predictions are then formulated into testable interventions in the wet lab to generate data for model refinement in the dry lab. It is this oscillation between wet and dry labs that is so potentially powerful, but at the same time so daunting. Broadly, two strategies have emerged to develop such models, by either top-down or bottom-up approaches (Figure 1).

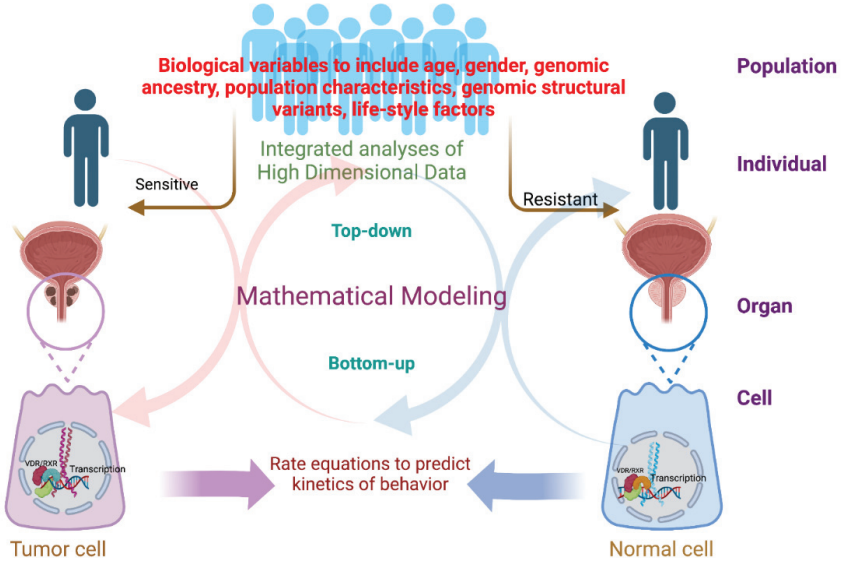

Figure 1. A workflow for top-down and bottoms-up system biology (SB) approaches to address prostate cancer health disparities. For top-down SB there is a plethora of high dimensional data including germline structural variants, population characteristics as serum-borne, and other clinical data that can be integrated through a range of approaches to deliver insights into how the prostate could either be either sensitive or resistant to the growth restraint properties of the VDR. From the bottoms-up perspective, more focused kinetic models can be developed for how the VDR functions and is disrupted between normal and tumor cells. Combining these approaches has the potential to develop a holistic understanding of how the VDR functions in the prostate gland and how this is impacted by genomic ancestry.

In the top-down approach, large datasets are interrogated with statistical methods to find patterns in the data with which to derive predictions on the system organization [19]. The models in top-down SB are phenomenological, meaning they are not directly mechanistically based and do not require knowledge about relationships between different molecular components. Identification of significant associations is used to develop a hypothesis on the nature of the molecule interactions and associations identified. These approaches naturally lend themselves to omics-derived data and develop hypotheses to be tested by wet lab analysis [20]. It is worth noting that the identified associations that are significant may not be causal, and in fact, themselves may not be true. Top-down approaches are often used with subsystems that have not yet been characterized to a high level of mechanistic detail and approaches such as Bayesian modeling are appropriate due to the missingness in the data from biological regulatory networks [21].

By contrast, the bottom-up approach begins with the hypothesis of biological mechanism and formulating equations on how the components of the system interact and then running simulations to generate predictions. This approach relies on experimental studies to determine the kinetic and chemical properties of the components, and starts with formulating system behaviors in rate equations, for example, expressed as differential equations, of the constituting parts of each system. These formulations are then integrated to predict the system behavior, with the goal to combine pathway models into a model for a larger system [21]. The data on the system under study are subjected to perturbations in the cell context and models are refined from the data. An example of a bottom-up approach is the silicon cell program where computational replicas of actual pathways are made to calculate system behavior [22].

The power of these approaches arises from being able to identify and direct experiments to test fundamental questions of a biological system. From the top-down perspective, these questions include identifying in a genome-wide manner the interactions of all components in a system to define the metabolic control that ultimately brings about cell, tissue, or organism behavior. Arising from this approach questions can be asked of a system. For example, what is the interconnectedness of the system and how does that change between health and disease states, or in different development or differentiation states? Which hubs in such networks are central and which are peripheral? Again, how does hub distribution shift in disease and development transitions? How do changes in gene and protein expression combine to control metabolism? How do germline or somatic structural variants change network topology and metabolic flux?

Similarly, from the bottom-up perspective, a model is curated from known biological interactions and expressed in mathematical terms to test behavior and make new predictions. For example, with any signaling system how is activation controlled and silenced? Given the ubiquitous role of enzymes in biology, a common question is how does changing the kinetics of activating/de-activating enzymes lead to altered signal amplitude? To what extent do germline or somatic structural variants in enzymes, or co-factors modulate enzymatic capacity and what effect does that exert on signal strength? More broadly, how are different signaling systems integrated and what is the quantitative effect of convergence? Does crosstalk generate synergistic events in regulatory complexes or are they merely convergent downstream at endpoints? What is the magnitude of control at each step and is there an order to control ranging from find-tuning to signal-independent activation? Finally, questions can be asked of the system in disease or development states and tested for pharmacological relevance including identifying which targets may be impactful but have deleterious side effects.

2. The Opportunities and Challenges of Applying Systems Biology Approaches to Studying the Vitamin D Receptor

A systems-level appreciation for vitamin D signaling has existed for several centuries, given that a description of rickets was first described in the 17th century [23]; rickets arises

from impaired bone mineralization due to insufficient signaling via the vitamin D receptor (NR1I1/VDR) (reviewed in [24]).

The VDR is a Type II member of the nuclear receptor superfamily, which binds the active hormone $1\alpha,25(OH)_2D_3$. Several features of how the VDR functions are important from an SB perspective. A feature of Type II receptors such as the VDR is that independent of ligand the receptor is significantly associated with the genome bound to cis-regulatory elements (CRE) and may exert repressive effects, which is reversed by ligand activation leading to genomic redistribution and transactivation [25–28]. Additionally, of interest from a systems perspective is that VDR interacts in distinct ways with a number of proteins. Firstly, it heterodimerizes with the RXRs (NR2B1/RXRα, NR2B2/RXRβ, NR2B3/RXRγ), which is also a central dimer partner for other Type II receptors including those retinoic acid receptors (NR1B1/RARα, NR1B2/RARβ, NR1B3/RARγ), and the peroxisome proliferator-activated receptors (NR1C1/PPARα, NR1C2/PPARβ, NR1C3/PPARγ) [reviewed in [24]]. Secondly, the VDR interacts with a range of coregulator proteins such as coactivators and corepressors that exert antagonist roles in the control of local chromatin structure, as well as components of the SWI/SNF complexes to remodel nucleosome positioning and other enzymes such as helicases [29,30] and splicing factors [31] to also facilitate transcription. The VDR appears to participate in protein–protein–DNA interactions, for example with other transcription factors, in a trans-regulatory mechanism to regulate transcription. Finally, there is emerging evidence for the VDR, like other nuclear receptors [32,33], to interact functionally with long non-coding RNAs (lncRNA) to control gene expression by directly affecting the DNA environment or other RNA binding proteins [34]. Indeed one such lncRNA, steroid receptor activator (SRA) coimmunoprecipitates with the coactivator NCOA1/SRC1 and may function more broadly as a scaffold for NR complexes (reviewed in [32]).

Finally, of central importance to the complete VDR signaling system is the generation of the ligand, $1\alpha,25(OH)_2D_3$, the levels of which are dynamically controlled in terms of synthesis of the precursor $25(OH)D_3$ which is held in a relatively tight range in the serum, and signs of deficiency in the VDR system can be seen when this serum level is diminished. The various metabolic steps that lead to the generation of $25(OH)D_3$, the active ligand $1\alpha,25(OH)_2D_3$, and a range of metabolites that ultimately lead to its catabolism are all tightly controlled by enzymatic reactions both in an endocrine and tissue-specific local intracrine manner.

2.1. Top-Down Approaches Applied to VDR Biology

From a top-down SB perspective, it is interesting to ask in what context the VDR itself, or coregulators, or ligand generation are identified as a central hub in gene networks associated with different cell phenotypes and disease states, and from a bottom-up SB perspective, it is attractive to develop models that accurately capture the cycling interactions of the VDR with different co-factor and predict how this relates to diverse transcriptional outputs, again across cell phenotypes and disease states.

High dimensional data approaches have been applied to ask questions about what the transcriptional effects are of adding $1\alpha,25(OH)_2D_3$, or deleting the VDR. From a top-down SB perspective, the question of VDR function is alternatively phrased to identify biological circumstances where the VDR is identified as a hub in the transcriptional network independent of any knowledge of the system. In this manner, analyses of across myeloid cells [35], granulocytes [36], and megakaryocytes [37] have identified significant control functions for the VDR to regulate specific cell differentiation outcomes. Interestingly, these studies support some of the earliest studies that explored the functions of $1\alpha,25(OH)_2D_3$ and revealed its capacity to initiate differentiation of leukemia cells [38,39]. These findings might suggest a role for the VDR to be mutated or deleted in leukemia and although there were numerous candidate studies of the VDR expression and genomic integrity, large-scale genomic approaches, for example in the TCGA leukemia cohort [40], suggest that the VDR is neither distorted to act as cancer-driver, nor has it been therapeutically exploited to date.

Together, these findings suggest that whilst the VDR is biologically impactful in the normal differentiation, for example, the process to monocytes, it is not itself so frequently disrupted in leukemia such that when it is mutated it acts in an oncogenic manner to disrupt normal progenitor differentiation, and it cannot be targeted to induce leukemia cell differentiation in patients. At first glance, these findings may appear contradictory, but most likely reflect the nature of redundancy in the system, and the role of VDR to undergo diverse protein interactions. Specifically, the VDR is known to interact with CEBPs [41] to induce leukemia cell differentiation [41], and indeed the CEBPs are a master regulator in the physiological transcriptional module containing the VDR to regulate monocyte differentiation [35]. However, CEPPs interact with multiple different nuclear receptors to regulate cell fates [42–45], and redundancy in the system limits the impact that altered VDR function alone can exert on cell fates. Finally, it is interesting to note that CEBPA mutations are in the top 33 mutational events in the TCGA leukemia cohort [40].

These identified roles for the VDR in monocyte differentiation reflect a broader function for the receptor to signal in differentiation processes [46] and immune phenotypes. Alongside the roles of vitamin D to prevent rickets, in the late 19th century the concept emerged of sunlight therapy, known as heliotherapy, to fight tuberculosis and other infections [47]. Indeed, unbiased transcriptomic analyses of the impact of *Mycobacterium tuberculosis* demonstrated a role for Toll-like receptors to initiate a signal transduction cascade in macrophages that upregulated the VDR and induced an antimicrobial response. Intriguingly, this response appeared to be dampened in people with African genomic ancestry perhaps associated with low serum levels of 25(OH)D_3 [48]. Similarly, *Mycobacterium tuberculosis* infection of lung cells identified VDR as a key regulated transcription factor [49]. Another infection of interest is the coronaviruses and in 2013 the lung cell response towards severe acute respiratory syndrome (SARS) arising from a coronavirus infection again identified the VDR as a key regulator of response [50]. More recently, COVID-19 infection of T cells from bronchoalveolar lavage demonstrated a role for the VDR to trigger super-enhancer activation and the control of innate immunity [51]. An unbiased identification of the role of the VDR in the regulation of innate immunity and immune phenotypes has also been identified by GWAS [52–55].

Viewed from the perspective of unbiased top-down statistical analyses has revealed critical roles for the VDR in innate immunity, control of inflammation, regulation of differentiation, and metabolic control [56–58], but not in some of the other phenotypes that are investigated at the pre-clinical and candidate level, notably including cancer; this was established in a previous structured literature search (reviewed in [59]). That is, no cancer-associated GWAS identifies structural variants in the VDR associated with cancer risk, and none of the TCGA papers (over 10,000 tumor samples across more than 30 tumor types) identify frequent VDR-associated structural variants or significantly altered expression that associates with cancer clinical phenotypes.

GWAS studies have continued to evolve in terms of the complexity of study design [60] and cohort sizes and different genomic ancestry, and methods have also developed to finding interactions between variants and also considering how non-coding variants withing regulatory regions may determine how efficiently a given transcription factor can function; so-called a cis-expression quantitative trait loci (eQTL). Certainly, there is evidence for significant enrichment of germline structural variants in VDR binding sites associated with genes that regulate immune phenotypes [61]. Likewise, there is evidence for such eQTLs are significantly impacted by genomic ancestry, for example in the case of the ex vivo response towards 1α,25$(OH)_2D_3$ in normal colon cells [62].

These methodological developments and the ever-increasing repertoire of high dimensional data are likely to increase the understanding of VDR and vitamin D signaling phenotypes and will most likely become ever more important to develop new hypotheses over how signaling occurs and where it is most biologically relevant. Perhaps this is readily illustrated by the application of Mendelian randomization methodology to test the factors such as levels of serum 25$(OH)D_3$ (either measured or predicted) and their causal relation-

ship with different clinical phenotypes. In this manner 25(OH)D_3 levels and components of the VDR axis are significantly casual in multiple sclerosis, hand grip strength, bacterial infection, and type 2 diabetes [63–66], but not for the prevention of COVID-19 infection, birth weight, or colon cancer [58,67,68].

2.2. Bottom-Up Approaches Applied to VDR Biology

Applying a bottom-up SB approach to VDR-dependent gene regulation requires constructing a kinetic model that captures the function of different VDR-containing complexes and the quantitative regulation patterns of VDR target genes. Such a model would combine spatial-temporal measurements for the generation of 1α,25$(OH)_2D_3$ in a target tissue, VDR genomic interactions, such as initial concentrations in the nucleus of the proteins involved, their half-life, and their associations, to explain how these actions predict the downstream transcriptional impact. Model refinement and validation can then be undertaken by empirical measurements of the cycling of VDR genomic interactions and transcriptional outputs.

The working assumption is that the transcription factor complex contains enzymes that remodel chromatin to allow signaling to the RNA polymerase complex and the initiation of transcription. In the case of a transcription factor such as the VDR, which has nuclear residence independent of ligand exposure, the complex exists in two states of genomic interaction, namely with and without ligand, and in each with qualitatively and quantitatively different cofactors, and also in terms of distribution (distal or proximal). The output of the model will then be to predict the kinetics of mRNA accumulation in a manner that reflects the different ligand-dependent and independent states, and spatial association to target genes. Of course, these components are most likely too simple to predict mRNA accumulation accurately. However, even such a simple model for different genes and the VDR could begin to define how much of the difference between mRNA accumulation could be explained by merely considering spatial and temporal factors for transcription factor residence on a gene. Model components could then be added to consider other well-understood biological components such as disease states, and chromatin states, as well as the impact of DNA helicases required for transcription, or even the role of non-coding RNA to impact mRNA accumulation.

To date, such an approach has been applied to several transcription factors in human cells. Within the nuclear receptor superfamily, kinetic modeling of ERα predicted a role of receptor phosphorylation to act as an underexplored feedback mechanism to predict RNA accumulation and was validated by empirical measurements [69]. Outside of nuclear receptors, other human transcription factor-centered models include describing the separate actions of NF-κB [70,71] and p53 [72], and their collective oscillatory behavior [73]. Separately, signal transduction events have also been modeled quite extensively, including the transduction events for RAS [74,75] and its interaction with other regulatory events such as the circadian clock [76] and EGFR signaling [77].

These approaches are powerful, and it is perhaps surprising why they have not been more widely applied to other nuclear receptors beyond the ERα, for example to the clinically relevant VDR. Several impediments no doubt include the time, resources, and interdisciplinary expertise required for model development and refinement. One impediment is establishing collaborations that bring insight from the wet and dry labs. To be successful these collaborations require sufficient data density of dynamic cellular events, such as precise measurements of the concentration of key proteins and RNA molecules in a cell, the frequency of transcription factor genomic interactions, and levels of mRNA accumulation to justify modelling in mathematical terms. Furthermore, these challenges are sometimes impeded by an uneasy relationship between the biological and mathematical communities [5]. The incentive for these intensive modelling efforts, most likely involving interdisciplinary collaborations, is that once such a model is built it has the potential to be translated to other transcription factor genome interactions, or across cell types, or disease states and allow speculation on the biological processes without having to undertake time-intensive and costly experiments in the wet lab.

3. Prostate Cancer and Health Disparities; An Exemplar of the Opportunities Arising from Systems Biology Approaches in Biomedicine

Men of African genomic ancestry in American (African American, (AA)) experience higher risks of developing more aggressive prostate cancer (PCa) than European American (EA) counterparts [78–81], which reflects underlying genetic [82–85] and epigenetic [86–91] drivers and biopsychosocial processes [89,92,93]. The role of the glucocorticoid receptor (GR) as a primary target for stress response has more recently been examined in the context of cancer (reviewed in [94]) and offers a potential functional explanation for how stress can be an accelerant of PCa in AA men [94–97]. A role for VDR signaling appears to be more high profile in the etiology of AA rather than EA PCa. Given that UVB radiation degrades folic acid as well as catalyzing vitamin D synthesis, a strong inverse correlation between skin pigmentation and latitude has arisen during ancestral adaptation [98,99], and amongst AA PCa patients there are significant associations between low serum vitamin D_3 levels and incidence and progression risks of PCa [100–115]. Furthermore, although vitamin D_3 supplementation in the VITAL cohort [116,117] had no overall impact on cancer incidence, the AA participants experienced a suggestive 23% ($p = 0.07$) reduction in cancer risk, indicating that larger cohorts may be more informative [118–120]. Strikingly, vitamin D_3 supplementation in AA and EA PCa patients only significantly modulated prostate gene expression in the AA patients [121], associated with the control of inflammation. This was supported by our recent study [122] in AA and EA cell models which have identified qualitatively and quantitatively distinct VDR actions in terms of VDR protein–protein interactions and more frequent VDR genomic interactions associated with significantly distinct target gene expression.

These data support the concept that one cell function that appears to be most highly responsive in a manner that reflects genomic ancestry is the role of VDR to modulate innate immunity [48,123–126], and reflecting this there are correlative findings that support a relationship between $25(OH)D_3$ levels and immune-modulatory factors such as interleukin (IL)-6 [127,128]. IL6 release into the serum is associated with activated macrophages and is elevated in Ghanaian and AA men [129–131]. Together these studies suggest that the biology of the prostate is the most sensitive VDR signaling in AA men, and more impacted by inadequate VDR signaling either as a result of molecular mechanisms or environments of low $25(OH)D_3$. One biologically impactful consequence of this is the loss of control of inflammatory signals. These studies also highlight that African ancestry is itself divergent, and is also further modified by admixture, for example in the AA population (refs).

The Potential Application of SB Approaches to Prostate Health and Disease

There are significant knowledge gaps in identifying how genomic ancestry, the environment, and lifestyle choices may combine to impact prostate health and disease. The application of SB modeling could be used to generate biologically plausible hypotheses to test specific relationships in these complex interactions. These knowledge gaps include whether germline structural variants impact the generation of $1\alpha,25(OH)_2D_3$, and the extent of relationships between serum $25(OH)D_3$ levels and PCa in a manner that reflects genomic ancestry, and if, and to what extent, this is impacted by admixture. In experimental models, it is emerging that the VDR genomic interactions differ between EA and AA prostate cell models (ref), and recently AR genomic interactions also appear different in EA and AA PCa samples. However, it is unclear if either the underlying sequence, for example at enhancer binding sites, or other mechanisms are impacting why these different genomic interactions arise. Finally, given the multi-parameter process of cis-regulatory interactions that drive transcription through to processed RNA and translation to protein, it is also unclear how genomic ancestry impacts these processes potentially in an emergent manner.

Together, these knowledge gaps can be combined in a hypothesis; namely that PCa risks in AA men reflect the interplay of genomic ancestry, including admixture, coupled with altered environmental signals that combine to drive qualitatively and quantitatively distinct functioning of the VDR and potentially other transcription factors and underscore

the health disparities in PCa. Potentially, this is also an attractive setting to develop a series of SB models with which to test these interactions in silico and generate predictions to validate in vitro and in vivo (Figure 1).

This provides an opportunity for both top-down and bottom-up SB modeling. From the top-down perspective, high dimensional data are available to test develop models that test how genomic ancestry significantly determines the associations between serum 25(OH)D_3 levels [118–120] and PCa structural variants [132,133], epigenetic states [86–91] and gene expression [87,91,134] and how this significantly relates to serum inflammatory markers. More specifically, Mendelian randomization approaches would be appropriate to test how germline structural variants are associated with serum 25(OH)D_3 levels depending on genomic ancestry.

To complement these approaches, other top-down strategies applied to high dimensional data sets could then determine epigenomes such as CpG methylation, histone modification, and chromatin accessibility [135,136] and transcriptome relationships in AA and EA PCa patients. Construction of a spatial matrix from epigenomic data, co-incidence with prostate cancer-specific enhancers [137,138], will be binned by accounting for orientation and distance to gene features. Machine learning approaches can then be applied to identify and test the significance of relationships between this matrix and PCa transcriptomes. For example, bootstrapping approaches can test the associations of the epigenome and patterns of observed gene expression compared to simulated data by random sampling [139]. In parallel, other approaches such as the Pareto optimization algorithm [140] can define the ordered correlation of relationships between the cistrome matrix and genesets, of which the strongest (positive or negative) are of greatest biological significance. Comparing the results of both approaches within and across genomic ancestry will reveal more significant cistrome–transcriptome features that are potentially driving PCa health disparities. Lasso and ridge regression can then be performed on the most significant cistrome–transcriptome relationship genes to identify gene expression patterns that predict clinical outcomes in publicly available data [141,142].

To meet these top-down approaches, bottom-up methods can be applied to build kinetic models that take into consideration the amount of the VDR and its key interacting factors in AA and EA prostate cells to simulate its ligand-dependent and independent genomic states and distribution across epigenetic states. The goal of this modeling would be to provide biological plausibility for why the VDR appears to be significantly transcriptionally divergent between AA and EA PCa. Such predictions would form the justification for validation in the wet lab using AA prostate cell line models and patient-derived xenografts. Finally, such modeling may well justify tailoring recommendations for healthy vitamin D serum levels that reflect genomic ancestry.

4. Summary

The VDR signaling system is physiologically impactful and when its functions are altered it is associated with several disease states. Systems biology approaches to the analyses of VDR functions have been applied in several top-down studies that have identified significant VDR functions. To date, however, no bottom-up approaches to kinetic modeling have been applied. The incentive in biomedicine to continue to apply SB approaches would be to develop insight into what are the most prominent contexts where the VDR system exerts biological impact and in turn how this can be exploited in diagnostic, prognostic, or therapeutic contexts. For example, the application of SB approaches, both from a top-down and bottom-up perspective, has the potential to be impactful in the context of prostate cancer health disparities.

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Review

Vitamin D and Its Target Genes

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Abstract: The vitamin D metabolite 1α,25-dihydroxyvitamin D_3 is the natural, high-affinity ligand of the transcription factor vitamin D receptor (VDR). In many tissues and cell types, VDR binds in a ligand-dependent fashion to thousands of genomic loci and modulates, via local chromatin changes, the expression of hundreds of primary target genes. Thus, the epigenome and transcriptome of VDR-expressing cells is directly affected by vitamin D. Vitamin D target genes encode for proteins with a large variety of physiological functions, ranging from the control of calcium homeostasis, innate and adaptive immunity, to cellular differentiation. This review will discuss VDR's binding to genomic DNA, as well as its genome-wide locations and interaction with partner proteins, in the context of chromatin. This information will be integrated into a model of vitamin D signaling, explaining the regulation of vitamin D target genes.

Keywords: vitamin D; VDR; target genes; chromatin; epigenome; transcriptome; vitamin D signaling

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1. Introduction

For 100 years, the term "vitamin D" has been used [1] for a molecule, the deficiency of which can lead to bone malformations, such as rickets [2]. More than 50 years ago, evidence accumulated that vitamin D_3 acts via its metabolites 25-hydroxvitamin D_3 (25(OH)D_3) and, in particular, via the nuclear hormone 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2$$D_3$) [3]. The emerging endocrinology of vitamin D was completed through the identification of vitamin D-binding proteins [4,5] and the cloning of VDR in different species [6,7]. VDR turned out to be an endocrine member of the nuclear receptor superfamily [8,9], suggesting that (similar to the steroid hormones estradiol, testosterone, progesterone, cortisol and mineralocorticoids, the vitamin A derivative all-*trans* retinoic acid and the thyroid hormone triiodothyronine) 1,25(OH)$_2$$D_3$ acts at nanomolar, or even picomolar, concentrations, as a direct regulator of specific target genes [10–12], in VDR-expressing tissues and cell types (www.proteinatlas.org/ENSG00000111424-VDR/tissue, accessed on 5 March 2022). The nearly ubiquitous expression of the VDR gene supports findings obtained during the past 30 years, that vitamin D regulates not only calcium homeostasis [13], but also immunity, cell growth and differentiation, as well as energy metabolism [14–16].

The genomic actions of vitamin D depend on the activation of VDR by 1,25(OH)$_2$$D_3$ and involve changes in the epigenome, leading to changes in the transcriptome and proteome. Therefore, in a typical in vitro vitamin D stimulation experiment, where supra-physiological concentrations of 10–100 nM 1,25(OH)$_2$$D_3$ are applied to a cell culture model, it takes (due to the time needed for RNA and protein synthesis) a few hours before the physiological effects of the nuclear hormone can be observed [17]. This is in contrast to the so-called non-genomic actions of vitamin D that happen within seconds to minutes and do not to involve VDR and changes in gene expression [18,19]. However, the timing of vitamin D signaling may not be of critical importance, since the physiology of vitamin D and its metabolites aims towards homeostasis, i.e., in vivo, there are no larger fluctuations in the concentrations of 1,25(OH)$_2$$D_3$ [20]. Thus, in net effect, the physiology of 1,25(OH)$_2$$D_3$ largely overlaps with the actions of the transcription factor VDR.

This review will outline VDR's DNA-binding modes, genome-wide locations, as well as its interaction with chromatin components and other partner proteins. This will provide the basis of a model of vitamin D signaling that explains the mode of action of vitamin D target genes and allows for their classification.

2. VDR: A Transcription Factor Activated by Vitamin D

Transcription factors are proteins that are able to bind sequence-specifically to genomic DNA and interact with other nuclear proteins, which modulates the activity of RNA polymerase II and mRNA production [21,22]. Some of the approximately 1600 transcription factors encoded by the human genome are constitutively active and regulated primarily by their expression, while most of them are activated by extra- and intracellular signals. A few of these signal-dependent transcription factors are located either in a latent form in the cytosol and activated through translocation into the nucleus, or—most of them—are found in the nucleus and modulated in their activity by post-translational modifications, such as phosphorylation or acetylation. Furthermore, some members of the nuclear receptor superfamily have an additional mechanism of activation, which is a ligand-induced conformational change on the surface of their ligand-binding domain (LBD) [23,24].

The inner surface of VDR's LBD forms a ligand-binding pocket, where 40, mostly non-polar, amino acids snugly enclose the molecule $1,25(OH)_2D_3$, so that it binds with an affinity of 0.1 nM [25]. This is a very high affinity, even in comparison with other nuclear receptors [26]. Ligand binding changes VDR's interaction profile with many of the more than 50 nuclear proteins that have been reported to cooperate with the receptor [27]. Some of these VDR-interacting proteins function as co-repressors, such as NCOR1 (nuclear receptor corepressor 1) [28] or COPS2 (COP9 signalosome subunit 2, also called ALIEN) [29], co-activators of the NCOA (nuclear receptor coactivator) family [30], or members of the Mediator complex, such as MED1 [31–33] (Figure 1). Other VDR partner proteins are chromatin-modifying enzymes, such as histone acetyltransferases (HATs) [30], histone deacetylases (HDACs) [29], lysine demethylases, such as KDM6B [34] and KDM1A [35] or chromatin remodeling proteins, such as BRD (bromodomain-containing) 7 and 9 [36]. The large variety of its protein interaction partners suggests that VDR is a dynamic member of a large nuclear protein complex [37] (Section 5).

Many in vitro studies indicated that VDR binds efficiently to genomic DNA in a complex with the nuclear receptor retinoid X receptor (RXR) [38–40]. The preferred binding sites for the heterodimeric VDR-RXR complex are RGKTSA (R=A or G, K=G or T, S=C or G) sequence motifs, arranged as a direct repeat with three spacing nucleotides (DR3) [41–43] (Figure 1). However, these genomic binding sites need to be accessible to VDR complexes; i.e., they need to be located within open chromatin, which is referred to as euchromatin (Section 3). Chromatin immunoprecipitation sequencing (ChIP-seq) is a next-generation sequencing method that is able to determine, in an unbiased fashion, the genome-wide binding pattern, the so-called cistrome, of a transcription factor, such as VDR. ChIP-seq for VDR had been performed in many cellular models (Section 4), which confirmed that DR3-type binding sites are the most enriched sequence motifs below the summits ($\pm$100 bp) of VDR peaks [44]. However, depending on the threshold settings of DNA motif-finding algorithms, such as HOMER [45], only 10–20% of all VDR binding sites contain DR3-type motifs [44,46]. Thus, in a genome-wide perspective, not all VDR-containing nuclear complexes contact genomic DNA via DR3-type binding sites, by some distance.

The low percentage of DR3-binding VDR complexes detected by ChIP-seq also suggests that there are a number of scenarios in which VDR acts independently of RXR. VDR may use other nuclear proteins as alternative cooperative binding partners on genomic DNA [47–49] or may bind indirectly to DNA, such as "backpack", to other transcription factors [50]. For example, below VDR ChIP-seq peak binding sites for the pioneer transcription factor PU.1 (purine-rich box-1) are enriched [44]. In fact, in THP-1 monocytic leukemia cells, the presence of PU.1 is observed on two-thirds of VDR's genomic binding sites [51]. This makes sense, since PU.1, VDR and the pioneer factor CEBPα (CCAAT

enhancer binding protein alpha) are the key transcription factors directing the differentiation of myeloid progenitor cells into monocytes and granulocytes, during the process of hematopoiesis [52]. Interestingly, THP-1 cells CEBPα [53], GABPα (GA-binding protein transcription factor alpha) [54] and ETS1 (ETS proto-oncogene 1, transcription factor) [55] also co-locate with VDR binding sites and act as pioneer factors for vitamin D signaling (Figure 1). Furthermore, in osteoblasts, vitamin D signaling is enhanced by the pioneer factors CEBPα and RUNX2 (RUNX family transcription factor 2) [56], while in T cells, this is mediated by the transcription factor BACH2 (BTB domain and CNC homolog 2) [57]. Thus, VDR uses help from RXR, but also from many other transcription factors, in order to form functional complexes with genomic DNA.

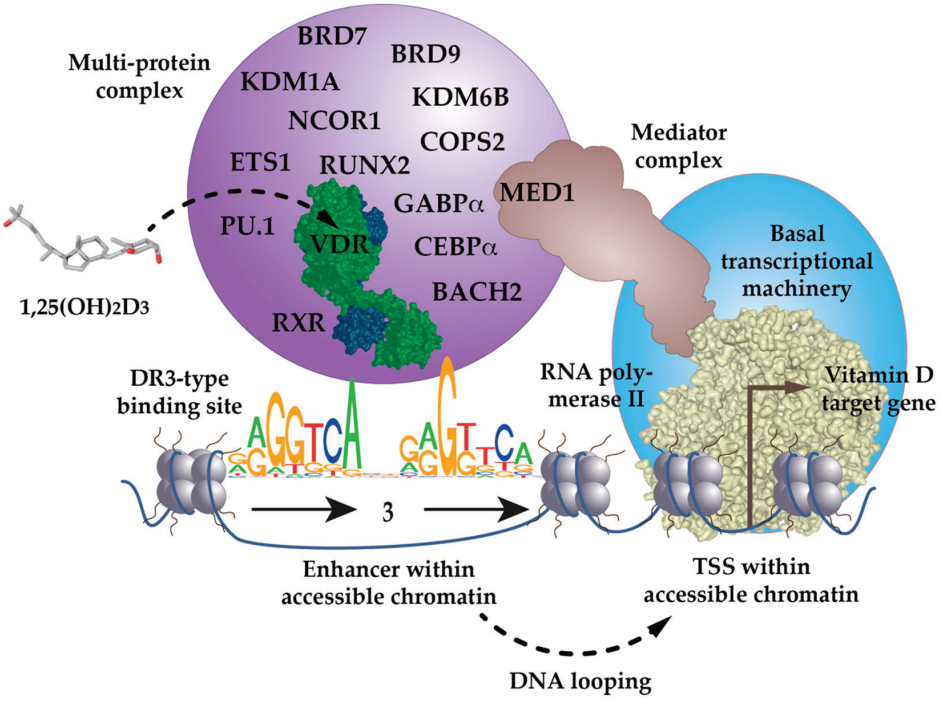

Figure 1. VDR as the key ligand-inducible component of a multi-protein complex. VDR is a part of a multi-protein complex that, e.g., contains co-receptors (RXR), pioneer factors (PU.1, CEBPα, GABPα, ETS1, RUNX2, BACH2), chromatin modifiers (KDM1A, KDM6B), chromatin remodelers (BRD7, BRD9), co-activators (MED1) and co-repressors (NCOR1, COPS2). The complex is activated through the binding of $1,25(OH)_2D_3$ to VDR and attaches preferentially to DR3-type binding sites within enhancer regions. The mediator complex connects the activated VDR complex with the RNA polymerase II waiting on transcription start site (TSS) regions of vitamin D target genes. In most cases the linear distance of enhancer and TSS region are multiple kb, so that the intervening genomic DNA forms a regulatory loop. In this way the expression of the vitamin D target genes is either increased or decreased.

3. Vitamin D Target Gene Regulation in the Context of Chromatin

Each of the approximately 20,000 protein-coding genes of the human genome has one or multiple transcription start sites (TSSs). The latter are core promoter regions, to which RNA polymerase II is directed by general transcription factors, such as TBP (TATA box binding protein) [58]. Depending on a direct interaction of this so-called basal transcriptional machinery, with signal-dependent transcription factors (Section 2) or an indirect interaction via members of the Mediator complex [59], the RNA polymerase

modulates the transcription rate of the respective gene, i.e., its expression (mRNA level) increases or decreases. Transcription factors, such as VDR, bind to enhancer regions [60], which are stretches of genomic DNA that contain specific binding sites (Section 2), for one or multiple transcription factors. The interaction of the protein complexes formed on TSS and enhancer regions is facilitated by DNA looping, in a so-called regulatory loop (Figure 1). Therefore, enhancers are equally likely found upstream and downstream of TSS regions [61]. However, both types of genomic regions need to be located within the same TAD (topologically associating domain), in order to efficiently interact. TADs are far larger loops of genomic DNA than regulatory loops, with a size of hundreds of kb to a few Mb [62]. They subdivide the human genome into at least 2000 units, which are functionally insulated from each other [63] (Figure 2). The borders of TADs are defined by the binding of the chromatin-organizing protein CTCF (CCCTC-binding factor) [64,65], forming together with cohesin and other proteins, so-called TAD anchors [66]. The interaction of the VDR-bound enhancers with genomic regions outside of a TAD are prevented by these insulating TAD borders. This is the reason why genes are regulated almost exclusively by enhancers that are located within the same TAD. This also implies that the linear distance between VDR-bound enhancers and TSS region(s) cannot be larger than the size of the respective TAD.

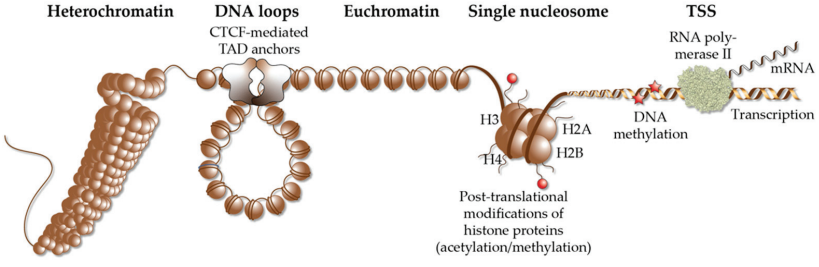

Figure 2. Elements of chromatin. Different elements of chromatin are shown, such as densely packed heterochromatin, DNA loops, such as TADs that are anchored by CTCF proteins, accessible euchromatin, the structure of a single nucleosome, chromatin modification via histone acetylation and methylation as well DNA methylation and a TSS, from which RNA polymerase II starts gene transcription into mRNA.

In a repeating unit of 200 bp, genomic DNA is packaged around nucleosomes, which are complexes of two copies of each of the histone proteins H2A, H2B, H3 and H4 (Figure 2). The complex of nucleosomes and genomic DNA is referred to as chromatin and can be interpreted as the physical expression of the epigenome [67]. Chromatin regions largely differ in their degree of packaging [68], so that transcription factors are limited in their access to genomic binding sites [69]. The accessibility of chromatin can be monitored genome-wide by the methods of DNase-seq (DNase I hypersensitivity sequencing) [70], FAIRE-seq (formaldehyde-assisted isolation of regulatory elements sequencing) [71] and ATAC-seq (assay for transposase-accessible chromatin using sequencing) [72]. The vast majority of the genome is covered in a cell- and tissue-specific fashion by densely packed heterochromatin, which is largely inaccessible, in order to prevent the unintentional activation of genes. In contrast, in an average differentiated cell, genomic DNA is accessible at less than 200,000 loci (representing only some 10% of the whole genome) that primarily comprise TSS and enhancer regions [61]. This has a key impact on vitamin D signaling, since VDR binds exclusively to accessible enhancer regions (Section 4) and activates only those genes, the TSS region of which are located within euchromatin.

The distribution of eu- and heterochromatin (including their specific epigenetic markers) of a given cell is referred to as its epigenetic landscape or epigenome [73]. The epigenome is determined by patterns of DNA methylation, post-translational modification of histone tails and 3-dimensional chromatin organization [74]. It depends on the activity of chromatin-modifying enzymes, such as DNA methyltransferases (DNMTs),

which add methyl groups to cytosines within genomic DNA, TET (Tet methylcytosine dioxygenase) proteins that initiate DNA methylation, or HATs, HDACs, lysine methyltransferases (KTMs) and KDMs, which add or remove acetyl and methyl groups to histones [75]. Histone acetylation is generally associated with transcriptional activation, but it is not important which exact amino acid is acetylated. In contrast, for histone methylation, the exact residue and its degree of methylation (mono-, di- or tri-methylation) is critical. Furthermore, the function of chromatin-remodeling enzymes is to shift or evict nucleosomes, in an ATP-dependent fashion. The projects ENCODE (www.encodeproject.org, accessed on 5 March 2022) [61] and Roadmap Epigenomics (www.roadmapepigenomics.org, accessed on 5 March 2022) [76] systematically assessed the epigenomes of more than 100 human cell lines, as well as primary cells, and serve as a reference for the epigenome of non-stimulated human tissues and cell types. However, in contrast to the static genome, the epigenome dynamically responds to intra- and extracellular signals, since chromatin modifying enzymes are often the endpoints of transduction cascades of peptide hormones, cytokines and growth factors [77]. Thus, the response of the epigenome to signals, such as $1,25(OH)_2D_3$, is even more important than its ground state.

Nuclear hormones, such as $1,25(OH)_2D_3$, affect the epigenome via direct interaction of their receptors with chromatin-modifying enzymes (Section 2), as well as through up- or down-regulating the genes encoding for chromatin modifiers. In this way:

1. Vitamin D affects histone markers for active chromatin, such as H3K27ac (acetylated histone H3 at lysine 27), and for TSS regions, such as H3K4me3 (tri-methylated histone H3 at lysine 4) [53,57,78];
2. VDR initiates the demethylation of its binding sites via interaction with TET2 [79];
3. The accessibility of thousands of VDR-binding enhancer and TSS regions is affected by $1,25(OH)_2D_3$ [80,81];
4. The binding of CTCF, to more than 1000 of its genomic sites, is modulated by $1,25(OH)_2D_3$ [82];
5. The organization of some 400 TADs is dependent on $1,25(OH)_2D_3$ [82], i.e., vitamin D affects the 3-dimensional chromatin structure.

Thus, there are multiple ways by which $1,25(OH)_2D_3$ modulates the epigenome of its target tissues. Interestingly, some $1,25(OH)_2D_3$-modulated chromatin loci, such as TSS regions, open only 2 h after ligand stimulation, while most sites take 24 h to reach maximal accessibility [78,80]. This suggests that many effects of vitamin D on the epigenome are secondary, i.e., they are mediated by genes and proteins that are primary vitamin D targets [83,84]. Nevertheless, the vitamin D-triggered effects on the epigenome facilitate the looping of VDR-bound enhancers, towards accessible TSS regions within the same TAD [85]. This assembly of enhancer and TSS regions enables the formation of a large protein complex, containing VDR, nuclear adaptor proteins, chromatin-modifying enzymes and RNA polymerase II, modulating gene transcription (Section 2) (Figure 1).

4. Genome-Wide Location of VDR

In human cellular systems, the VDR cistrome had been determined in B cells (GM10855 and GM10861) [86], T cells [87], macrophages (lipopolysaccharide-polarized THP-1) cells [44], peripheral blood dendritic cells [79], colorectal cancer cells (LS180) [88], prostate epithelial cells (RWPE1) [89], hepatic stellate cells (LX2) [90] and human kidney tissue [91]. However, the VDR cistrome was studied in most detail in monocytes (undifferentiated THP-1 cells) [46,92]. For comparison, the mouse VDR cistrome was obtained in pre-adipocytes (3T3-L1) [93], osteocytic cells (IDG-SW3) [94], pre-osteoblastic and differentiated osteoblastic cells (MC3T3-E1) [95], as well as in mouse intestine [96], mouse kidney [91] and bone-marrow-derived mesenchymal stem cells [56]. In the presence of $1,25(OH)_2D_3$, the VDR cistrome comprises 5000–20,000 genomic loci, which represents a 2- to 10-fold increase compared to respective unstimulated cells. However, this implies that the VDR cistrome contains a lower number of persistent loci that remain constantly occupied, while their occupancy significantly changes after stimulation with a ligand [46]. These persistent VDR

binding sites are the primary contact points of the human genome with $1,25(OH)_2D_3$ and are considered as "hotspots" for vitamin D signaling (Section 6). These sites coordinate the functional consequences of ligand stimulation over time, i.e., these sites best represent the spatio-temporal response of the (epi)genome to the extracellular changes in vitamin D levels. In addition, there are transient VDR-binding loci that modulate the response of the epigenome to vitamin D and support persistent VDR sites. Thus, the genome-wide, ligand-induced binding of VDR, to its preferred loci, is the most prominent of all the epigenome-wide effects of vitamin D.

5. Model of Vitamin D Signaling

The here-presented model of vitamin D signaling describes the sequential activation of vitamin D target genes [97]. Typically, protein-coding target genes of vitamin D are considered, but there are also some non-coding RNA genes that are known to be modulated by vitamin D [98–100]. VDR does not act as an isolated protein but functions in the context of a larger, dynamically composed protein complex that contains RXR, other possible co-receptors, pioneer factors, such as PU.1, CEBPα, GABPα, ETS1, RUNX2 and BACH2, co-factors, chromatin modifiers and chromatin remodelers (Figure 1).

1. The pioneer factors within the complex may take the first contact to enhancer regions. With the help of chromatin-remodeling proteins, they optimize the access of VDR to suitable binding motifs within the enhancer region, including the demethylation of genomic DNA.
2. Chromatin modifiers within the complex then leave marks, such as H3K27ac, to the local chromatin region.
3. Although VDR may not be the first protein of the complex making contact with the enhancer region, its specific activation by $1,25(OH)_2D_3$ drives the activity of the other members of the complex. This may explain the epigenetic effects of $1,25(OH)_2D_3$, such as chromatin opening, histone marks and the recruitment of pioneer factors.
4. When the complex is established on the enhancer region, DNA looping events to TSS regions within the same TAD region, which are complexed with a basal transcriptional machinery, become stabilized. Via $1,25(OH)_2D_3$-triggered effects on CTCF-dependent TAD anchor formation, this also affects the structure of the whole TAD.

In terms of net effect, the epigenome-modulating functions of vitamin D will increase or decrease the activity of RNA polymerase II, so that the mRNA expression of the respective gene(s) changes. On persistent VDR binding sites, i.e., enhancer with residual VDR binding, even in the absence of a ligand, the above-described multi-step process happens more efficiently, explaining why these loci are the primary sites of $1,25(OH)_2D_3$-dependent gene expression.

6. Vitamin D Target Genes

Vitamin D target genes are detected via a statistically significant change in their expression, within a given time frame (often 24 h), after ligand stimulation. Long-time known vitamin D target genes, such as *BGLAP* (bone gamma-carboxyglutamate protein, also called osteocalcin) [101] or *CAMP* (cathelicidin antimicrobial peptide) [102], were deduced based on the observation of the physiological effects of vitamin D, e.g., on calcium homeostasis or the defense against microbe infection, respectively. On the level of mRNA changes, vitamin D target genes were analyzed, initially, by single gene approaches, using northern blotting or quantitative PCR. After the completion of the human genome, microarrays became popular, which are able to detect the transcriptome-wide effects of vitamin D stimulation in one assay [103,104]. However, for some 10 years, RNA sequencing (RNA-seq) has been the method of choice for describing the vitamin D-dependent transcriptome [105]. Studies in a large number of cellular models led to a tremendous increase in the number of putative vitamin D target genes. For example, a microarray in THP-1 cells reported, in one experimental setting, 3372 significantly (false discovery rate (FDR) < 0.05) regulated genes [92], while in another microarray, in the same cellular model, 4532 genes passed the

statistical threshold [106]. Interestingly, 1227 genes were common and more than half of them (695) were confirmed by RNA-seq [46,80]. Thus, in a given cellular model, it is more likely that a few hundred genes respond to vitamin D than thousands of gene candidates. Interestingly, a meta-analysis of transcriptome-wide investigations of vitamin D target genes in 94 different human and mouse cell models resulted in only two common targets, *CYP24A1* (cytochrome P450 family 24 subfamily A member 1) and *CLMN* (calmin) [107]. Thus, vitamin D target genes are largely tissue specific.

Time course analysis of vitamin D target genes allows one to classify them into rapidly responding (4–8 h) "primary" target genes and delayed-reacting "secondary" targets (Figure 3A). Primary target genes are directly regulated by $1,25(OH)_2D_3$-activated VDR, as described in the model of vitamin D signaling (Section 5); i.e., these genes need to have, within the same TAD, a VDR-binding enhancer. In contrast, secondary target genes may be regulated by transcription factors, co-factors or chromatin modifiers, which are encoded by primary vitamin D target genes (Figure 3A). Suitable proteins encoded by primary target genes are the transcription factors BCL6 (B-cell CLL/lymphoma 6), NFE2 (nuclear factor, erythroid 2), POU4F2 (POU class 4 homeobox 2) and ELF4 (E74-like factor 4) in THP-1 cells [83], as well as IRF5 (interferon regulatory factor 5), MAFF (MAF BZIP transcription factor F), MYCL (MYCL proto-oncogene, BHLH transcription factor), NFXL1 (nuclear transcription factor, X-box binding-like 1) and TFEC (transcription factor EC), as well as the transcriptional co-regulators MAMLD1 (mastermind-like domain-containing 1), PPARGC1B (PPARG coactivator 1 beta), SRA1 (steroid receptor RNA activator 1) and ZBTB46 (zinc finger and BTB domain-containing 46) in human PBMCs (peripheral blood mononuclear cells) [108]. In this way, secondary target genes do not have to carry a VDR-binding enhancer within their TADs. For example, although the time course study in human PBMCs used a strict statistical approach (threshold testing applying a fold change (FC) > 1.5 and 2), 662 vitamin D-responding genes were identified (FDR < 0.05), 179 of which are primary and 483 secondary targets [108]. An alternative classification of the same set of genes suggests that 293 of them are direct and 369 indirect targets of vitamin D (Figure 3B). Irrespective of the timing of their response to $1,25(OH)_2D_3$, the expression change of direct targets is driven by VDR-bound enhancers, while indirect targets are primarily stabilized in their expression by $1,25(OH)_2D_3$ and its receptor, against an up- or down-regulation by other transcription factors and/or their epigenetic effects.

The model of vitamin D signaling (Section 5) illustrates the mechanisms of up-regulation of primary vitamin D target genes. However, the majority of vitamin D target genes are down-regulated, in particular, when cells are stimulated with $1,25(OH)_2D_3$ for 24 h or longer. The down-regulation of a gene by vitamin D is only possible when the gene is first up-regulated by other transcription factors or epigenetic effects, mediated by chromatin modifiers. The most, likely mechanism of down-regulation of a gene is to block one or several of its up-regulating factors. Accordingly, the majority of down-regulated target genes could be classified as indirect targets, i.e., vitamin D counteracts their up-regulation rather than prominently down-regulating their expression [108]. For example, VDR antagonizes pro-inflammatory transcription factors, such as NFAT, AP1 and NFκB, in immune cells [109]. However, since each gene is up-regulated by an individual set of transcription factors and chromatin modifiers, there are also individual mechanisms of its down-regulation. Thus, there is no general model for describing the mechanism of the down-regulation of vitamin D target genes.

The expression of the majority of vitamin D target genes is less than 5-fold, up- or down-regulated (after a stimulation for 24 h with $1,25(OH)_2D_3$); i.e., only a few genes respond with huge expression changes to vitamin D. For example, in THP-1 cells, the top five are the up-regulated genes *CYP24A1*, *CAMP*, *TSPAN18* (tetraspanin 18), *CD14* (CD14 molecule) and *FBP1* (fructose-bisphosphatase 1), with an FC of expression ranging from 47 to 402 [80]. In human PBMCs, *CYP24A1* and *CAMP* also have an FC of 409 and 158, respectively, the top up-regulated vitamin D target genes, but *STEAP4* (STEAP4 metalloreductase), *NRG1* (neuregulin 1) and *CXCL10* (C-X-C motif chemokine ligand 10) show

an FC of 471, 450 and 158, respectively, meaning comparable levels of down-regulation of their expression [108]. This prominent down-regulation of expression is more remarkable than the up-regulation, since it is far easier to increase a very low basal expression than a down-regulation of a highly expressed gene. Nevertheless, one should remember that these in vitro $1,25(OH)_2D_3$ experiments are designed for maximal effects and do not reflect the reality of the endocrinology of vitamin D in vivo [110,111].

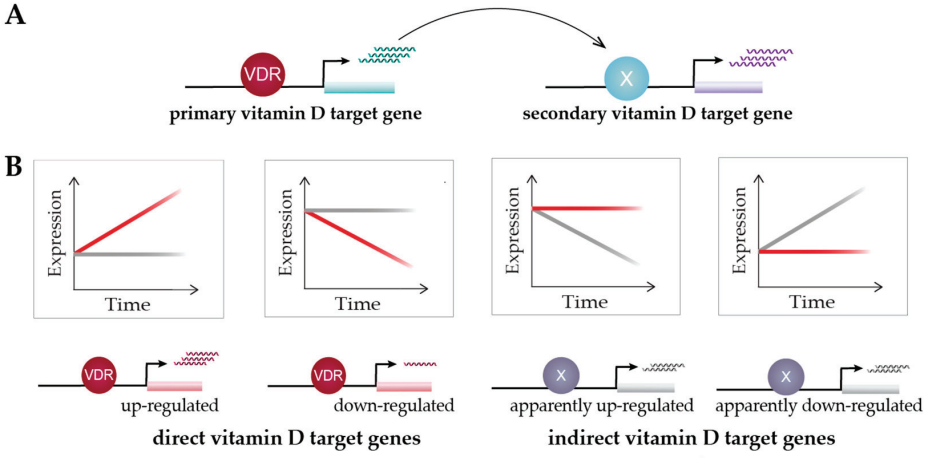

Figure 3. Classification of vitamin D target genes. Primary vitamin D target genes are directly regulated by VDR, while secondary vitamin D targets are controlled by transcriptional regulators that are encoded by primary targets (**A**). Time course analysis allows to differentiate vitamin D target genes in four different types based on cause and direction of expression change [108] (**B**). This suggests an alternative view on vitamin D signaling: $1,25(OH)_2D_3$ either directly induces or reduces the expression of its target genes via VDR or prevents their expression change mediated by other factors. Red and grey lines indicate gene's expression level in the presence or absence of $1,25(OH)_2D_3$, respectively.

7. Functional Profile of Vitamin D Target Genes

The most important thing to consider in the analysis of lists of hundreds of vitamin D target genes is the identification of the underlying biological processes. This is often evaluated by gene ontology analysis, where the list of target genes is assessed for statistically significant enrichment of a predefined list of terms, concerning (i) the molecular function, i.e., the molecular activity of a gene, (ii) the biological process, i.e., the cellular or physiological role carried out by a gene in the context of other genes, and (iii) the cellular component, i.e., the location where the gene's product functions in the cell. For example, in THP-1 cells, the biological processes "neutrophil activation", "inflammatory response", "neutrophil degranulation", "negative regulation of T cell proliferation" and "positive regulation of cytokine secretion", are most significantly associated with the list of 695 vitamin D target genes [84]. This fits with the known key functions of vitamin D in monocytes [112]. Since monocytes are the most vitamin D responsive cell fraction of PBMCs, gene ontology analysis of the 662 vitamin D target genes in this primary tissue suggests similar functions, such as neutrophil degranulation", "inflammatory response", "cytokine-mediated signaling pathway", "extracellular matrix organization" and "positive regulation of angiogenesis" [108]. For example, vitamin D down-regulates 10 of 12 *HLA* (human leukocyte antigen) class II genes and five *S100A* (S100 calcium-binding protein A) genes encoding for alarmins, as well as modulating the expression of six members of the *CXCL* gene family, encoding for chemokines [108]. The resulting vitamin D-triggered immune tolerance leads to the induction of regulatory T cells, which down-regulate the activity of other cells in the immune system [57,79]. This is the central mechanism for

how vitamin D dampens chronic inflammation and autoimmunity in diseases, such as inflammatory bowel disease [113] and multiple sclerosis [114].

VDR is expressed in many cell types (Section 1), but most of them differ majorly in their respective epigenetic landscape and, thus, expression of vitamin D target genes. Therefore, there are a large variety of biological processes being regulated by $1,25(OH)_2D_3$, i.e., in summary of all VDR-expressing tissues, vitamin D has rather ubiquitous functions.

8. Conclusions and Future View

Although vitamin D was discovered through its critical role in calcium homeostasis, being essential for proper bone formation, vitamin D signaling is studied most intensively, nowadays, in the immune system [115]. Therefore, the present molecular understanding of vitamin D signaling (Section 5) is mainly based on the integration of data obtained in immune cells [97].

The main challenge for future investigations of vitamin D target genes is their analysis in a human in vivo setting. The first studies involving a transcriptome- and epigenome-wide analysis, performed directly after isolation of PBMCs from vitamin D_3-supplemented human donors, have already begun [116–118]. These, and similar types of investigations, may provide us with a further enhanced understanding of the action of VDR and its target genes, in particular, in the context of immunity.

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Review

Vitamin D and Its Receptor from a Structural Perspective

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Abstract: The activities of 1α,25-dihydroxyvitamin D3, 1,25D_3, are mediated via its binding to the vitamin D receptor (VDR), a ligand-dependent transcription factor that belongs to the nuclear receptor superfamily. Numerous studies have demonstrated the important role of 1,25D_3 and VDR signaling in various biological processes and associated pathologies. A wealth of information about ligand recognition and mechanism of action by structural analysis of the VDR complexes is also available. The methods used in these structural studies were mainly X-ray crystallography complemented by NMR, cryo-electron microscopy and structural mass spectrometry. This review aims to provide an overview of the current knowledge of VDR structures and also to explore the recent progress in understanding the complex mechanism of action of 1,25D_3 from a structural perspective.

Keywords: vitamin D; vitamin D receptor; 3D structure; structural analysis; molecular recognition; protein–ligand interactions; coregulators

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1. Introduction

Upon sun exposure, the secosteroid prohormone vitamin D is two-step hydroxylated in the liver and the kidney into the biologically hormonal form, 1α,25-dihydroxyvitamin D3 (1,25D_3) [1]. Most effects of 1,25D_3 are mediated via its binding to the vitamin D receptor (VDR, also termed NR1I1), a ligand-dependent transcription factor (TF) that belongs to the nuclear receptor (NR) superfamily. VDR was cloned and sequenced in mammalian and avian species in the late 1980's [2–5]. VDR acts as a heterodimer with one of the three retinoid X receptor (RXR) isotypes (RXRα, NR2B1; RXRβ, NR2B2; and RXRγ, NR2B3). VDR and its ligand control calcium metabolism, cell growth, differentiation, anti-proliferation, apoptosis and adaptive/innate immune responses [6–8]. VDR is expressed in the different tissues of the body and many of these tissues were not originally considered target tissues for 1,25D_3. Conversion of 25D_3 to 1,25D_3 also occurs in many non-renal tissues and cells, including the skin, parathyroid glands, bone cells, both cardiovascular and immune cells, and many others [9,10]. The discovery of these autocrine/paracrine activities of 1,25D_3 has markedly increased our appreciation of the wide effects of 1,25D_3. An additional layer of complexity came with the identification of alternative vitamin D pathways and the major enzymes involved that produce other natural vitamin D metabolites, with some of them showing potent VDR activation [11,12]. Deregulation of VDR function may lead to severe diseases, such as cancers, psoriasis, rickets, renal osteodystrophy, and autoimmunity disorders (multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, type I diabetes) [13]. Despite the large number of potential applications, clinical use of the native hormone 1,25D_3 is limited by calcification of soft tissues (hypercalcemia). However, synthetic analogs have been developed and some of the highly active and non-calcemic VDR ligands have found clinical applications in the standard topical treatment of psoriasis, secondary hyperparathyroidism or osteoporosis [14,15].

In this review, we discuss the general mechanism of action of VDR and what we have learned from structural studies of VDR complexes from isolated domains to full length

proteins focusing on $1,25D_3$ action. Recent studies that aimed to describe how coregulators interact with VDR will also be discussed. These studies have significantly advanced our understanding on the molecular mechanism by which $1,25D_3$ mediates transcription regulation. However, important questions remain regarding the basic mechanism of the cell-specific action of ligands and possible cross-talks with other NRs and TFs. Finally, the ongoing effort to characterize the structures, dynamics and relationships with the function of large VDR coregulatory complexes will be discussed.

2. Structure and Mechanism of Activation of the Vitamin D Receptor

VDR shares two main features with other members of the NR superfamily, principal mechanism of action and structural organization. Simplified regulation of target gene expression by VDR can be presented as follows (Figure 1): VDR forms a heterodimer with RXR and binds to specific DNA sequences of controlled genes called VDR response element (VDRE). These VDREs are often localized thousands of base pairs from the coding region of the regulated gene [16,17]. Typically, in the absence of ligands or in the presence of antagonists, corepressors with histone deacetylase activity are recruited to VDR bound to their target genes, while binding of agonist ligands induces a change in the structure of the NR that allows interaction with coactivators [8,16]. Recruitment of coactivators with enzymatic activities, such as histone acetyl-transferases (HAT), prepares target gene promoters through decondensation of the chromatin. HATs can further be replaced by the mediator complex that provides a link with the basal transcriptional machinery.

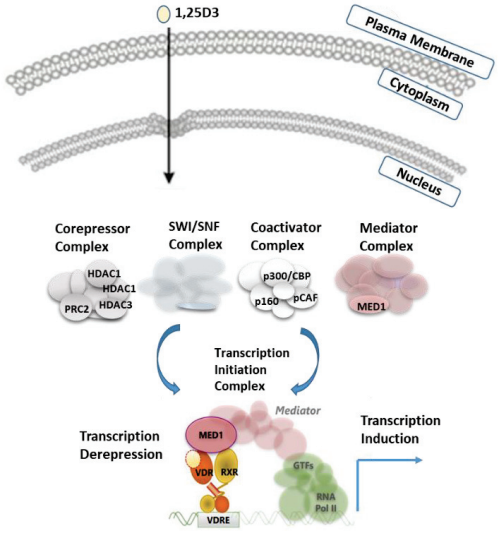

Figure 1. Schematic model for VDR regulation. Upon $1,25D_3$ binding to VDR, VDR translocates into the nucleus, binds as a heterodimer with RXR to DNA and interacts with various coregulators, leading to the activation of transcription or relief of constitutive repression.

The second feature shared by VDR and other NRs is its structural organization. VDR is a molecule of approximately 50–60 kDa, depending on species. The human VDR has two potential start sites with a common polymorphism (Fok 1) that alters the first ATG start site to ACG, leading to a VDR that is three amino acids shorter (424 AA vs. 427 AA), a polymorphism correlated with reduced bone density [18]. The VDR has a modular organization (Figure 2A) that consists of a short variable and flexible N-terminal domain, a highly conserved DNA-binding domain (DBD), a conserved ligand-binding domain (LBD) and a hinge region connecting the DBD to LBD. In addition, VDR has a unique feature among NRs, with its long insertion region within the LBD that is in a disordered state [19].

Since the first crystal structures of VDR LBD in 2000 [20] and of VDR DBD in 2002 [21] (Figure 2B,C), numerous studies have investigated the isolated domains of VDR, mainly by X-ray crystallography complemented by NMR and hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS).

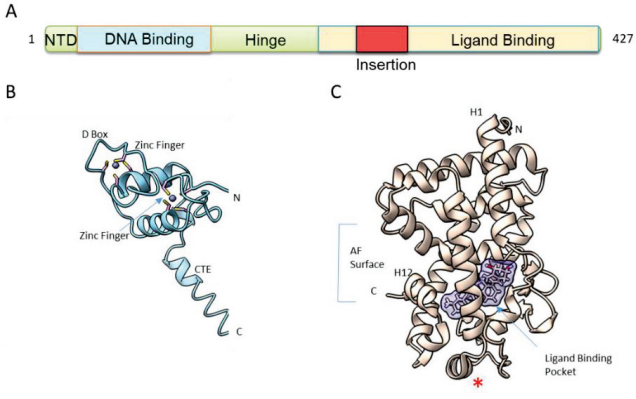

Figure 2. VDR structure. (**A**) Modular organization of VDR. NTD: N-terminal domain. DBD: DNA binding domain. LBD: ligand binding domain. (**B**) Overall structure of hVDR DBD monomer (PDB ID: 1KB4 [21]). Zn atoms are represented by spheres. CTE: C-terminal extension. (**C**) Overall structure of the hVDR LBD bound to $1,25D_3$ (PDB ID: 1DB1 [20]). LBP: ligand binding pocket. AF: activation function. The red star corresponds to the position of the truncation of the insertion domain.

3. DNA Binding

The DBD, the most conserved domain in VDR from different species and among the NRs, is comprised of two zinc fingers (Figure 2B). The first zinc finger is important for specific DNA binding to the VDREs, while the second one is involved in heterodimer interaction. Steric constraints of the VDR–RXR complex determine the optimal heterodimer binding site within VDRE as a direct repeat of the sequence RGKTSA (R = A or G, K = G or T, S = C or G), separated by three nucleotides (DR3) [22–24]. RXR binds to the upstream half site, while VDR binds to the downstream site. The crystal structure of the VDR DBD homodimer on DR3 [21] has revealed that the key interactions between the VDR DBD and the DNA, including four conserved residues in the recognition helix, Glu42, Lys45, Arg49 and Arg50, make sequence-specific base contacts in the major groove of the half-site. ChIP-seq studies in various cell types (see references [25–31] among others) have confirmed that the DR3 are the most enriched motifs upon ligand treatment but that represent only 10–20% of all VDR binding sites and most of VDREs spread over the whole genome [17]. A prerequisite for VDR DNA binding is the accessibility of the binding site through the action of pioneer factors and coactivators to open the chromatin and to modify chromatin topology [17,24,25].

4. Ligand Binding

NR is a highly dynamic scaffold protein and VDR LBD (Figure 2C) in a similar way to other NRs, is dynamic and only stabilized into a fixed conformation upon ligand binding. The dynamics of ligand binding process by VDR has been investigated by NMR [32] and HDX-MS [33–35]. These studies revealed that the entire C-terminal of VDR LBD in its apo state is very dynamic, with 80% of amide hydrogen exchange. The region forming the ligand binding pocket (LBP) (Figure 2C) also showed a high exchange rate, while the central layer of the α-helical sandwich appeared to be protected. Binding of $1,25D_3$ has been shown to lead to significant protection from hydrogen amide exchange, not only for the LBP but also in regions remote from the LBP.

Detailed information on the binding mode of $1,25D_3$ has been obtained by the elucidation of the crystal structure by X-ray crystallography of its complex with the human VDR LBD [20]. For the crystallization of the hVDR–$1,25D_3$ complex, a truncated form of the hVDR LBD was used that lacks the insertion domain (Figure 2A). This region is characterized by poor sequence conservation between VDR family members, is predicted to be disordered and does not play a major role in receptor selectivity for $1,25D_3$ [19,36]. The general fold of VDR LBD (PDB IDs: 1DB1 and 7QPP) consists of a three-layered α-helical sandwich composed of twelve helices (H1 to H12), three two-turn helices (H3n, H4n and Hx) and a three-stranded β-sheet (Figure 3A). The LBP is surrounded by helices H2, H3, H5, H6, H7, H10 an H12. The residues of each of β-sheet strands also form contacts with the ligand.

The ligand occupies 56% of the volume of the LBP (697 $Å^3$) with some water molecules near the position 2 of the A-ring of $1,25D_3$ (Figure 3B). The ligand adopts a chair B conformation with the 19-methylene "up" and the 1α-OH and 3β-OH groups in equatorial and axial orientations, respectively, while the aliphatic chain at position 17 of the D-ring adopts an extended conformation. The ligand is anchored in the LBP through three pair of hydrogen bonds formed between three hydroxyl groups of the ligand and polar residues: 1-OH group with Ser237 (H3) and Arg274 (H5), 3-OH group with Ser278 (H5) and Tyr143 (loop H1-H2) and 25-OH group with His305 (loop H6-H7) and His397 (H11) (Figure 3B). In addition, the ligand interacts with the hydrophobic residues lining the LBP (Figure 3C).

The LBD also contains the regions necessary for heterodimerization to RXR, comprising H9 and H10 and the loop 8–9, and for coactivator interaction through the activation function AF-2 formed by H3, H4 and H12. Helix 12 closed the LBP and is stabilized by two interactions with the ligand. Helix 12 is also stabilized by several hydrophobic contacts with residues of H3, H5 and H11 and two polar interactions with residues of H3 and H4. Some of these residues contact the ligand, thus indicating an additional indirect ligand-control of the position of helix H12.

The structures of $1,25D_3$ in the complex with VDR of other species were also described for *Rattus norvegicus* (rVDR) [37], *Danio rerio* (zebrafish, zVDRα) [38] and *Petromyzon marinus* (sea lamprey (l)) [39], the most basal vertebrate showing the most divergent VDR sequence. In the case of the rVDR LBD complex (PDB ID: 1RK3), the same truncation of the large insertion region connecting helices H1 to H3 as for hVDR was applied. For the zVDR (PDB ID: 2HC4) and lVDR (PDB ID: 7QPI), the wild-type LBD was used. The binding mode of $1,25D_3$ to VDR LBP is similar in all VDR structures, indicating a conserved ligand selectivity of VDRs across vertebrate species. While the differences are small between the structures of h, r and z VDR LBDs and primarily involve the loops, some significant differences are observed for lVDR around the linker regions between H11-H12 and H9-H10. These differences explain the weaker AF-2 stabilization and weaker RXR dimerization [39], and consequently the lower efficacy to activate lVDR compared to higher vertebrate VDRs [40,41]. Interpretation of the VDR structures and VDR–ligand interaction differences in a phylogenetic context allow for significant progress towards understanding the molecular activities. Indeed, structural and sequence co-evolution analysis allow to identify the conserved residues to maintain their functional integrity, and pairs of amino acids that coevolved to accommodate novel functionalities. Coevolving residues are located in H9 and in the insertion domain [39], accounting for the increased sensitivity to RXR and coregulators during evolution and leading to the increase in transactivation responses to $1,25D_3$ and VDR to be fully activated by $1,25D_3$ and to respond to lithocholic acid in higher vertebrates, facilitating novel functions.

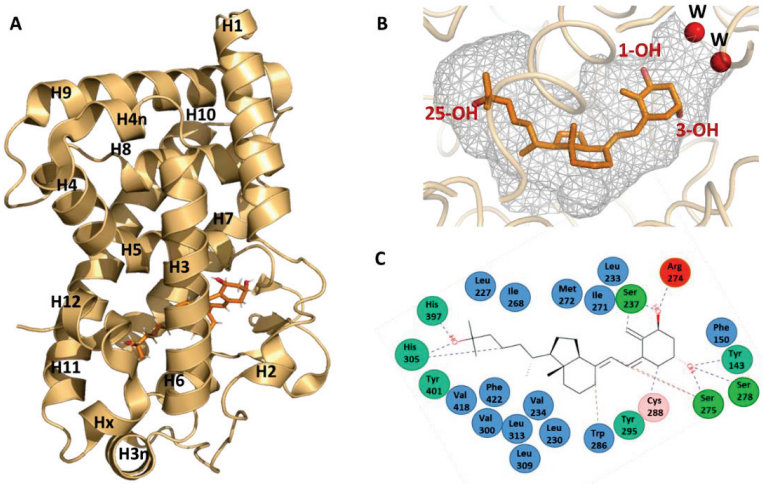

Figure 3. 1,25D_3 recognition by VDR. (**A**) Overall structure of the hVDR LBD. The hVDR LBD bound to 1,25D_3 is composed of 15 helices and 1 β-sheet (PDB ID: 1DB1 and 7QPP) [20,39]. (**B**) Conformation of 1,25D_3 in the VDR LBP shown as a grey surface. (**C**) Interaction map of 1,25D_3 in the LBP of hVDR.

This structural knowledge has been pivotal in the comprehension of VDR action and to understand the impact of mutations found in hereditary vitamin D-resistant rickets (HVDRR) patients showing a phenotype connected with softening and weakening of the bones [42,43]. The alterations in the VDR gene are caused by mutations that result in suboptimal gene regulatory responses, despite the presence of 1,25D_3 in the body. To date, 12 missense mutations have been identified in hVDR DBD, all associated with alopecia [43]. In the LBD, 24 amino acids of the hVDR were found mutated in HVDRR patients, some associated with alopecia [43]. The mutations result in alterations of VDR functions as ligand- or DNA-binding, heterodimerization with RXR or coregulator-binding. However, the overall effect of the mutation may result from the combination effects of all four functions at the same time. Among those mutations, the mutations involved residues forming hydrogen bonds with the hydroxyl groups of 1,25D_3, including Arg274Leu/His [44–46], His305Gln [47] and His397Pro [48], the last one being associated with very severe HVDRR. The discussion of the structural implications of these missense mutations were presented in the studies [49,50].

In addition, the crystal structures of VDR LBD have provided significant information for the characterization and the design of more specific analogs. A large part of the VDR LBP remains unoccupied when bound by 1,25D_3 (Figure 3B), providing additional space for the fitting of the modified moieties of the hormone and some analogs induce significant conformational changes, enlarging the original LBP (review in [15,51,52]). Not only seco-osteroidal analogs, but also other VDR ligands with non secosteroidal structures, such as lithocholic acid derivatives and mimics of 1,25D_3, have been reported. Hundreds of analogs in the complexes with VDR LBD from human, rat or zebrafish species were crystalized. The binding mode of some of these analogs to VDR LBP and their mechanism of action were discussed in [15,51,52]. In addition to the development of molecules targeting the VDR LBP and acting as agonist or antagonist, small molecules that target VDR–coregulator binding and act as protein-protein inhibitors are being developed [53]. Atomic-level understanding allows for the development of compounds with more original chemical structures and more specific action. However, due to the complexity of VDR signaling, these compounds remain largely unsuccessful in reaching therapeutic applications thus far.

5. Structure of Full-Length VDR Complex

Until now, only the VDR LBD monomer has been crystallized. The lower affinity of VDR for RXR compared to other NRs, such as PPAR or RAR [39,54], may explain the difficulty to crystallize the VDR–RXR LBDs heterodimer. DNA binding stabilizes the VDR heterodimer and $1,25D_3$ is required for high affinity binding and activation. On other hand, RXR ligand, 9-cis retinoic acid, has been shown to either inhibit [55] or activate [56,57] -$1,25D_3$ stimulation of gene transcription. In the absence of a full-length heterodimer crystal structure, solution methods using small angle X-ray scattering (SAXS) [58], cryo-electron microscopy (cryoEM) [59] and HDX-MS [60] have provided information on the full length VDR–RXR–DNA complex. These methods render it possible to visualize how the DBD and the LBD/heterodimerization domains are arranged relative to one another and how their binding to ligand, DNA, and coactivators influence one another.

The LBD and DBD domains in the full length structure is structurally conserved, compared to those of previously solved individual domain structures. The solution structures of VDR–RXR show that DBDs and LBDs are separated and positioned asymmetrically (Figure 4). The relative position of the domains and the observed asymmetry of the overall architecture both point to the essential role played by the hinge domains in establishing and maintaining the integrity of the functional structures. While the RXR hinge does not have a well-defined structure, the hinge domain of VDR forms an α-helix that stabilizes the whole complex, thus facilitating the positioning of the LBD and the surface to be accessible by the coregulators [58,59]. The 3D structures of several NRs truncated by their NTDs and in complex with DNA have now been obtained by X-ray crystallography or cryoEM for PPAR–RXR [61], HNF4 [62], LXR–RXR [63], RAR–RXR [64], EcR–USP [65] and AR [66]. These studies suggest that NRs are rather flexible macromolecules, adapting several conformations. In the VDR–RXR complex, even with the relatively separated positioning, there is evidence of long-range allosteric connections between the VDR LBD, DBD and DNA [60]. HDX-MS was used to understand the conformational plasticity and allosteric/dynamic communications in VDR complexes and has revealed cooperative effects between the VDR DBD and LBD to fine tune transcriptional regulation by the ligands and the DNA [67]. Upon $1,25D_3$ binding to full VDR–RXR, the differential HDX experiment has revealed a profile very similar to the binding to VDR LBD alone with a stabilization of VDR H12. Interestingly, an increase in solvent exchange in the DBD of VDR was also observed upon ligand binding, indicating that the ligand impact on the DBD conformation. Upon ligand binding, a stabilization of the heterodimer interface was also observed. The HDX profile of $1,25D_3$ binding in the presence of RXR ligand was similar to that of $1,25D_3$ alone. Allosteric communication is ligand dependent and bidirectional. DNA binding modulates both DBD–DBD and LBD–LBD interactions and influences coactivator recognition and binding. Conformational dynamics indicate that the binding of VDR–RXR to DNA results in significant alterations in the conformation of the LBD within the region important for interactions with coactivators, VDR H12 and RXR H3, and dimer interface H10–H11. Difference in DNA sequences modulate the receptor dynamics in remote regions, such as the coactivator binding surfaces [60,67]. A similar effect was observed for the RAR–RXR–DNA complex [68].

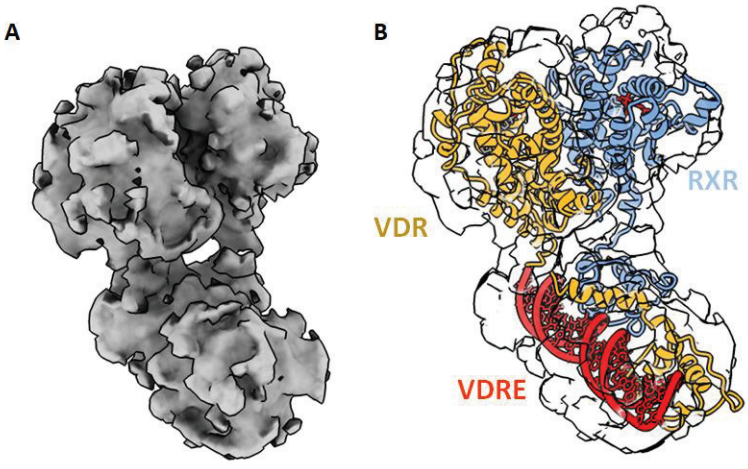

Figure 4. Cryo-EM structure of the VDR–RXR on DNA. (**A**) Electron density map shown as surface. (**B**) Fitted atomic model of the heterodimer in the EM map [59].

6. VDR–Coregulatory Complexes

The successful regulation of transcription by NRs requires the recruitment of coregulators to genomic loci, an event that directly affects the transcriptional rate. Hundreds of NR coregulators have been reported and include coactivators and corepressors [69]. For VDR, the mechanism of coactivation is now well understood, while VDR corepression is less studied. The major VDR coactivators are the NCoAs and mediator complexes [70,71]. The NCoAs (NCoA1, NCoA2 and NCoA3) recruit secondary coactivators CBP/p300 and p/CAF that have histone acetyl transferase activity and interact with VDR in a $1,25D_3$-dependent manner [71]. Another important VDR coactivator is MED1, a subunit of the mediator that links the TFs to the transcription machinery [72].

Most of the coactivators that can be recruited to the NRs in a ligand-dependent way contain the conserved LXXLL motif or NR box [73]. An analogous sequence motif (LXXH/IIXXXI/L) was identified in corepressors [74–76]. NR boxes are often located within intrinsically disordered regions of the coactivators and corepressors. NCoAs contain several domains separated by long disordered regions, the N-terminal part contains a highly conserved basic helix-loop-helix (HLH) and a signaling PAS (Per/Arnt/Sim) domain that mediates protein–protein interactions. The NRs interact with NCoAs via the receptor interaction domain (RID) that contains three LXXLL motifs. Each domain in NCoAs specializes in recruiting various TFs or other coregulators of transcription, including protein-modifying enzymes and chromatin remodelers. VDR has been shown to preferentially interact with the second and third LXXLL motifs of the NCoA RID [77]. MED1 contains two NR boxes in its central RID that differentially bind to NRs [78]. The second motif has been shown to preferentially bind to VDR [77]. These coregulators are not specific to VDR, but interact with a large number of other NRs and transcription factors. The mediator complex can be found in large numbers of loci and are known to be involved as super enhancers [79]. In addition, other domains of MED1 outside the RID has been shown to interact with NRs [80].

Multiple biochemical and structural studies have mapped the interaction regions of NRs with the coactivator NR box [73,81–83] and numerous X-ray structures of NR LBDs with bound coregulator LXXLL peptides are nowadays deposited in the PDB. Analogous recognition region in the corepressors, CoR-NR box, binds in the same hydrophobic groove on LBDs. Therefore, the recruitment of coactivators and corepressors is mutually exclusive. The intrinsically disordered properties of the coregulators that are important to adapt

and interact with many different transcription factors also limit their studies by X-ray crystallography or cryoEM.

MED1 NR2 as well as NCoA1 NR2, NCoA2 NR2 and NR3 were crystallized with liganded VDR LBD [37,39,84,85], revealing the binding mode of the LXXLL motifs to the VDR LBD. The LXXLL peptide formed a short a-helix that binds to a surface formed by helices H3, H4 and H12 of the LBD. The interaction of MED1 NR2 that buries about 507 $Å^2$ of the receptor's surface involves the three leucines that are buried within the pocket and surrounded by hydrophobic residues. The peptide is additionally locked through the formation of hydrogen bonds between conserved lysine residue in H3 and a glutamate residue in H12 that define a charge clamp.

Structures of coregulatory complexes with large fragment or full-length coactivators have been less studied due to their dynamic conformations. Several biophysical and solution structural studies in solution have been performed for some NRs bound to coactivator RIDs and recent developments in cryoEM have started to provide structural information on NR–coactivator complexes, including Erα-NcoA3-p300 [86] and AR-NcoA3-p300 [87]. However, NR–coregulator complexes structures have not reached atomic resolution yet, due to the conformational dynamics of the transcriptional complexes. For VDR, mass spectrometry structural methods combined with biophysical and structural methods have provided important details of VDR complex assembly and conformational plasticity and allosteric/dynamic communications within the complexes [60,67,88].

Differential HDX-MS has been used to study the interaction of VDR–RXR with the NCoA1 RID that contains the three LXXLL motifs [60,67]. As expected in the absence of both ligands, no coactivator interaction was observed and the separate addition of the $1,25D_3$ or RXR ligand, 9-cis retinoic acid, allow the coactivator to interact in a ligand-specific manner and independently. In the presence of $1,25D_3$, only RXR AF-2 within the full VDR–RXR complex was insensitive to the binding of NCoA1 RID, indicating that it is primarily associated to VDR AF-2. However, RXR and its ligand modulate the interaction when VDR is liganded and NCoA1 RID binding stabilizes not only VDR AF-2 but also RXR H3 and H10-H11 [60]. Helices 3 and 4 of VDR that are part of the coactivator binding cleft cannot be further stabilized, since they achieve maximal stabilization upon $1,25D_3$ binding. For RXR, the loop between helices 10 and 11 is important in the formation of the hydrophobic groove facilitating coactivator binding. In addition, DNA binding modulates the interaction of NCoA1 with the heterodimer. Mutations of each LXXLL motifs and luciferase transactivation assays suggested that the third motif was associated to VDR and the first one to RXR [60]. Mutations on residues of VDR and RXR involved in the coactivator charge clamp indicate that the coactivator binding surface of each receptor is important for NCoA1 RID (Figure 5A). These data are in contrast with the SAXS data showing that MED1 RID mainly interacts with RXR's heterodimeric partner [58]. Importantly, to fully understand the binding of MED1 to VDR–RXR, it was necessary to use a larger fragment of MED1 that not only contains the RID but also the N-terminal domain (50–660) [89]. Significant differences could be observed in comparison to the NCoA1 RID complex [89] by integrative structural methods combining SAXS, NMR and structural mass spectrometry. Differential HDX-MS and crosslink mass spectrometry confirmed that VDR interaction with MED1 motif NR2 is driving the complex formation but also demonstrated that other VDR–RXR regions outside the VDR AF-2, as well as MED1 regions other than RID, modulate the association and form an extended interaction surface (Figure 5B). Both LXXLL motifs of MED1 were perturbed upon formation of the complex with VDR–RXR, suggesting that while the NR1 box is not accommodated within the classical coactivator binding site, it could be either interacting with an alternative site of the receptors or stabilized allosterically. In addition to RID, the structured N-terminal domain of MED1 is also affected upon binding to VDR–RXR and is likely interacting with both VDR and RXR LBDs; in particular, the MED1 region 243–255 is largely stabilized in the complex with the receptor heterodimer. Crosslink MS also confirmed that this MED1 region is in physical proximity to the RXR. Among other novel MED1-interacting regions within the VDR–RXR heterodimer is the

flexible insertion domain in the VDR LBD located between H1 and H3. Conformational changes upon interaction with MED1 was observed by NMR. This effect was not observed in the HDX-MS experiment; however, the observed difference could be attributed to the different temporal resolution of the two methods.

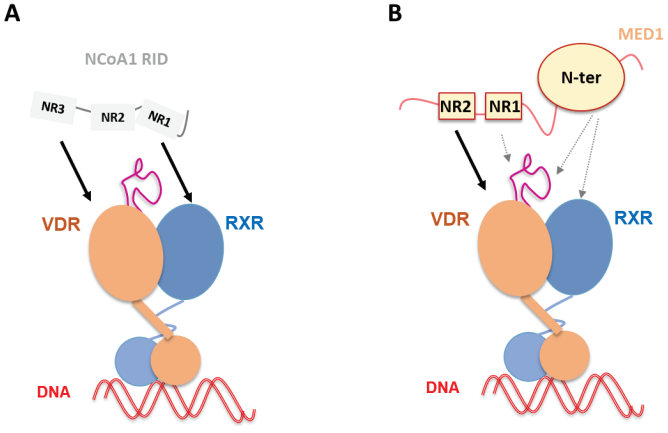

Figure 5. Schematic representation of NCoA1 and MED1 binding to liganded VDR–RXR–DNA complex. (**A**) NCoA1 RID binding to liganded VDR–RXR in complex with and without DNA [60]. The NCoA1 NR3 motif is associated to VDR and the NR1 to RXR. NCoA1 RID binding has been shown to stabilize not only VDR AF-2 but also RXR H3 and H10-H11. (**B**) MED1 (50–660) binding to liganded VDR–RXR–DNA complex [89]. Complex formation is primarily driven by strong ligand-dependent MED1 NR2 binding to the VDR AF-2, but other MED1 regions including NR1 and the structured N-terminal domain are involved in the interaction, as well as alternative sites of the receptors, including VDR insertion domain and RXR.

Differences in the binding modes could serve as molecular determinants of how the NRs discriminate between the coactivators. These studies were performed on large domains of coactivators but future studies should be carried out on the heterodimer complex with full length coactivators, as other domains, as shown for the N-ter domain of MED1, may modulate interactions directly or allosterically.

7. Conclusions and Perspectives

Our understanding of the molecular and structural insights for $1,25D_3$ action via its master regulator VDR has continuously advanced in the last twenty years, through the elucidation of the atomic structures of VDR DBD and LBD in complex with $1,25D_3$ and analogs. In addition, few structural studies on full length DNA bound VDR complexes have provided information on the allosteric effects driven by other domains and other effectors as DNA and coregulators. Important questions remain regarding the basic mechanisms of cell-specific action of ligands and possible cross-talks with other NRs and TFs. A deeper understanding of the interactions of VDR with specific coregulators will also be essential to better understand VDR action and may likely impact the future of drug development. However, full-length coregulators have large unstructured regions that remain a major hurdle for their structural characterization. CryoEM has started to provide structural information and will be essential to understand the structural dynamics of coregulatory complexes. The new structural biology tools, such as structural mass spectrometry, single molecule cryoEM with new processing tools and 3D classification, and cryo-tomography, will ultimately provide details on the structures of VDR complexes with coregulator complexes in a physiological environment.

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Conflicts of Interest: The author declares no conflict of interest.

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Review

The Centennial Collection of VDR Ligands: Metabolites, Analogs, Hybrids and Non-Secosteroidal Ligands

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Abstract: Since the discovery of vitamin D a century ago, a great number of metabolites, analogs, hybrids and nonsteroidal VDR ligands have been developed. An enormous effort has been made to synthesize compounds which present beneficial properties while attaining lower calcium serum levels than calcitriol. This structural review covers VDR ligands published to date.

Keywords: metabolites; analogs; hybrids and VDR nonsecosteroidal ligands

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1. Introduction

Since the chemical structure of vitamin D_3 (**1**, Figure 1 [1–36], cholecalciferol) was established in 1932, successive studies have shown it to be essential in physiological processes. Two hydroxylations of **1** are necessary before attaining its most biologically active form. The first is a 25-hydroxylation, which occurs mainly in the liver and produces the most abundant circulating metabolite, 25-hydroxyvitamin D_3 (**11**, Figure 1, 25-hydroxycholecalciferol, calcidiol, $25OHD_3$) [12]. Subsequently, a second hydroxylation at the 1α position generates the vitamin D hormone, 1α,25-dihydroxyvitamin D_3 (**13**, Figure 1, 1α,25-dihydroxycholecalciferol, calcitriol, $1,25(OH)_2D_3$) [14]. This is a pleiotropic hormone that exerts genomic actions by binding to its specific receptor (the vitamin D receptor, VDR), which is present on target cells and found in more than 200 different tissues.

The biological role of $1,25(OH)_2D_3$ has been related to calcium and phosphorus homeostasis. However, the effects of vitamin D are not limited to mineral homeostasis, skeletal health maintenance, or immune modulation. In addition, this hormone also has fundamental effects on cellular proliferation and differentiation, regulating genes involved in the cell cycle and apoptosis both in normal and tumor cells. These properties and its wide distribution have led to the study of its effects on various pathologies, such as osteoporosis and cancer, thus arousing interest in the field of health and the pharmaceutical industry. Unfortunately, the therapeutic use of $1,25(OH)_2D_3$ also leads to an increase in the concentration of calcium in blood (hypercalcemia), which can cause significant side effects. Therefore, numerous attempts have been made to synthesize noncalcemic analogs of $1,25(OH)_2D_3$ for use in health treatment.

In recent decades, structure–function relationships (SARs) have been determined to support the chemical modifications of the secosteroid structure of $1,25(OH)_2D_3$. The novel structures' goal is to reduce their calcemic activity in comparison with calcitriol while exerting their interesting biological properties. A huge synthesis effort has been carried out, yielding interesting chemical reviews in this regard [2]. The current review updates the scientific information on the structural library of VDR ligands and incorporates nonsteroidal VDR ligands.

2. Materials and Methods

All compounds contained in this review were collected from published papers and patents. Most of the materials were freely accessible via the Internet, and paper copies

were available in other cases. After careful reading, relevant structures were drawn using CHEMDRAW software [3]. No database was generated. A structural analysis of this collection may require future elaboration of a database.

3. Results

We found 1778 VDR compounds, which are displayed chronologically in 31 figures. All of these compounds are ligands that specifically bind to their VDR receptor. This binding allows the interaction of the 1,25$(OH)_2D_3$-VDR complex with target genes in the cell nucleus, modulating their expression and mediating a biological response. The following color scheme was used in the figures: dark blue corresponds to marketed compounds (Figures 1, 3, 4, 8 and 9), light violet to outstanding compounds with interesting properties (Figures 2, 4–10, 12–15, 17–21 and 23–31), and dark green to non-secosteroidal VDR ligands (Figures 9, 11, 12, 15 and 20–27).

Vitamin D is closely associated with calcium and phosphorus homeostasis. No scientific rational has yet been found for the calcemic properties of a compound in comparison with calcitriol. Therefore, structure–function relationships (SARs) were carried out in order to validate the key modifications in the structure of 1,25$(OH)_2D_3$ that may alter biological and calcemic properties. After more than 50 years of study, some hints have been obtained. For example, it is known that C-19 methylene deletion yields low calcemic analogs; it is also known that deletion/substitution of the steroidal cycles de-A ring, de-C ring, and/or de-D ring may yield low calcemic analogs. Lowering the calcemic side effects of the vitamin D analogs is important; however, we must not lose sight of other modifications that may increase the antiproliferative and prodifferentiation activity (side-chain modification with extra double and or triple bonds) as well as increase the metabolic stability (fluorine atom incorporation). In summary, the following main structural topics are covered in the current review:

- C-21 Methyl epimerization;
- C-19 Methylene deletion;
- Incorporation of fluorine atoms;
- Deletion/substitution of steroidal cycles: de-A ring, de-C ring, and/or de-D ring;
- C-2 Functionalization;
- C-3 Epimerization;
- Side-chain modification with extra double and/or triple bonds, heteroatoms, and/or branched hydrocarbons.

What is novel in this collection is the incorporation of non-secosteroidal VDR ligands (dark green). In 1999, Boehm [4] hypothesized that "non-secosteroidal VDR ligands might display different profiles of activity and metabolism than do secosteroidal l,25$(OH)_2D_3$, analogs, including less calcemic properties, which might render them attractive as both topical and oral pharmaceuticals for treating a variety of diseases. This hypothesis was based in part on the success that nonsteroidal androgen receptor (AR) and estrogen receptor (ER) modulators have had as drugs. Nonsteroidal compounds have been synthesized that modulate the activity of these receptors and show enhanced tissue selectivity in comparison to the steroids".

Figure 1 (1931–1978) [1–36]. Vitamin D_3 (**1**, cholecalciferol) [1] was discovered in 1922, but it was not chemically characterized until 1931. Dihydrotachysterol$_2$ (**5**) [10] was introduced in 1934, and it is still on the market as an antitetanic agent AT-10. In 1968, the most abundant metabolite of vitamin D_3 was discovered as 25-hydroxyvitamin D_3 (**11**, 25-hydroxycholecalciferol) [18], and in 1971, 1α,25-dihydroxyvitamin D_3 **13**, 1α,25-dihydroxycholecalciferol, calcitriol, 1α,25$(OH)_2D_3$ [21], the vitamin D_3 hormone, was identified. Later, 1α-hydroxyvitamin D_3 (**21**, Alfacalcidiol) [25], a synthetic analog, was marketed for the treatment of secondary hyperparathyroidism (2HPT), renal failure, and osteoporosis.

Figure 2 (1978–1982) [37–52]. 25-Hydroxyvitamin D_3 26(23)-lactones (**58**–**61**) were discovered in 1980 [50–52], and they behave as antagonists of gene transcription induced by VDR. They were the first compounds discovered to have antagonist properties.

Figure 3 (1982–1987) [53–78]. 26,26,26,27,27,27-Hexafluoro-1α,25-dihydroxyvitamin D_3 (**70**, Falecalcitriol) [60] is used in the treatment of 2HPT and osteoporosis. 1α,25-Dihydroxy-22-oxavitamin D_3 (**100**, Maxacalcitol) [76] is used in 2HPT and psoriasis.

Figure 4 (1987–1991) [78–94]. **111** (Calcipotriol, MC903) [79] is marketed as a treatment with exceptional clinical response in psoriasis. 1α,25-Dihydroxy-22(23)-didehydrovitamin D_3 (**116**) [83] has shown potent antiproliferative activity. 2β-(Hydroxypropoxy)-1α,25-dihydroxyvitamin D_3 (**131,** ED-71) [85] is used in osteoporosis treatment.

Figure 5 (1991–1992) [95–117]. Compound **186** [107] is an important analog functionalized at C-11 that may allow the synthesis of haptens, without disturbing the VDR ligand anchoring groups (1α-OH, 3β-OH and 25-OH).

Figure 6 (1993–1994) [118–136]. Compounds **225** [93] and **208** [108] were independently developed by different research groups and are important analogs functionalized at C-18 and C-11, respectively. They may allow the synthesis of haptens without disturbing the VDR ligand anchoring groups.

Figure 7 (1994–1997) [136–148]. Compounds **308** and **309** [147] present an interesting property by exhibiting only nongenomic rapid effects at physiological concentrations. Moreover, 1α-hydroxyl group addition (**309**) does not alter the sensitivity of nongenomic effects of **308**.

Figure 8 (1997–1999) [149–158]. 1α-Hydroxyvitamin D_2 (**325**, Doxercalciferol) [151] is marketed as a 2HPT treatment. (22*E*,24*E*)-Diene-24,26,27-trishomo-19-nor-1α,25-dihydroxyvitamin D_3 (**348**, Ro 25-8584) [152] represents an outstanding compound inhibiting the proliferation in myeloid leukemia cell lines. When 2-methylene-19-nor-1α,25-dihydroxyvitamin D_3 (**349**, 2MD) [156] is given as oral therapy, it is at least 100 times more potent than 1α,25$(OH)_2D_3$ in stimulating bone mass increase. A randomized clinical trial showed that **349** increased bone turnover but not BMD (bone mass density) in postmenopausal woman with osteopenia.

Figure 9 (1999) [158–168]. 24*R*,25-Dihydroxyvitamin D_3 (**388**, Tacalcitol) [160] is prescribed for psoriasis. 24,26,27-Trishomo-1α,25-dihydroxyvitamin D_3 (**406**, Seocalcitol, EB 1089) [163] acts as a powerful antiproliferative used in breast, colon, or pancreas tumor models.

Figure 10 (2000–2001) [169–182]. 1α-Hydroxy-26(27)-dehydro-25-(butylcarboxylate)-vitamin D_3 (**433**, ZK159222) and 1α-hydroxy-26(27)-dehydro-25-(ethylpropenoate)-vitamin D_3 (**434**, ZK168281) [170] have been identified as VDR antagonists, though **434** is more potent than **433**. Both compounds selectively stabilize an antagonist conformation of the VDR-LBD (ligand-binding domain). 1α,25-Dihydroxy-21-(3-hydroxy-3-methylbutyl)-vitamin D_3 (**435**, Gemini) [171] has emerged as the lead compound with superior gene transcription activity and tumor-cell-line inhibition.

Figure 11 (2001–2002) [183–196]. 1α,25-$(OH)_2$-16-ene-20-epi-23-yne-3-epi-D_3 (**493**), 1α,25$(OH)_2$-16-ene-23-yne-hexafluoro-3-epi-D_3 (**494**), and 1α,25$(OH)_2$-16-ene-3-epi-D_3 (**495**) are potent inducers of apoptosis of HL-60 cells. Their 3-natural (3β-OH) analogs have been shown to be potent modulators of HL-60 cell growth and differentiation [184]. This is the first report to demonstrate that the epimerization of the hydroxyl group at C-3 of the A-ring of 1α,25$(OH)_2D_3$ plays an important modulation role for HL-60 cell differentiation and apoptosis. 2,2-Difluoro-1α,25-dihydroxyvitamin D_3 (**507**) [185] is similar to 1,25$(OH)_2D_3$ in terms of in vitro antiproliferative activity, but it is different in terms of transcriptional activity. In addition, **507** is 2–3 times more transcriptionally active than calcitriol, with similar in vivo calcemic activity. 2,2-Dimethyl-1α,25-dihydroxy-19-norvitamin D_3 (**509**) [186] is 7.5 times less transcriptionally active than calcitriol and considerably less calcemic. Moreover, **509** strongly suppresses parathyroid hormone (PTH) secretion.

Figure 12 (2002) [197–204]. Seco-C-9,11-bisnor-17-methyl-26,26,26,27,27,27-hexafluoro-20-epi-1α,25-dihydroxyvitamin D_3 (**533**, WY1112) [197] and seco-C-9,11,21-trisnor-17-methyl-23(24)-didehydro-26,26,26,27,27,27-hexafluoro-1α,25-dihydroxyvitamin D_3 (**559**, CD578) [198] display high differentiation ratios between antiproliferative and calcemic affects.

26,27-Bishomo-1α-fluoro,25-hydroxy-23-en-vitamin D_3 (**582**, Ro-26-9228) [203] is used for treatment of osteoporosis.

Figure 13 (2003–2004) [205–218]. Dienyne **646** [215] represents the first locked side-chain analog of calcitriol with remarkable VDR transcriptional activity. Lactone **657** [217] showed one order of magnitude higher antagonist activity than lactone **66** (Figure 2).

Figure 14 (2004–2006) [218–223]. Further development in double side-chain vitamin D analogs, the Gemini series, made it possible to assess the steric VDR requirements of drug candidates. Compounds **684–695 [220]** present two different side chains at C-20 that improve their toxicity profiles and pharmacokinetic drug performance.

Figure 15 (2006–2007) [224–240]. C-20 cyclopropyl vitamin D_3 analog **755** [233] showed high MLR (mixed lymphocyte reaction) activity for the suppression of interferon-γ release with no calcemic activity. Immunomodulatory activity was measured by suppression of interferon-γ release in mixed lymphocyte reaction cells. The inhibition of clonal proliferation was evaluated in the leukemia HL-60, breast cancer MCF-7, prostate PC-3, and LNCaP cell lines. Significant separation of the immunomodulatory activity from hypercalcemic effects (MTD, maximum tolerated dose) was observed. Compound **747** was 2900 times more active and 100 times less hypercalcemic than 1α,25$(OH)_2D_3$, while **755** was 29 times more active and 100 less hypercalcemic. In the breast cancer MCF-7 cell line, compounds **753**, **754**, **755**, and **757** were ten thousand times more active but equally or less hypercalcemic than 1α,25$(OH)_2D_3$. Metabolism of 16-ene-20-cyclopropyl compounds is arrested at the 24-keto stage, which explains the increased biological activity of the 16-ene variants.

Figure 16 (2006–2008) [241–253]. Intensive research activity was carried out on the leading structures with outstanding biological properties, i.e., Gemini compounds **799–803** [246,247]. These studies focused on the structural modifications of Gemini that influenced the differentiation-inducing, antiproliferative, and transcriptional activity of the compounds in human leukemia cells. The cyclopropyl modification at the pro-*R* side chain decreased the activity of the compound compared to 1α,25$(OH)_2D_3$, and further A-ring modifications did not restore this activity. Cyclopropyl modification at the pro-*S* side chain of Gemini increased the VDR-induced transcriptional activity. In addition, privileged VDR antagonists lactones **804–832** and **833–864** [243,244] were studied. The antagonistic activity was markedly affected by the structure of the lactone ring, including length of the alkyl chain and the stereochemistries on the C23 and C24 positions. The VDR binding affinity of the (23*S*,24*S*)-24-alkylated vitamin D_3 lactones increased 2.3–3.7-fold as compared to the unsubstituted lactones **64**–**67** (Figure 2). The antagonistic activity of (23*S*,24*S*)-isomers were enhanced to be 2.2-,3.5-, 1.8-, and 1.7-fold higher compared to the unsubstituted lactones **64**–**67** (Figure 2).

Figure 17 (2008–2009) [254–264]. 2-Methylene-19-nor-(20*S*)-1α-hydroxy-bishomopregnacalciferol **942** [20(*S*)-2MbisP] [263] were able to suppress PTH at levels that did not stimulate bone resorption, intestinal calcium, or phosphate absorption and may have potential for use in the treatment of 2HPT in chronic kidney disease.

Figure 18 (2009–2010) [265–270]. Hybrid compounds **1020** (26,27-bis-nor-25-bishomo-19-nor-25′-oxo-25″-methylcarboxamide-1α-hydroxyvitamin D_3) and **1022** (26,27-bis-nor-25-homo-19-nor-25′-(2aminophenyl)-carboxamide-1α-hydroxyvitamin D_3) [270] showed antiproliferative activity against AT84 carcinoma cells; neither of them induced hypercalcemia even at concentrations 100-fold higher than those tolerated for 1,25D. This demonstrates that it is possible to create a wide range of bifunctional molecules that possess VDR agonism and HDACi (histone deacetylases inhibitor) activity. Structural latitude is significant with a wide variety of ZBGs (zinc-binding group) amenable to incorporation into the side chain of vitamin D-like secosteroids. Importantly, several of these molecules function as antiproliferative agents against AT84 cells in vitro, while possessing minimal hypercalcemic activity in vivo.

Figure 19 (2009–2010) [271–283]. Intensive research activity was carried out on Gemini compounds **1053**–**1069** [273]. Calcitriol was implicated in many cellular functions including cell growth and differentiation. It was shown that Gemini compounds were active in gene

transcription induction with enhanced antitumor activity. Fine tuning of their structurally derived biological properties would be required for therapeutic use.

Figure 20 (2010–2012) [284–298]. 25-Diethylphosphite-1α-hydroxy-23(24)-didehydrovitamin D_3 **1131** [290] was tested for antiproliferative effects on several human and murine tumor cell lines: human squamous cell carcinoma HN12, human glioma T98G, and Kaposi sarcoma SVEC vGPCR cell lines. Furthermore, in human glioma T98G and human squamous cell carcinoma HN12 cell lines, the antiproliferative effects exerted by compound **1131** were greater than those elicited by 1α,25$(OH)_2D_3$. Visual observation of internal animal organs such as liver, duodenum, lungs, and kidneys showed no macroscopic morphological alterations after treatment with this compound. This compound appears to be well tolerated even at high doses. Altogether, these results suggest that compound **1131** exerts considerable antiproliferative activity at nonhypercalcemic dosages and may have therapeutic potential for the treatment of various hyperproliferative disorders. Non-secosteroidal VDR ligand (4-{1-ethyl-1-[4-(2-hydroxy-3,3-dimethyl-butoxy)-3-methyl-phenyl]-propyl}-2-methyl-phenoxy)-hydroxyacetamide **1173** [295] was confirmed to significantly prevent bone loss after daily treatment without inducing hypercalcemia. These types of compound are potent inhibitors of the Hh (Hedgehog) signaling pathway. Studies show that, contrary to secosteroidal hybrids, the optimal location for incorporating the highly hydrophilic hydroxamic acid corresponds to the portion of the molecules that serve as secosteroidal A-ring mimetics. The best hybrid, **1173**, is a full VDR agonist, as assessed by several criteria, and an efficacious antiproliferative agent against both 1,25D-sensitive (SCC25, AT84) and 1α,25$(OH)_2D_3$-resistant (SCC4) squamous carcinoma cell lines. Importantly, the activity in 1α,25$(OH)_2D_3$-resistant SCC4 cells required both the VDR agonism and HDACi activity of **1173**. This study revealed the remarkable flexibility in the conversion of calcitriol analogs into fully integrated bifunctional molecules, suggesting that it may be possible to extend fully integrated bifunctionalization to other pharmacophores.

Figure 21 (2012–2013) [298–313]. 24*S*-Methyl-21-epi-2-methylene-22-oxa-1α,25-dihydroxyvitamin D_3 (**1191**, VS-105) [306] bound to VDR is highly inductive of functional responses in vitro and effectively suppresses PTH in a dose range that does not affect serum calcium in 5/6 NX uremic rats. [6-(4-{1-Ethyl-1-[4-((*E*)-3-ethyl-3-hydroxy-1-pentenyl)-3-methylphenyl]propyl}-2-methylphenyl)pyridin-3-yl]acetic acid (**1218**) [308] showed excellent ability to prevent BMD loss in mature rats in an osteoporosis model, without severe hypercalcemia and with good PK profiling.

Figure 22 (2013–2014) [313–316]. Compounds 1**247**–**1301** (non-secosteroidal VDR ligands) [315] were analyzed and presented better therapeutic efficacy when compared to 1α,25$(OH)_2D_3$ in experimental models of cancer and osteoporosis with less induction of hypercalcemia, a major potential adverse effect in the clinical application of VDR ligands. Compounds **1302**–**1313** [316] were analyzed for their binding affinity and inhibitory activity against CYP24A1 (24-hydroxylase; this mitochondrial protein initiates the degradation of 1α,25$(OH)_2D_3$ by hydroxylation of the side chain), and the imidazole styrylbenzamides **1305**–**1309** were identified as potent inhibitors of CYP24A1, with similar or greater CYP27B1 (1α-hydroxylase; the protein encoded by this gene it hydroxylates 25OHD_3 at the 1α-position, producing 1α,25$(OH)_2D_3$) selectivity than standard ketoconazole. Further evaluation of the 3,5-dimethoxy (**1308**) and 3,4,5-trimethoxy derivatives (**1309**) in chronic lymphocytic leukemia cells revealed that cotreatment of 1α,25-dihydroxyvitamin D_3 and inhibitor upregulated GADD45α (growth arrest and DNA damage 45 gen) and CDKN1A (cyclin-dependent kinase inhibitor 1A gen).

Figure 23 (2014) [317–321]. Intensive research activity was carried out on Gemini compounds **1338**–**1364** [320].

Figure 24 (2014–2015) [322–336]. 1α,20*S*,24*R*-Trihydroxyvitamin D_3 (**1410**) [332] showed a higher degree of activation, anti-inflammatory activity, and antiproliferative activity than vitamin D_3 receptor.

Figure 25 (2015–2017) [337–351]. 1α,25-Dihydroxy-21-(3-hydroxy-3-methyl-1-methylenebutyl)vitamin D_3 (**1428**, UV1) [337] presented potent antitumoral effects over a wide panel

of tumor cell lines without inducing hypercalcemia or toxicity in vivo. The first vitamin D analog carrying an *o*-carborane in the side chain **1436** [340] showed that the substitution of hydroxyl group at C-25 by this apolar bulky group was possible. VDR binding was half of calcitriol's, the transcriptional activity was similar, and the calcemic induction was significantly lower. **1436** is an outstanding B-carrier containing 10 boron atoms, which notably bind to VDR, a nuclear receptor. This suggests that **1436** may be interesting as a BNCT (boron neutron capture therapy) drug.

Figure 26 (2017–2018) [351–358]. 1,1′-([4-(3-[4-(3-Hydroxypropoxy)-3-methylphenyl] pentan-3-yl)-1,2-phenylene]bis(oxy))bis(3,3-dimethylbutan-2-ol) (**1503**) [358] displayed efficient inhibitory activity against collagen deposition and fibrotic gene expression in chronic pancreatitis. It also showed physicochemical and pharmacokinetic properties with antitumor activity, highlighting its potential therapeutic applications in cancer treatment.

Figure 27 (2018) [359–364]. (1*R*,3*S*,*Z*)-5-{(*E*)-3-[3-(6-Hydroxy-6-methylheptyl)phenyl] pent-2-en-1-ylidene}-4-methylenecyclohexane-1,3-diol (**1573**) [359] exhibited significant tumor growth inhibition and increased survival in SCID mouse models implanted with MDA-MB-231 breast tumor cells. Des-C-ring aromatic D-ring analog **1587** [363] showed remarkable lack of calcemic activity together with its significant antiproliferative and transcriptional activities in breast cancer cell lines, suggesting the therapeutical potential of **1587** for the treatment of breast tumors.

Figure 28 (2018–2019) [365–378]. 21-nor-17(*S*)-Methyl-20(22),23(24)-didehydro-26,26, 26,27,27,27-hexafluoro-1α,25-dihydroxyvitamin D_3 (**1600**) [368] bound strongly to VDR ligand binding domain and induced VDR transcriptional activity. Hybrid **1619** [371] was found to be a potent inhibitor of Hh (Hedgehog) signaling pathway.

Figure 29 (2019–2020) [379–383]. It is known that 25(OH)D3, down-regulates SREBP (sterol regulatory element-binding protein) independently of VDR. A screening of over 250 vitamin D congeners was carried out for their ability to inhibit the activity of an SREBP-responsive luciferase reporter. This is a VDR-responsive reporter assay. A comparison of the relative activity of the six compounds revealed **1639 [379]** as the VDR-selective activator.

Figure 30 (2020–2022) [384–389]. Des-C-ring aromatic D-ring analogs **1712** and **1713** [373] showed a remarkable lack of calcemic activity together with significant antiproliferative and transcriptional properties in breast cancer cell lines, suggesting a therapeutical potential for **1712** and **1713** in breast tumor treatment.

Figure 31 (2021–2022) [390–392]. KK-052 (**1746**) [391], was found to be the first vitamin D-based SREBP (sterol regulatory element-binding proteins) inhibitor that mitigates hepatic lipid accumulation without calcemic action in mice. KK-052 maintained the ability of 25-hydroxyvitamin D_3 to induce the degradation of SREBP but lacked VDR-mediated activity. KK-052 serves as a valuable compound for interrogating SREBP/SCAP in vivo and may represent an unprecedented translational opportunity for synthetic vitamin D analogs.

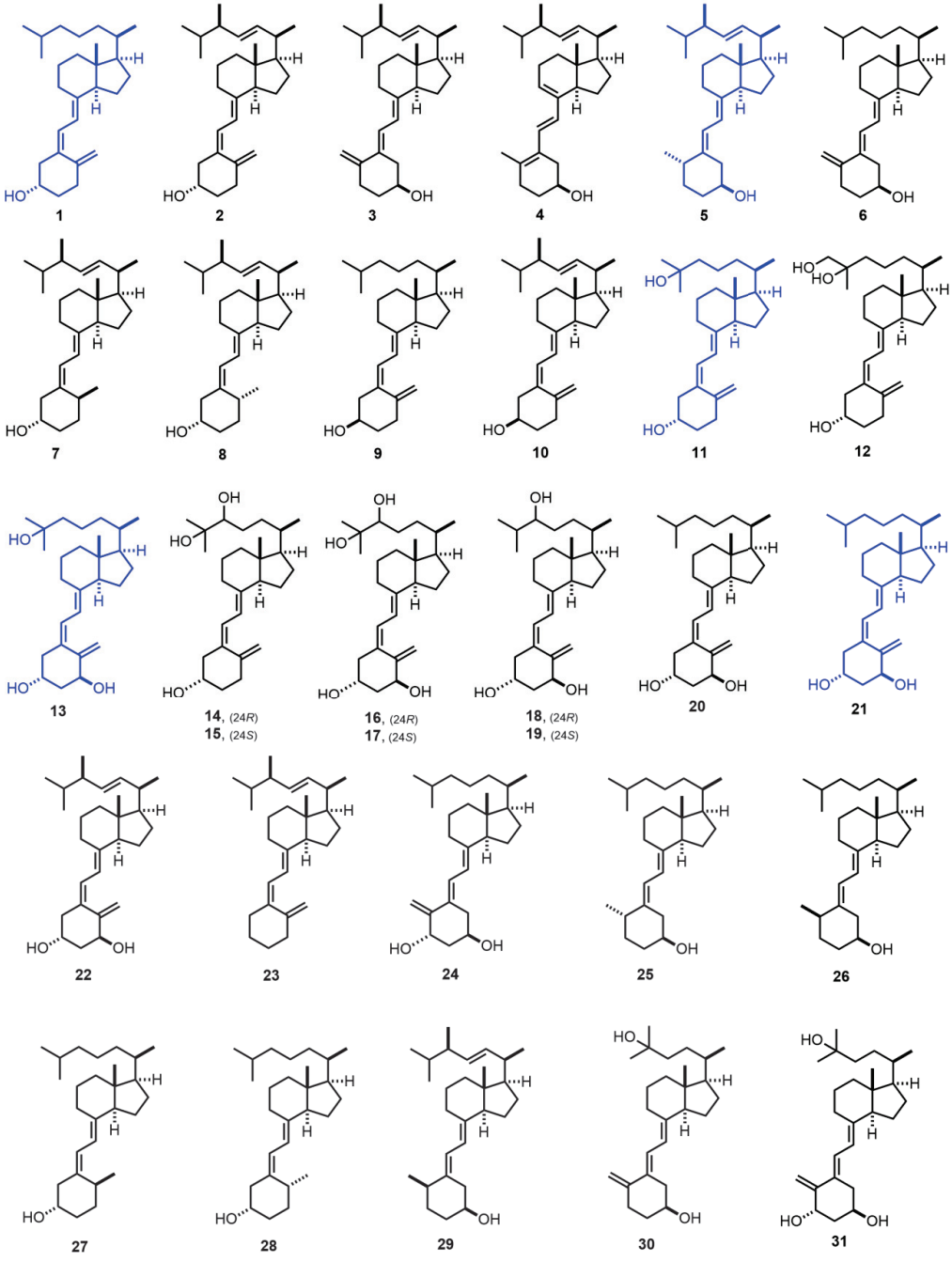


Figure 1. (1931–1978) [1–36].

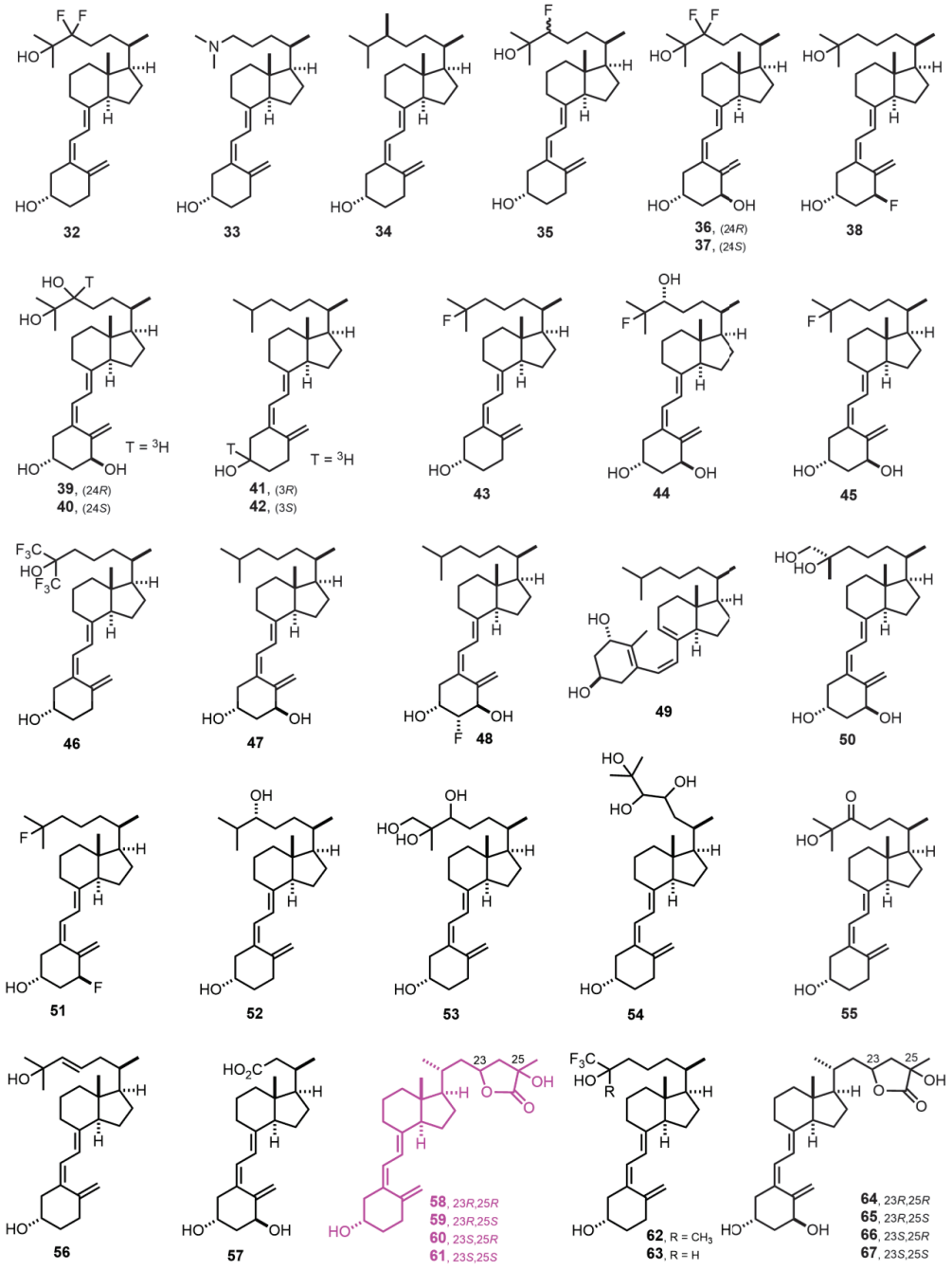
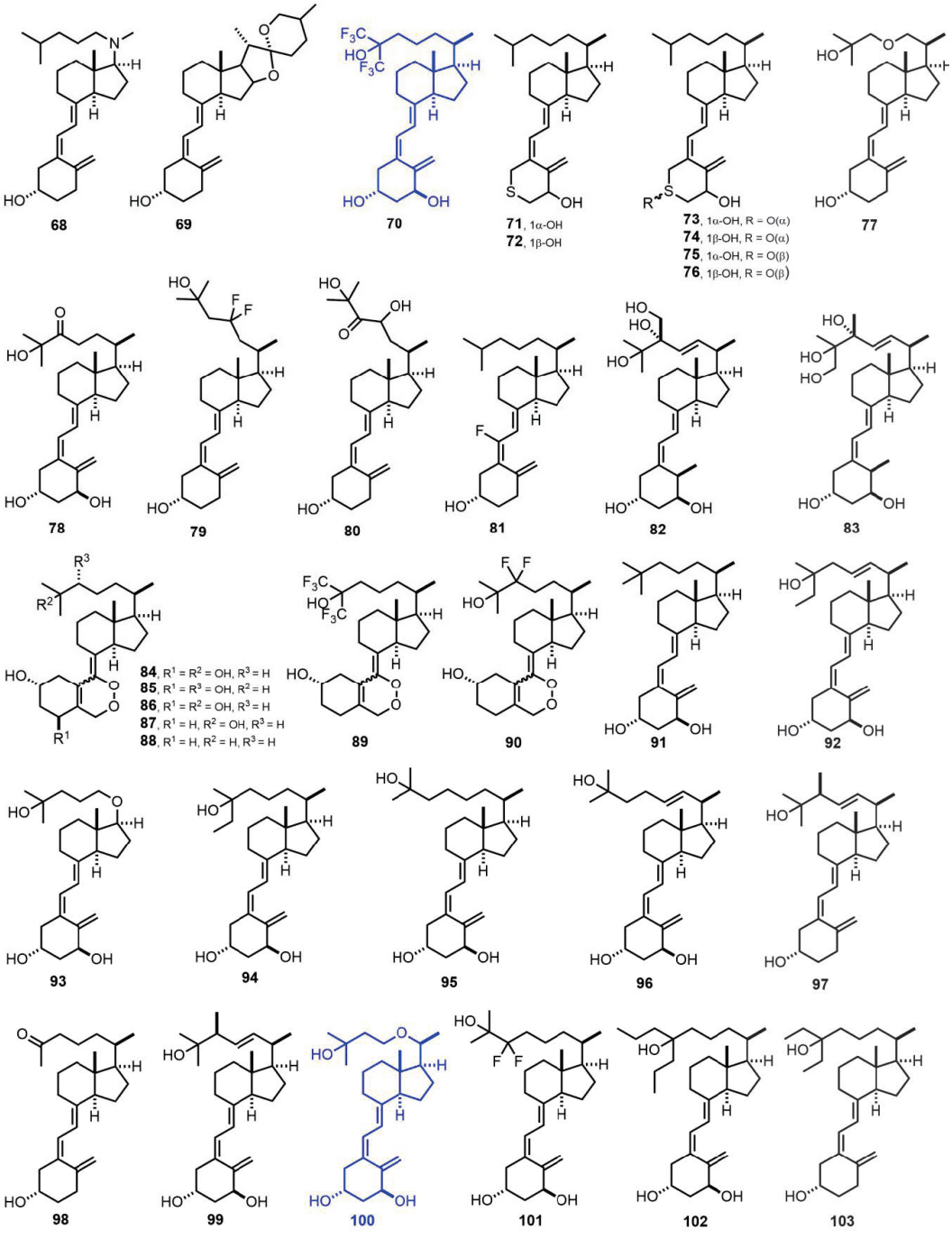


Figure 2. (1978–1982) [37–52].



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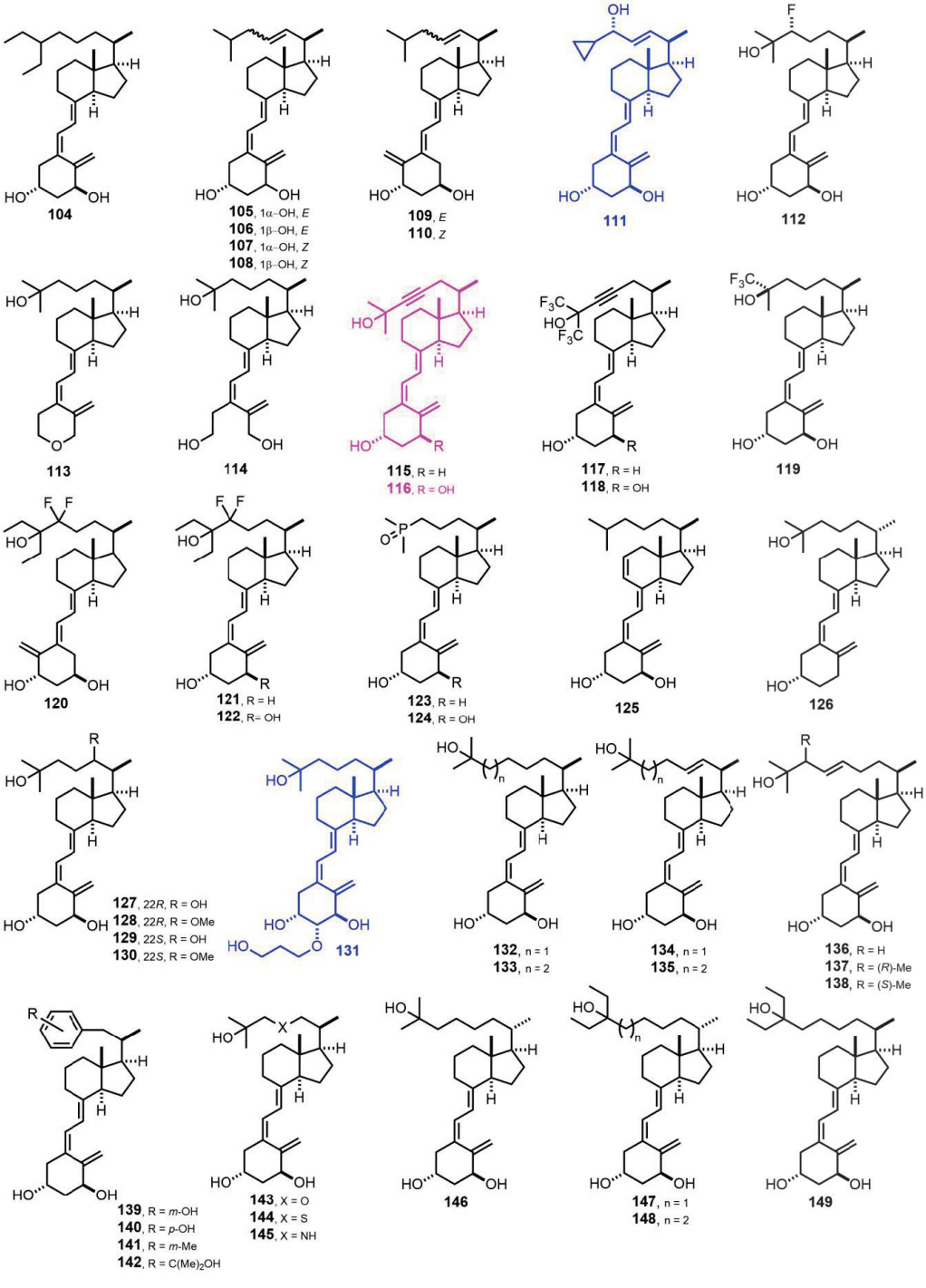
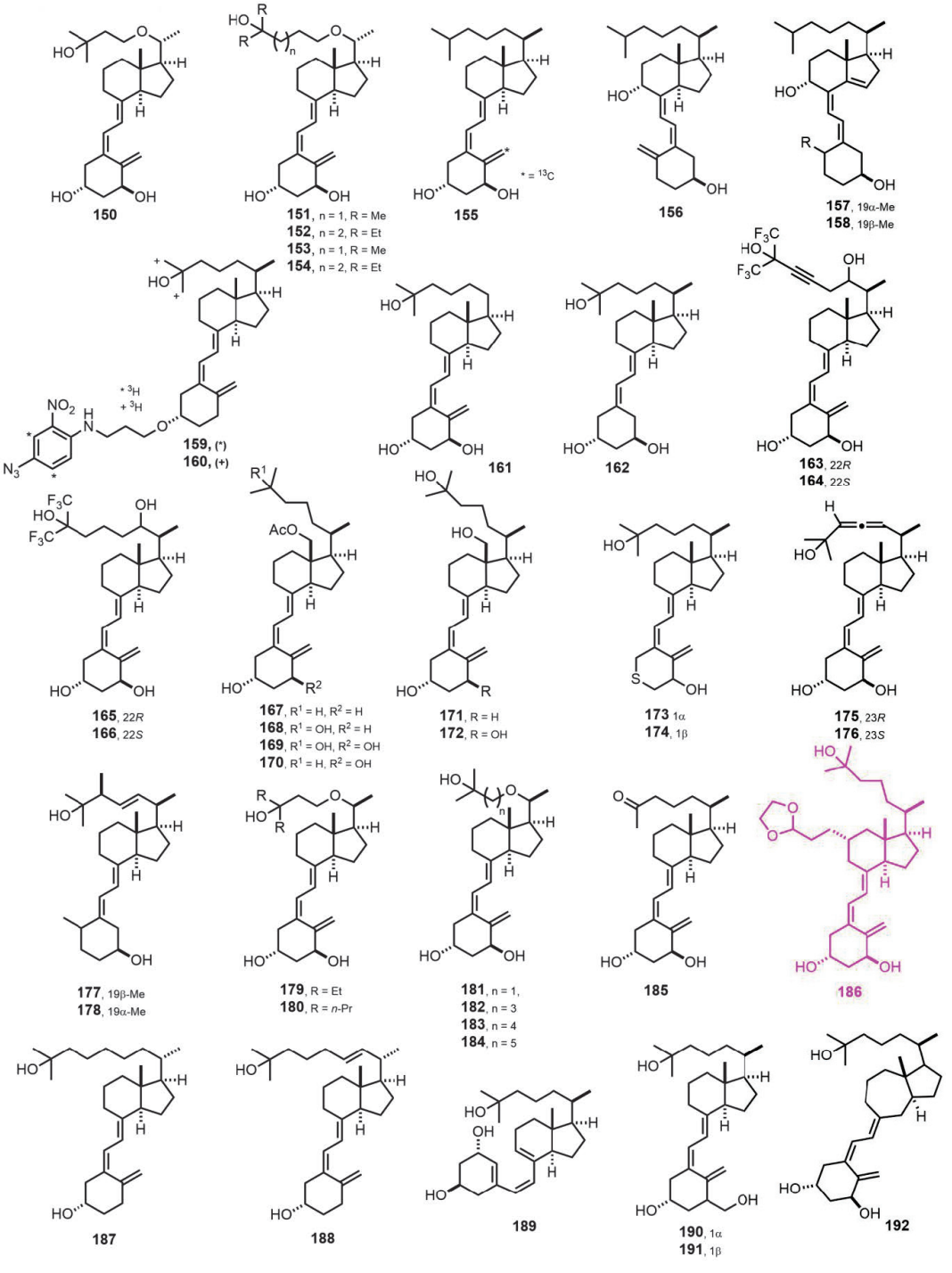


Figure 4. (1987–1991) [78–94].



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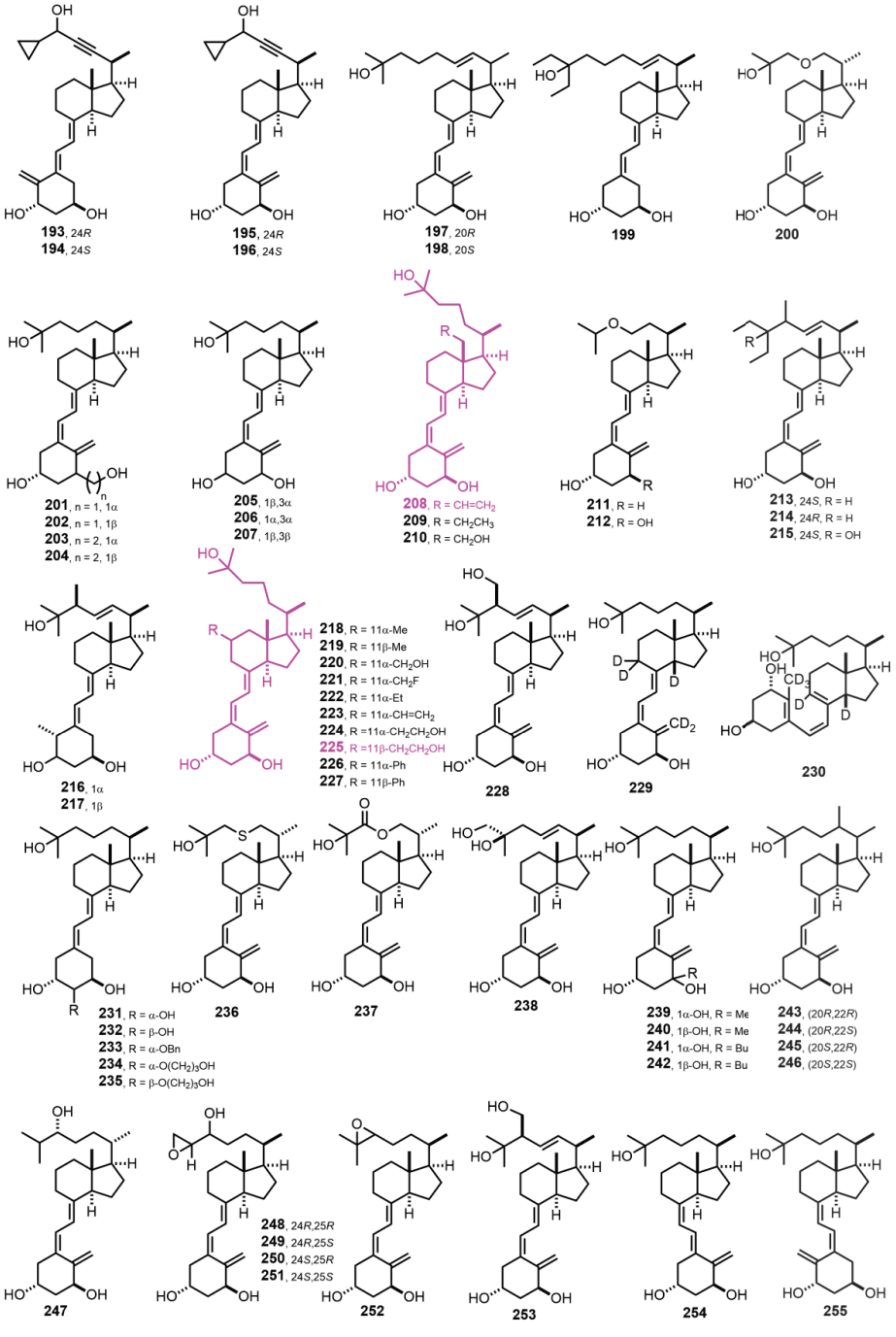

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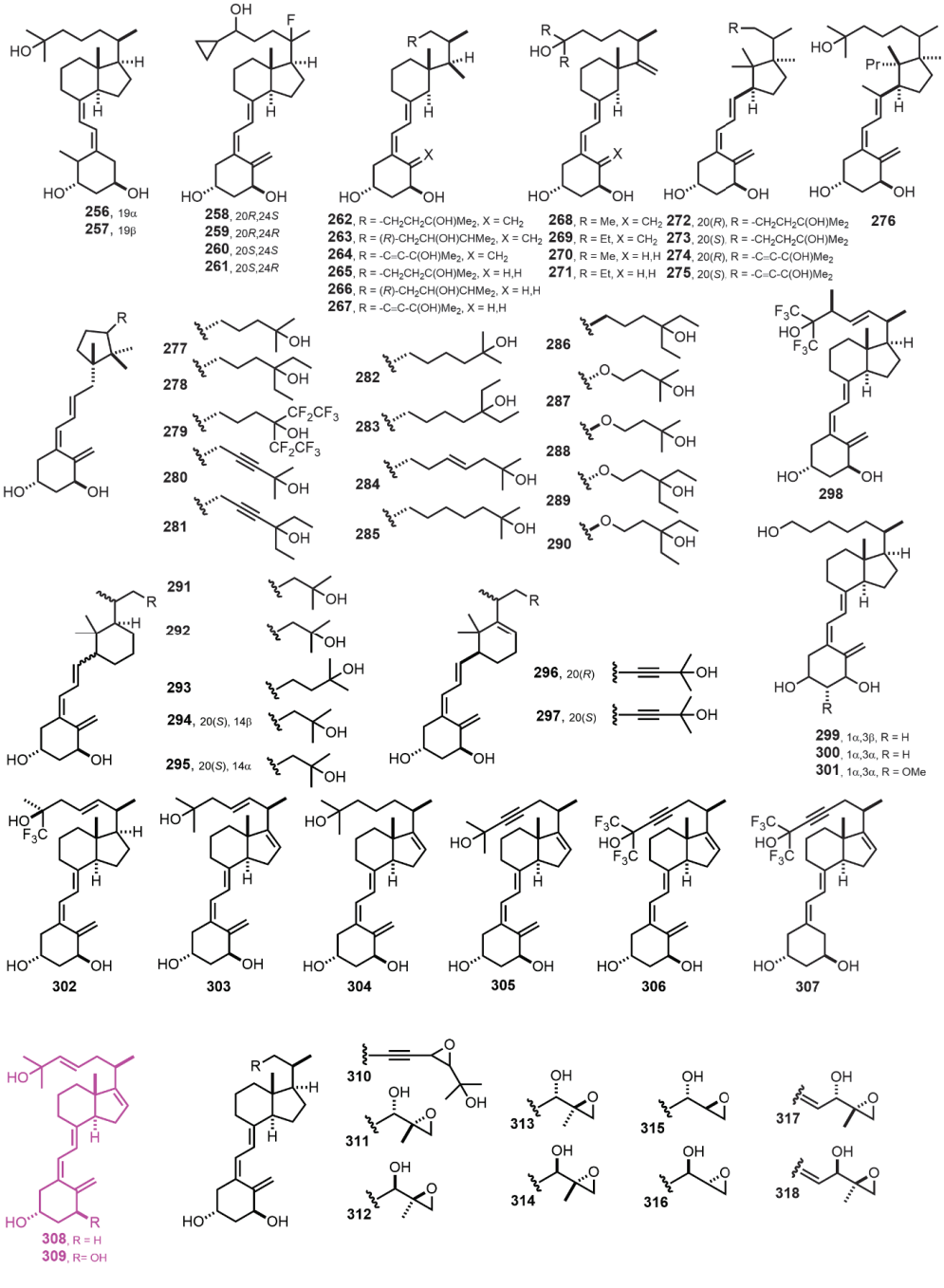
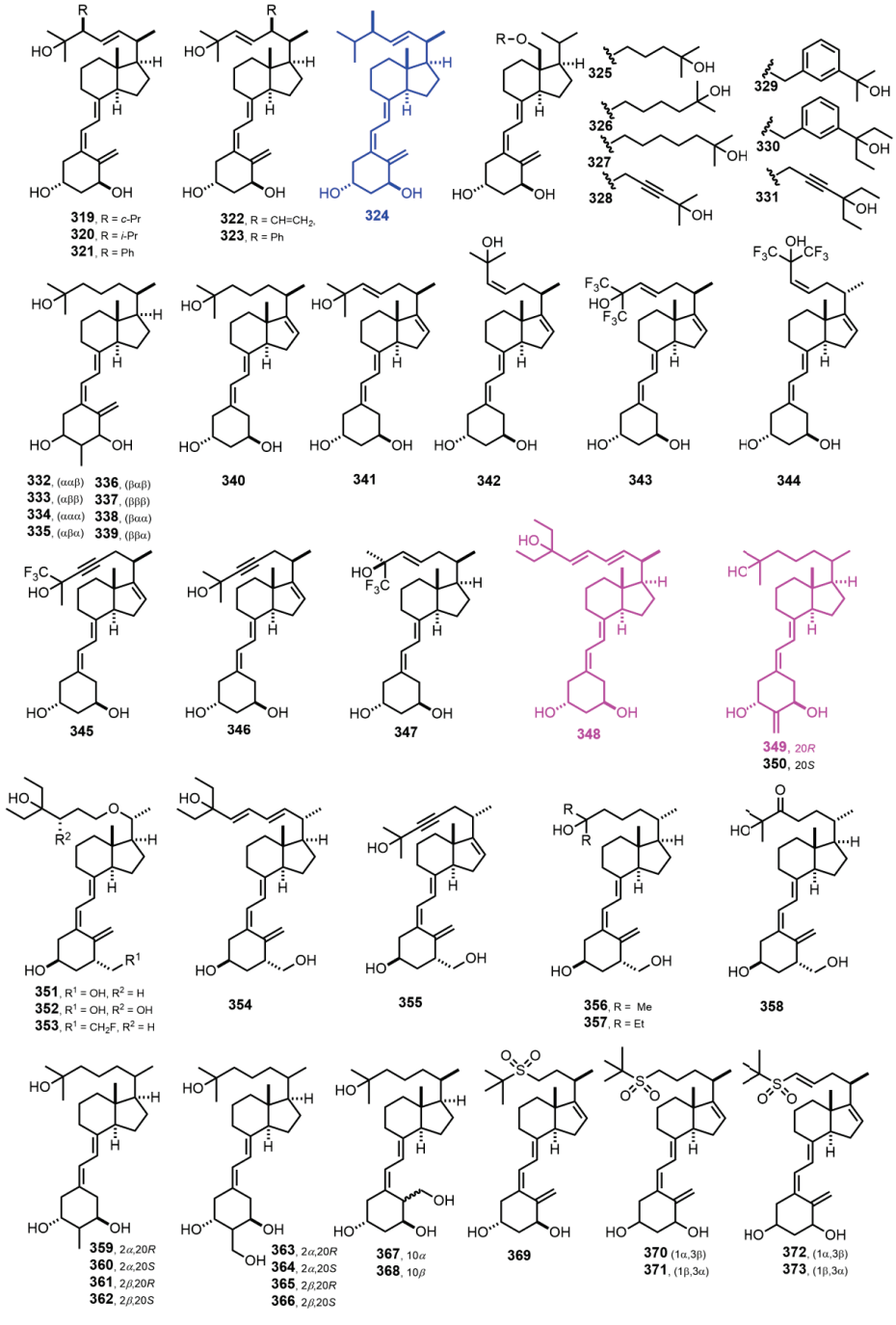


Figure 7. (1994–1997) [136–148].



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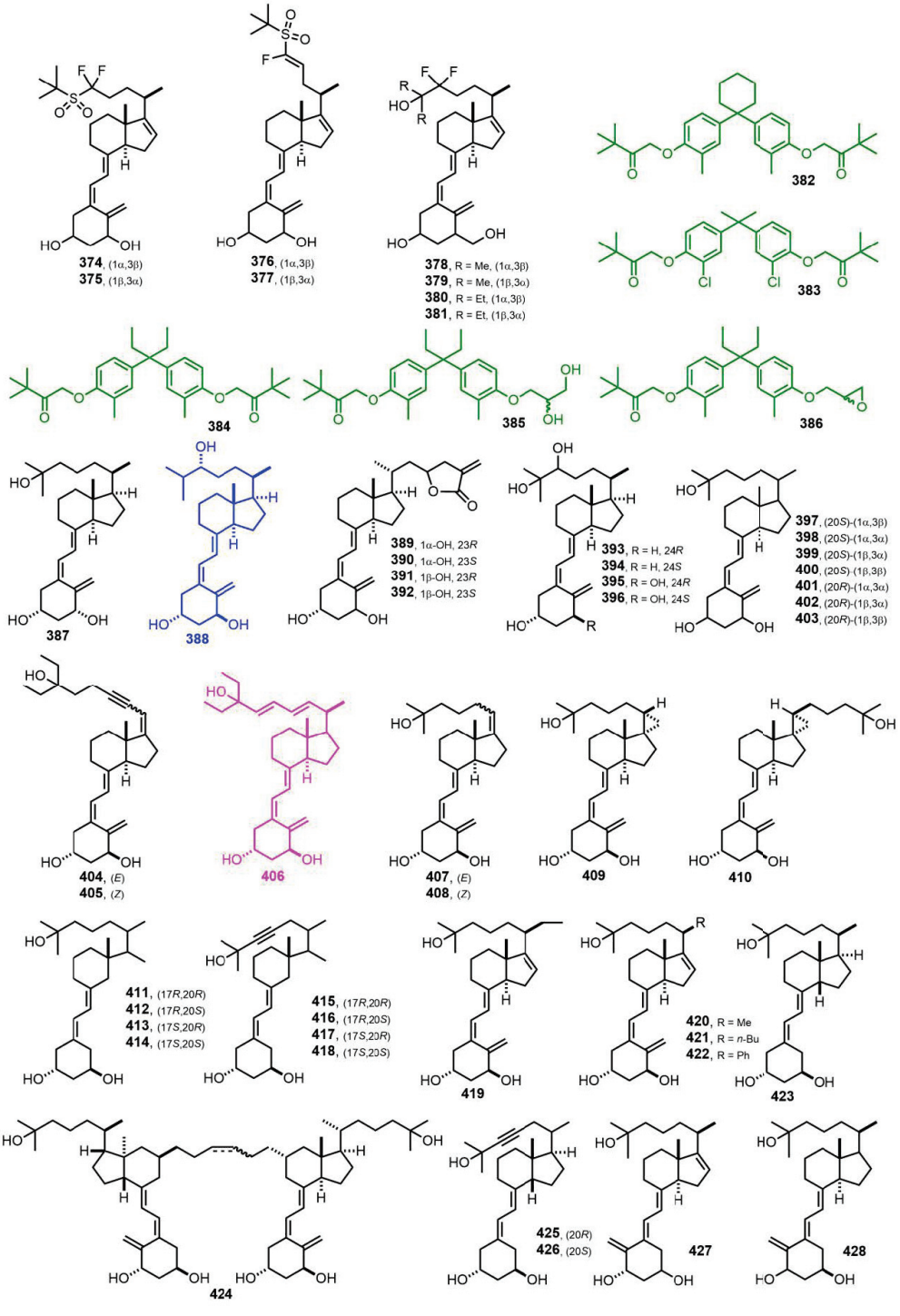

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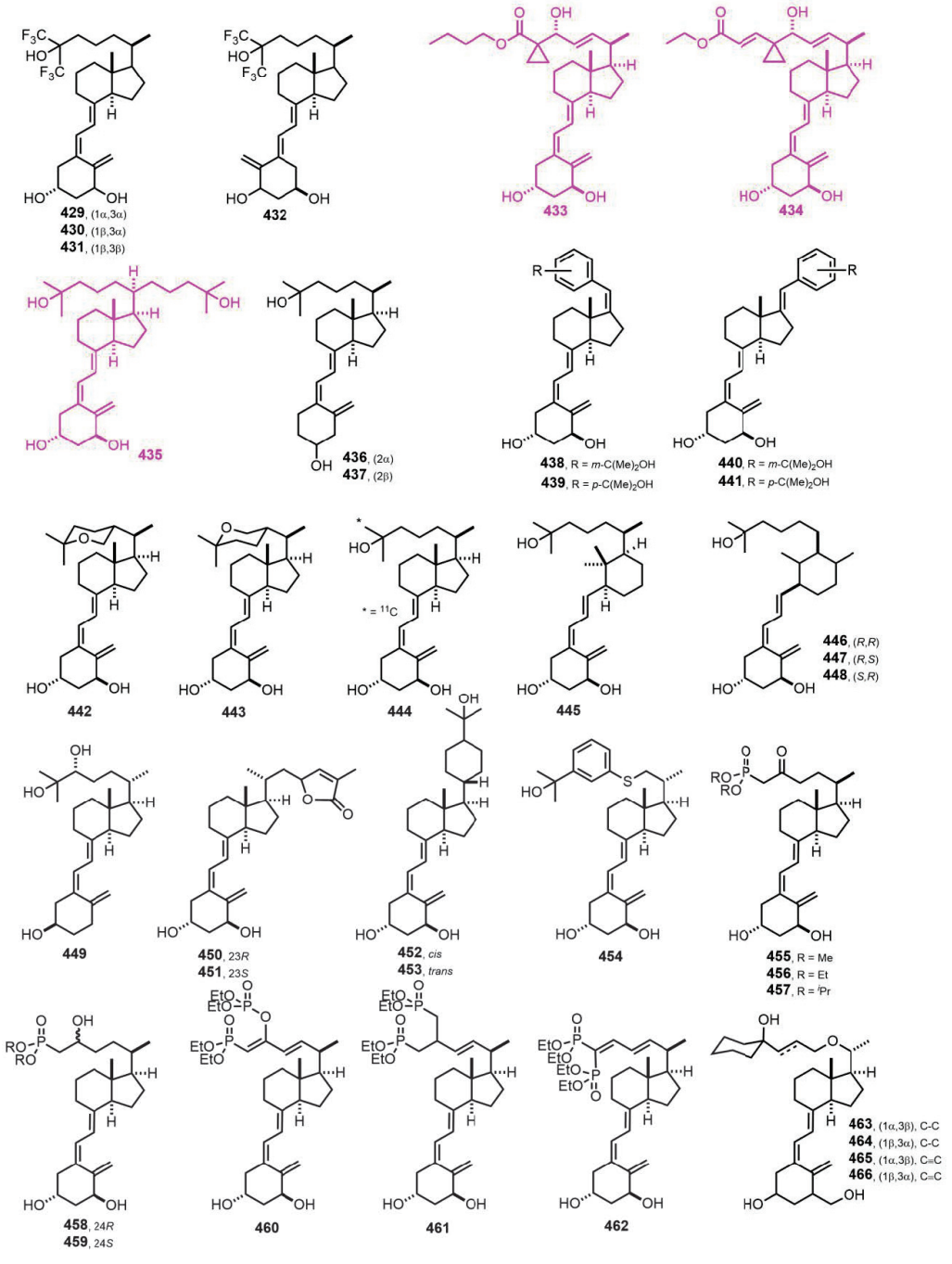

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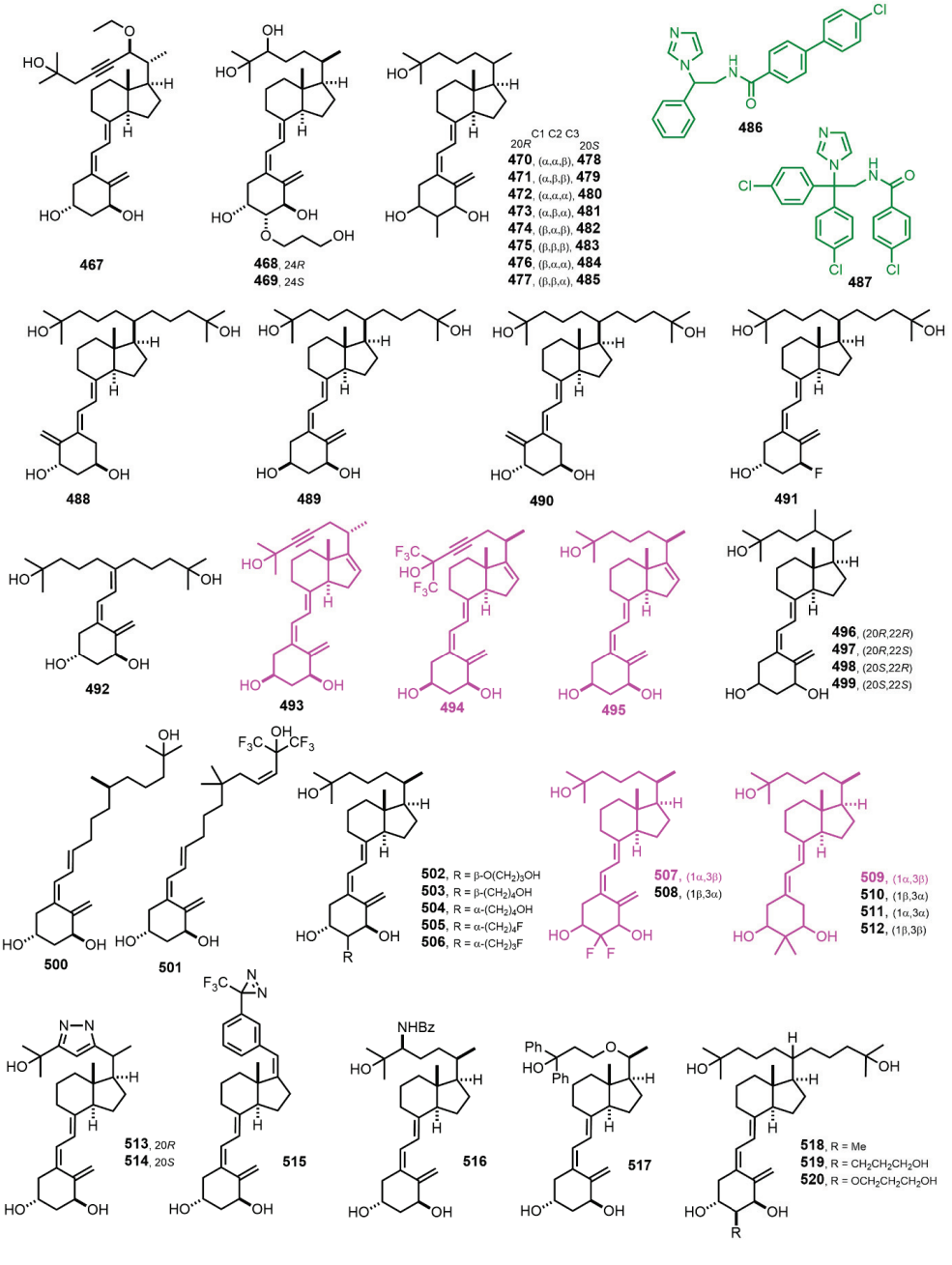

Figure 11. (2001–2002) [183–196].

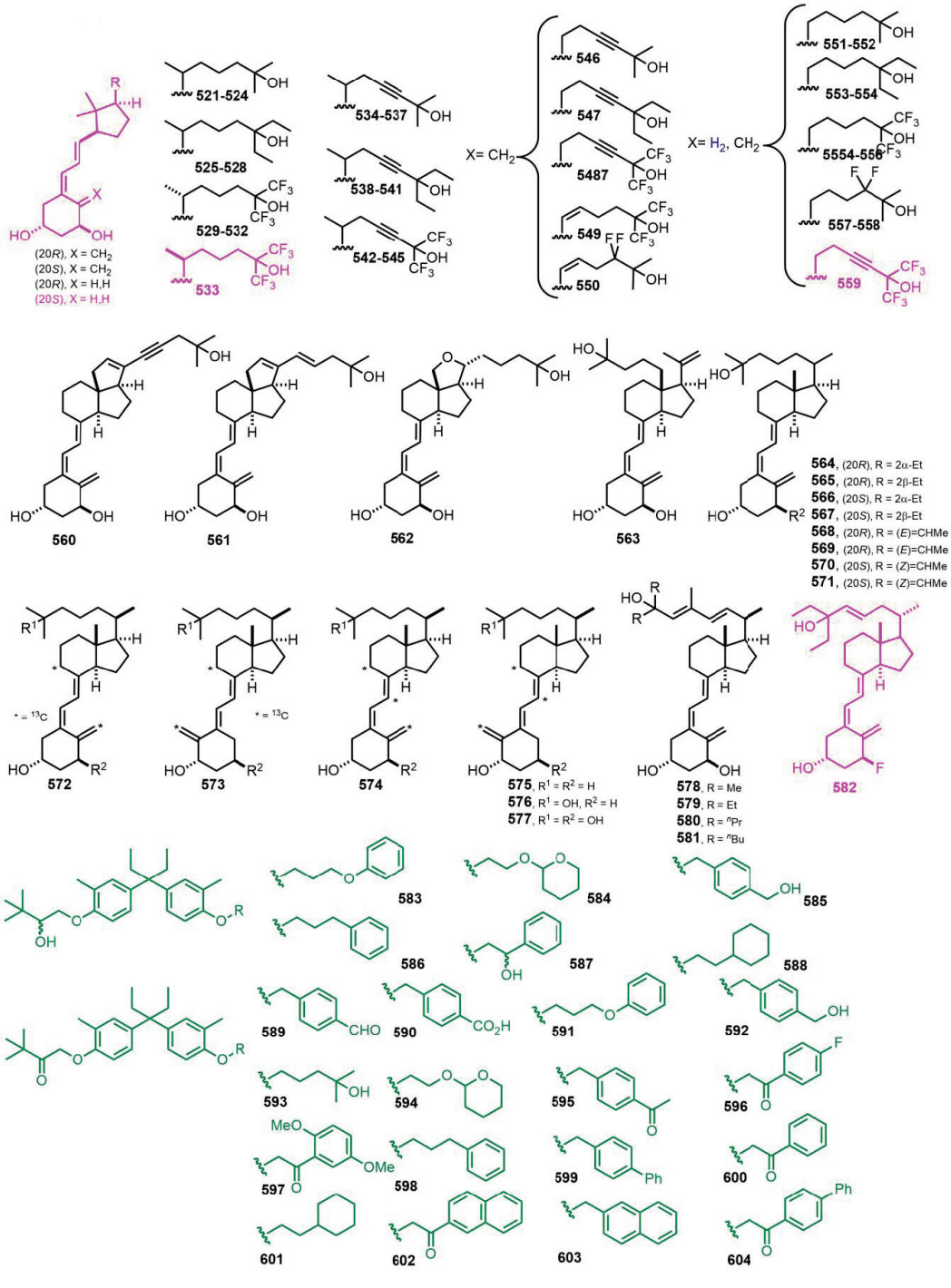

Figure 12. (2002) [197–204].

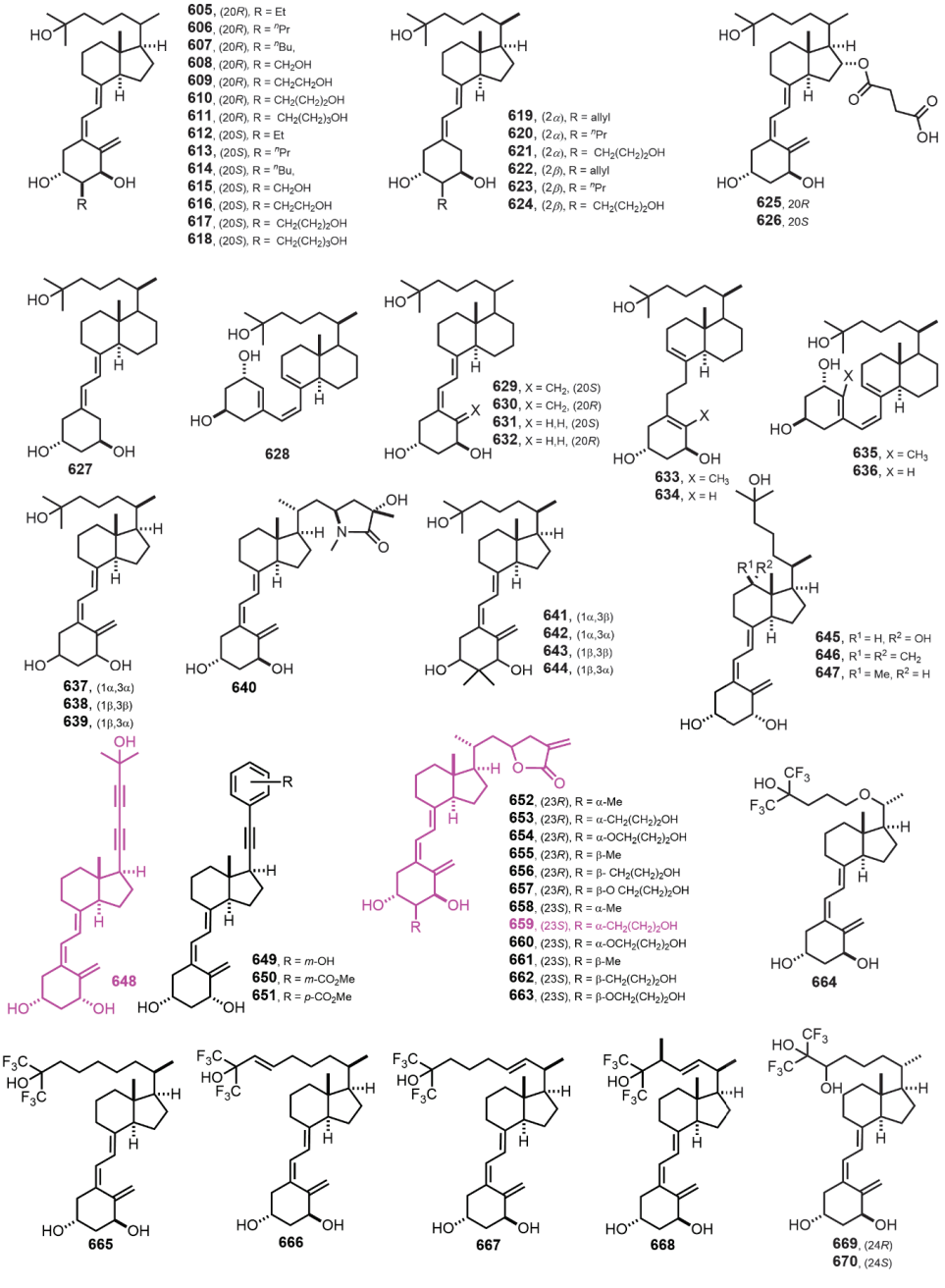

Figure 13. (2003–2004) [205–218].

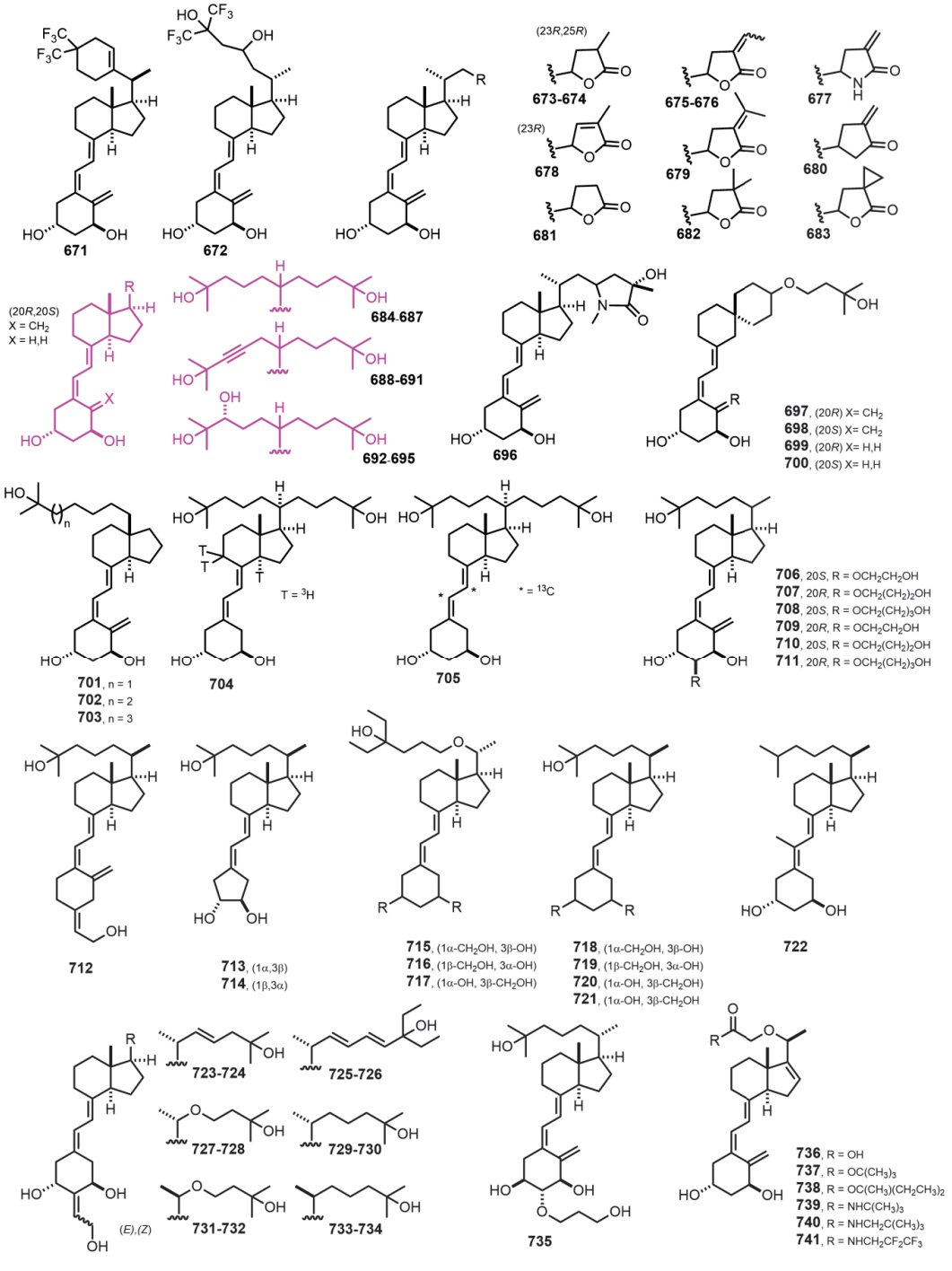

Figure 14. (2004–2006) [218–223].

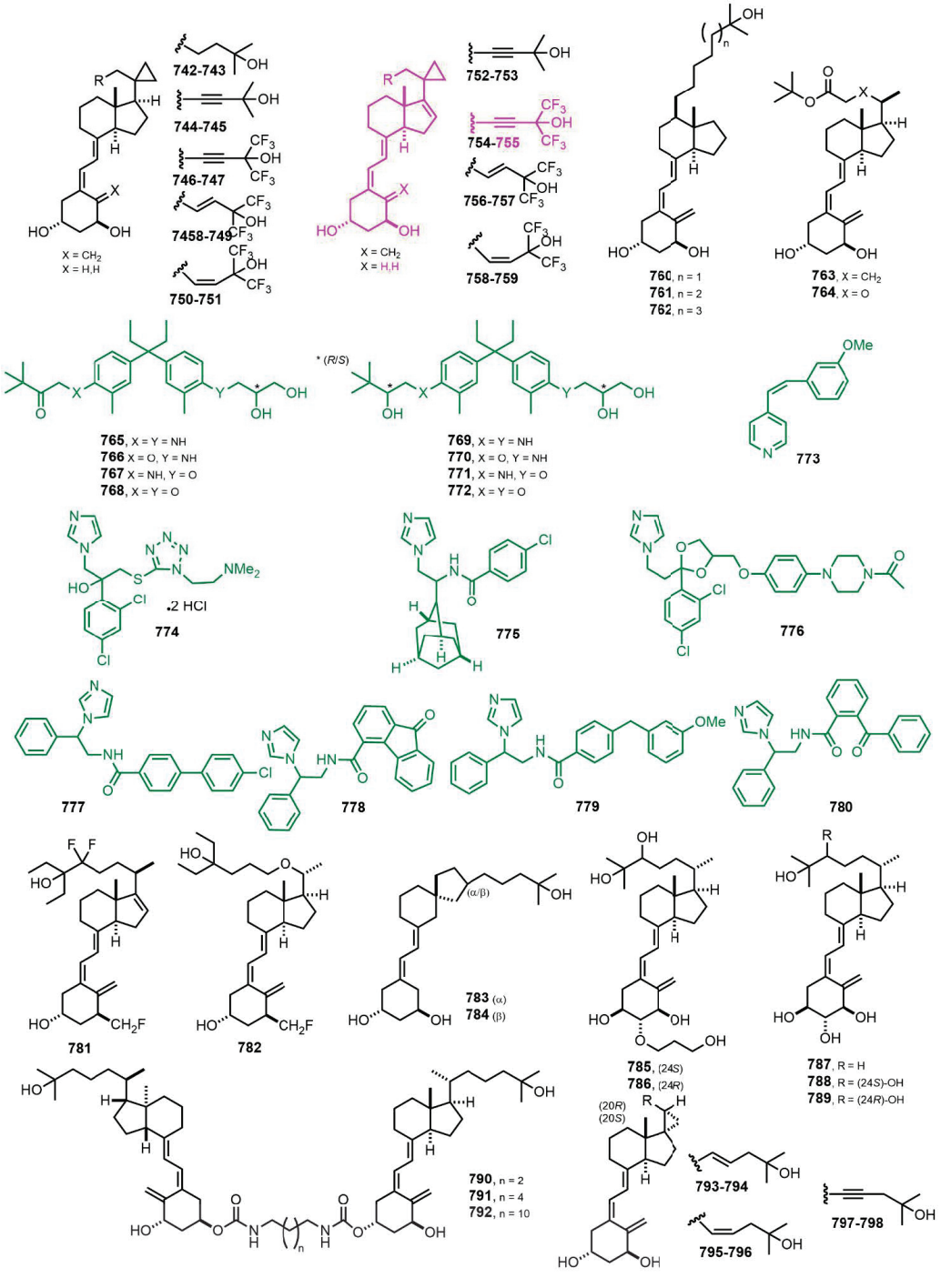

Figure 15. (2006–2007) [224–240].

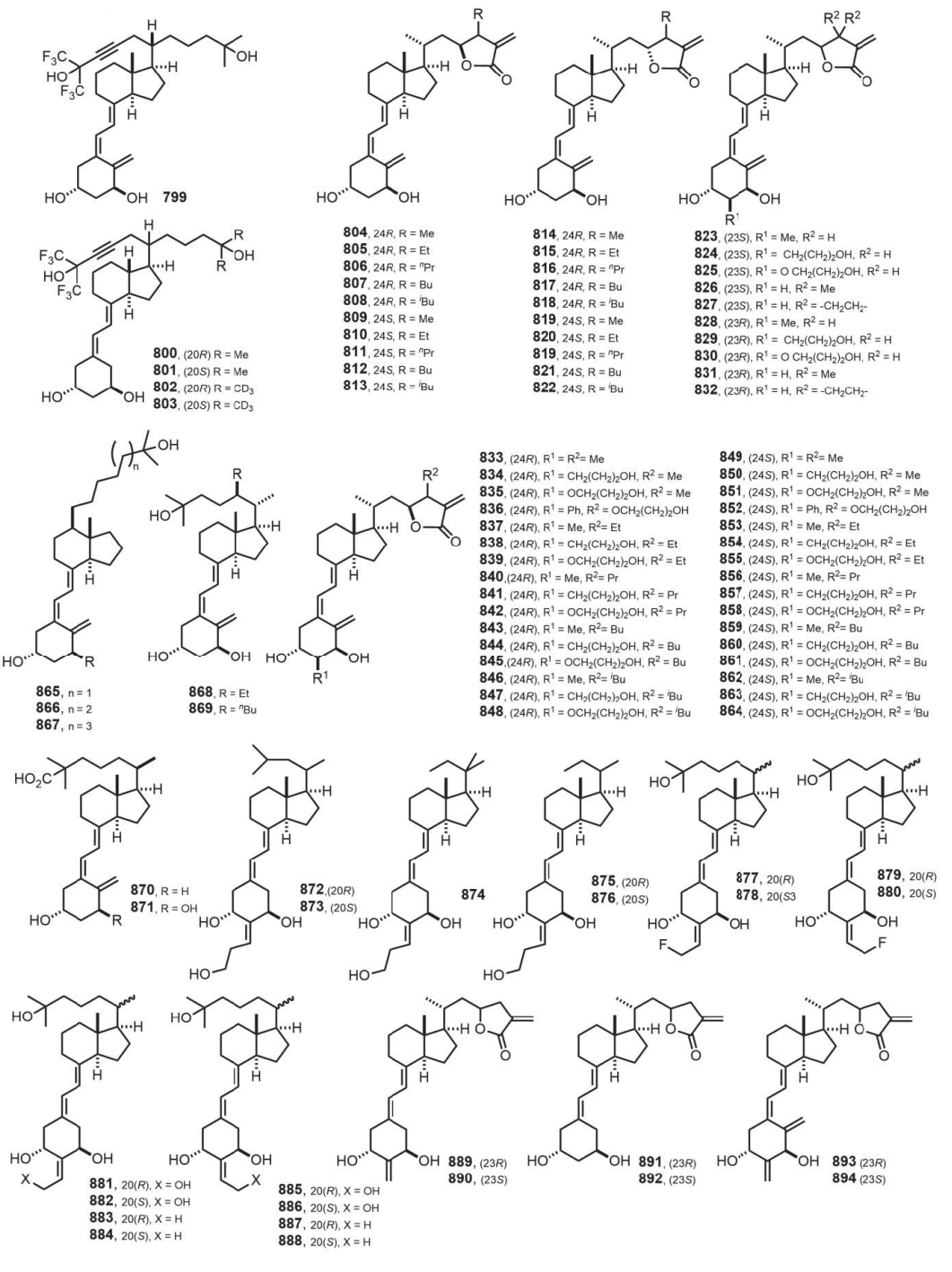

Figure 16. (2006–2008) [241–253].

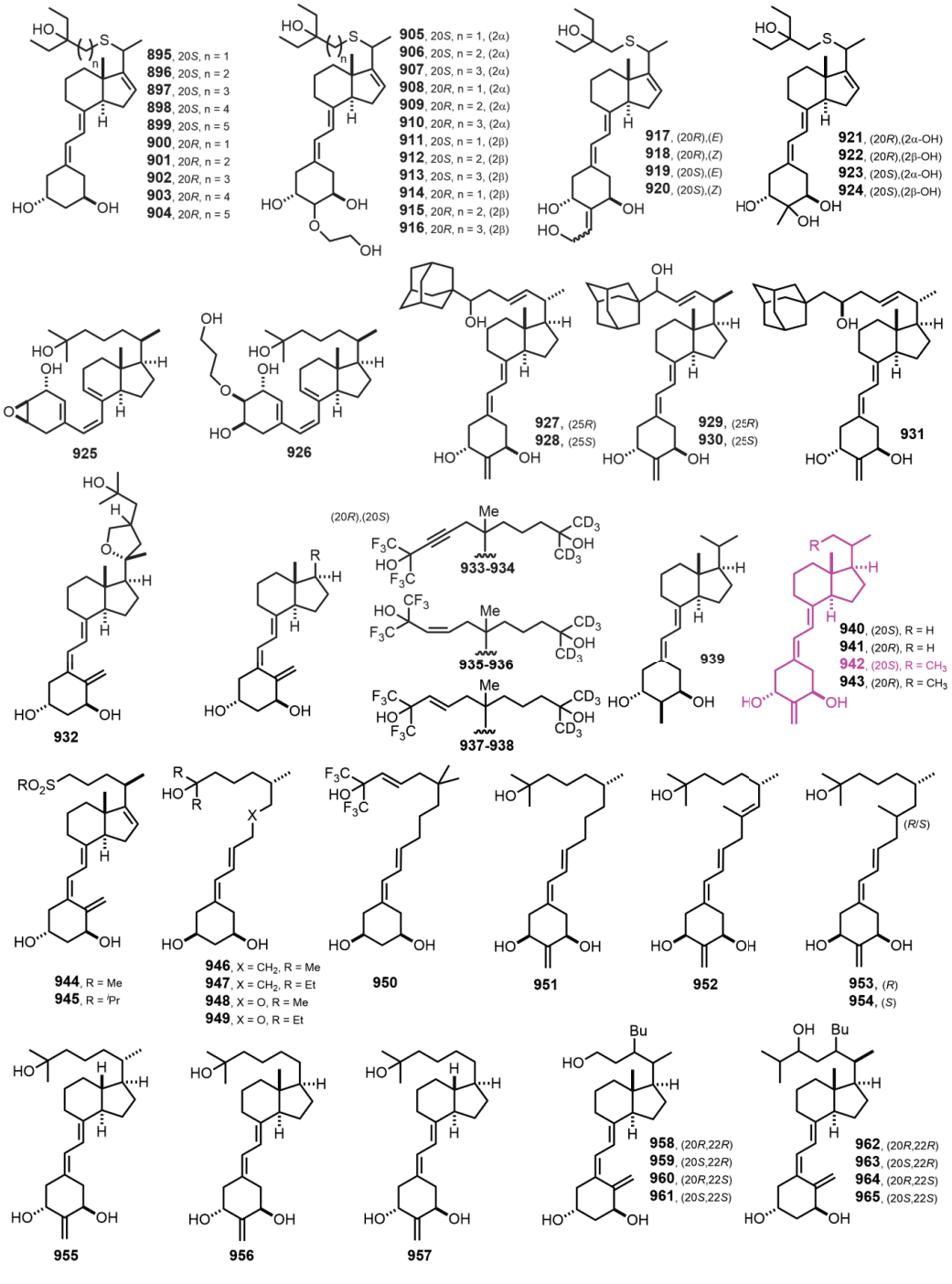

Figure 17. (2008–2009) [254–264].

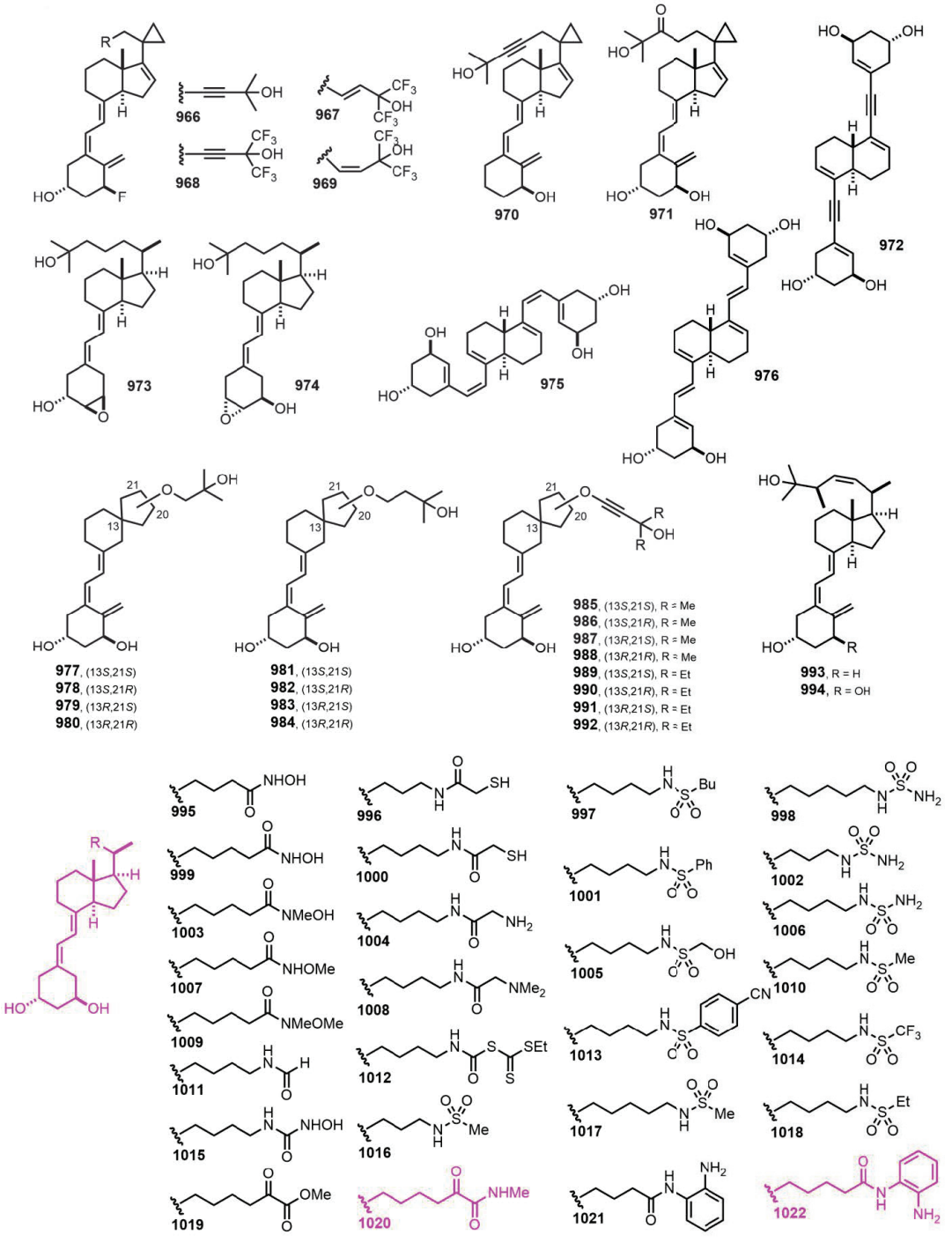

Figure 18. (2009–2010) [265–270].

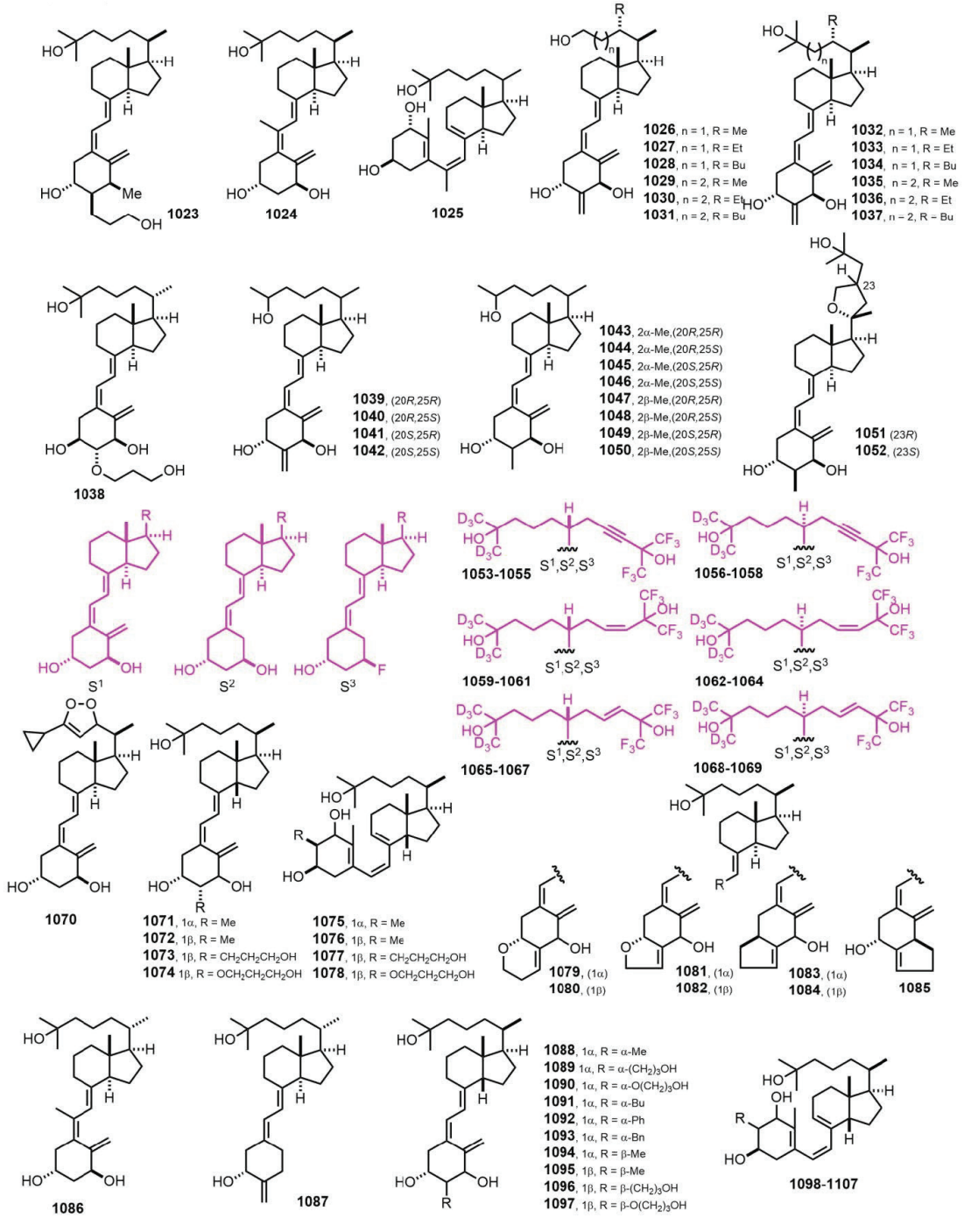

Figure 19. (2009–2010) [271–283].

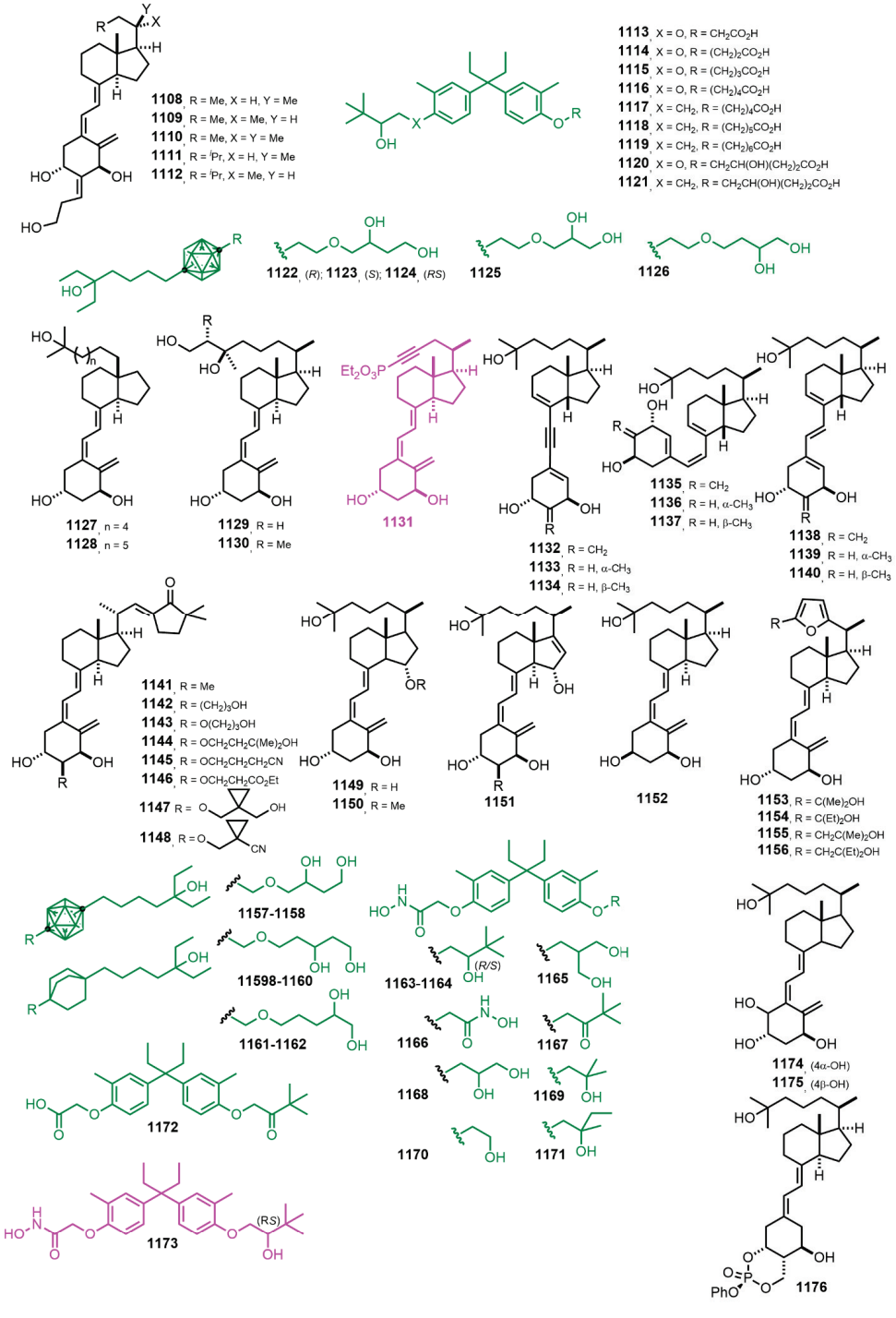

Figure 20. (2010–2012) [284–298].

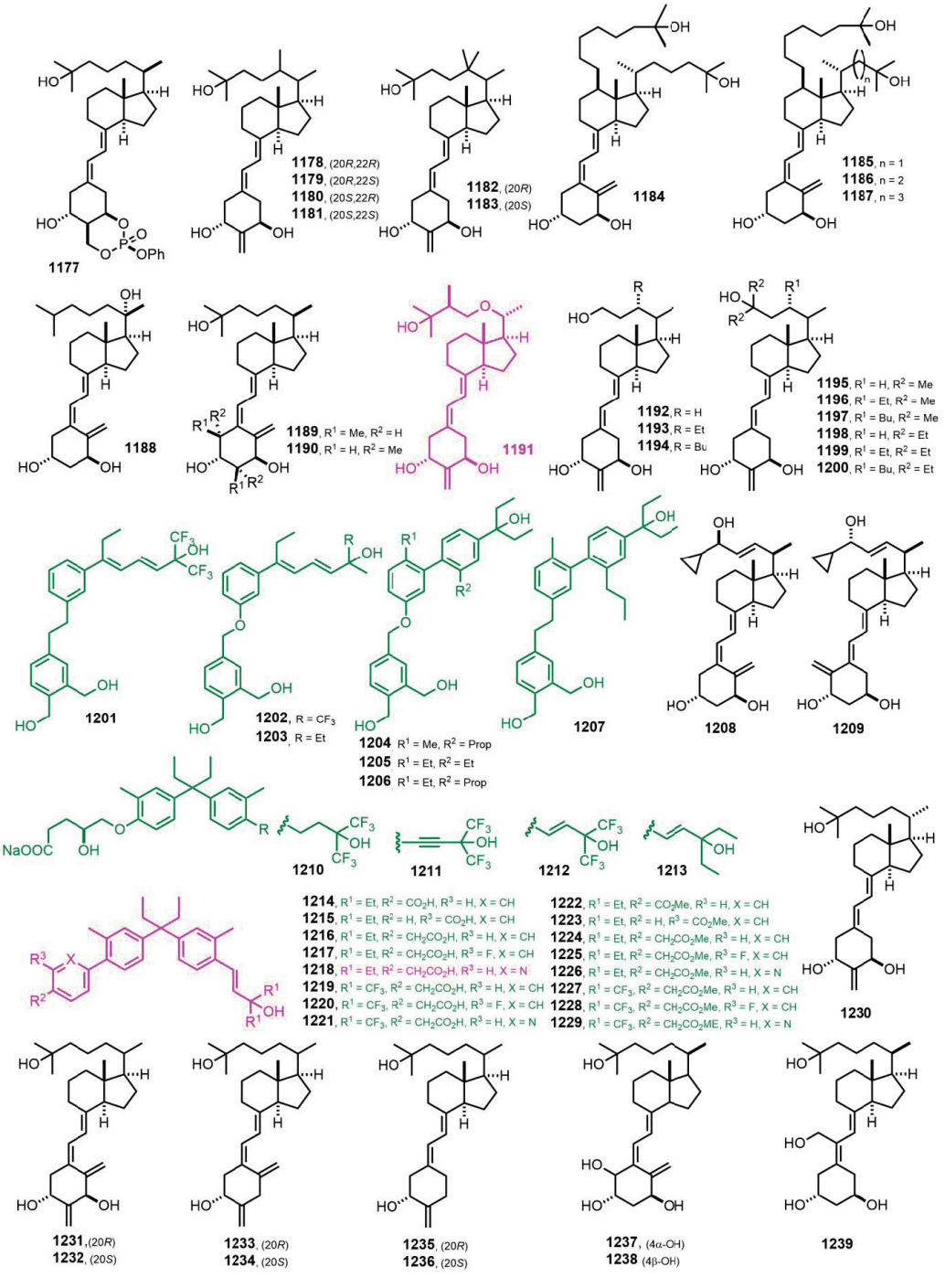

Figure 21. (2012–2013) [298–313].

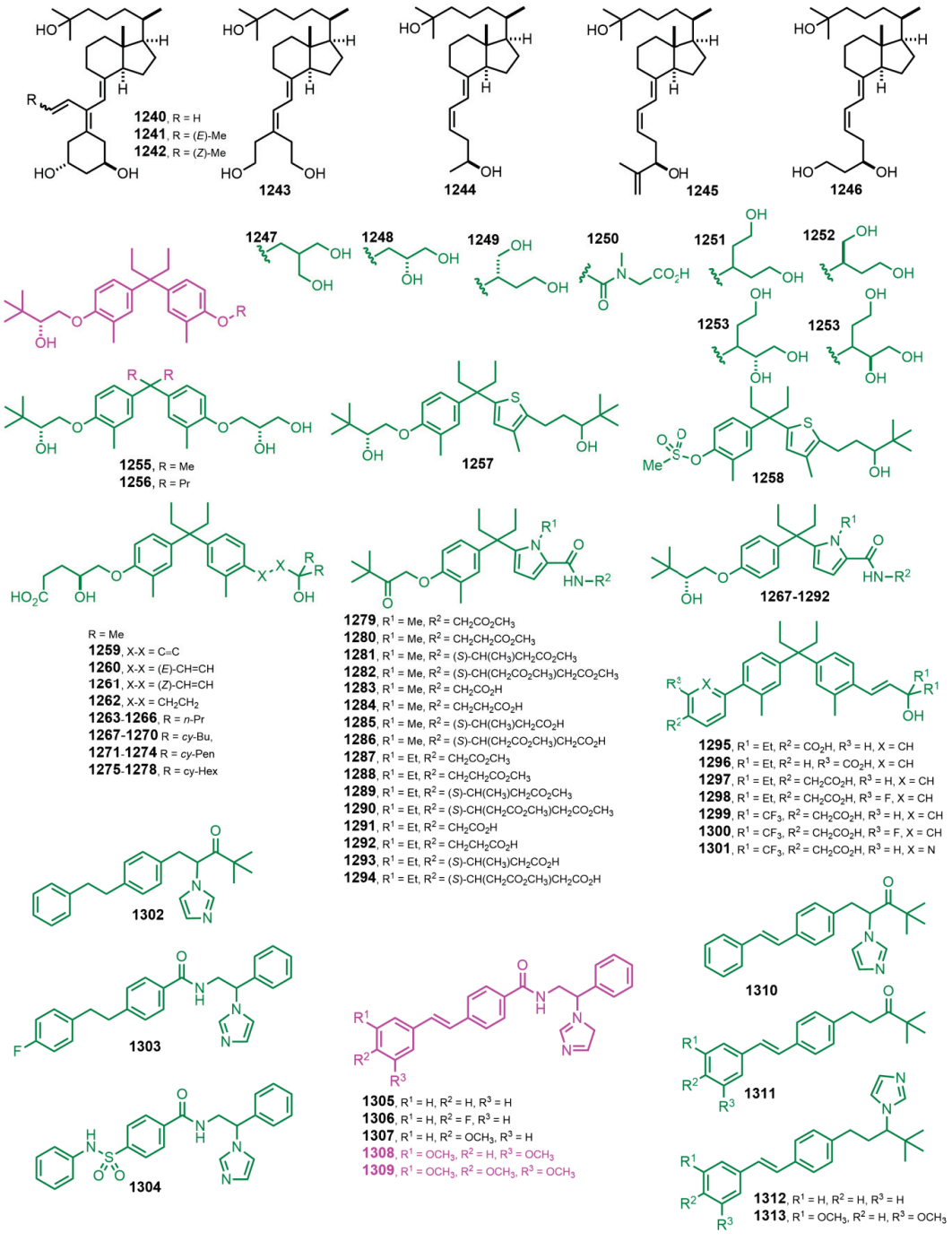

Figure 22. (2013–2014) [313–316].

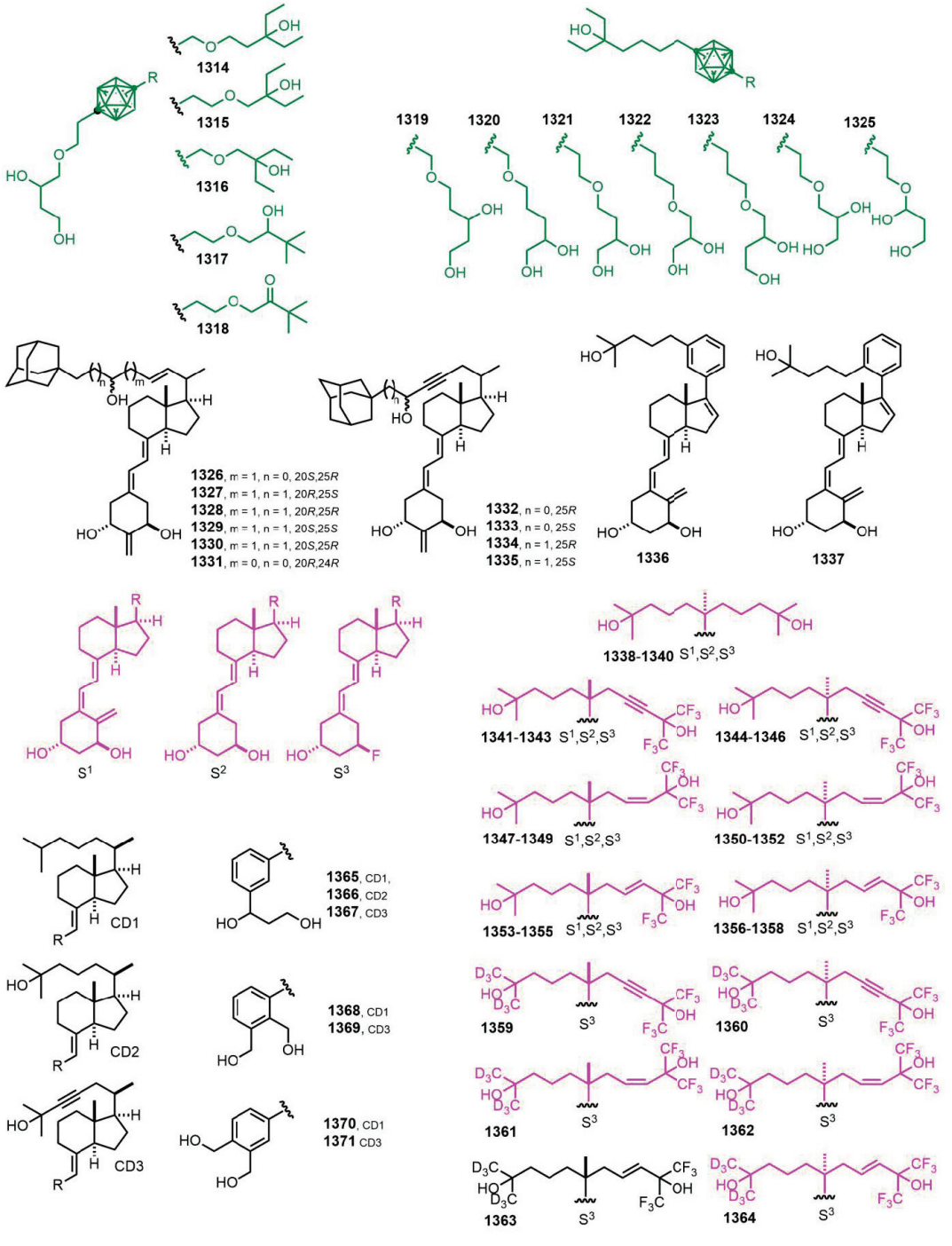

Figure 23. (2014) [317–321].

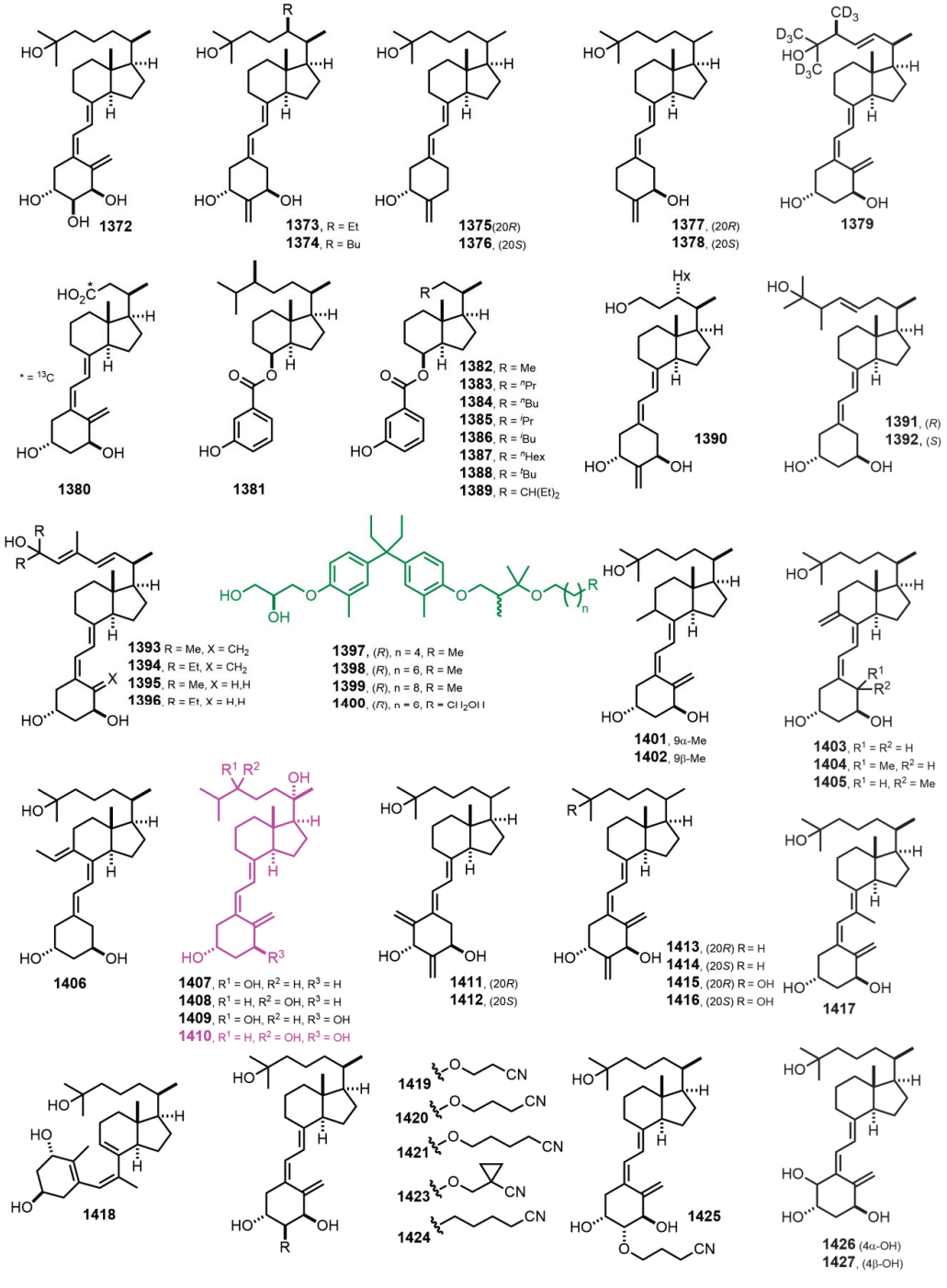

Figure 24. (2014–2015) [322–336].

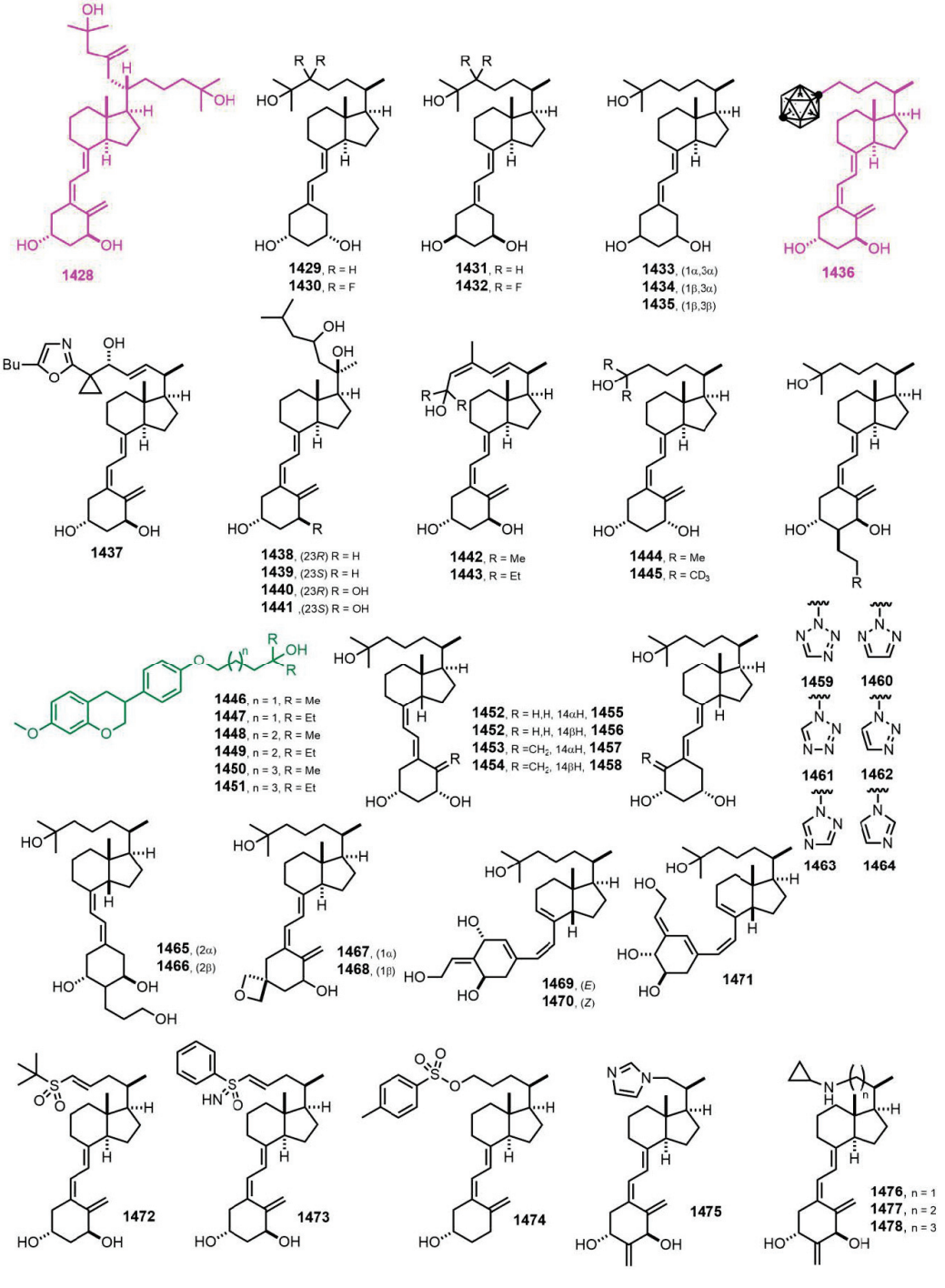

Figure 25. (2015–2017) [337–351].

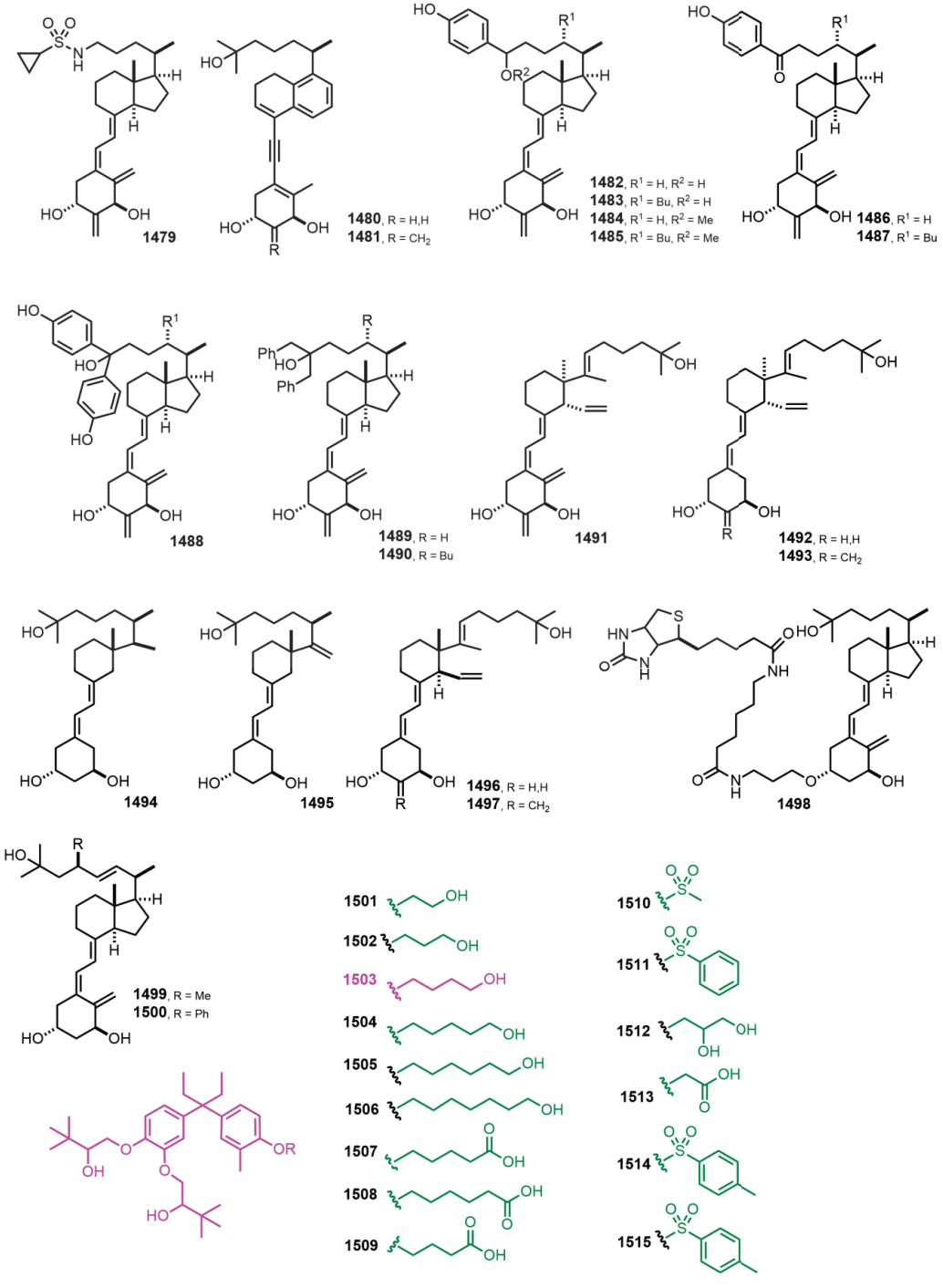

Figure 26. (2017–2018) [351–358].

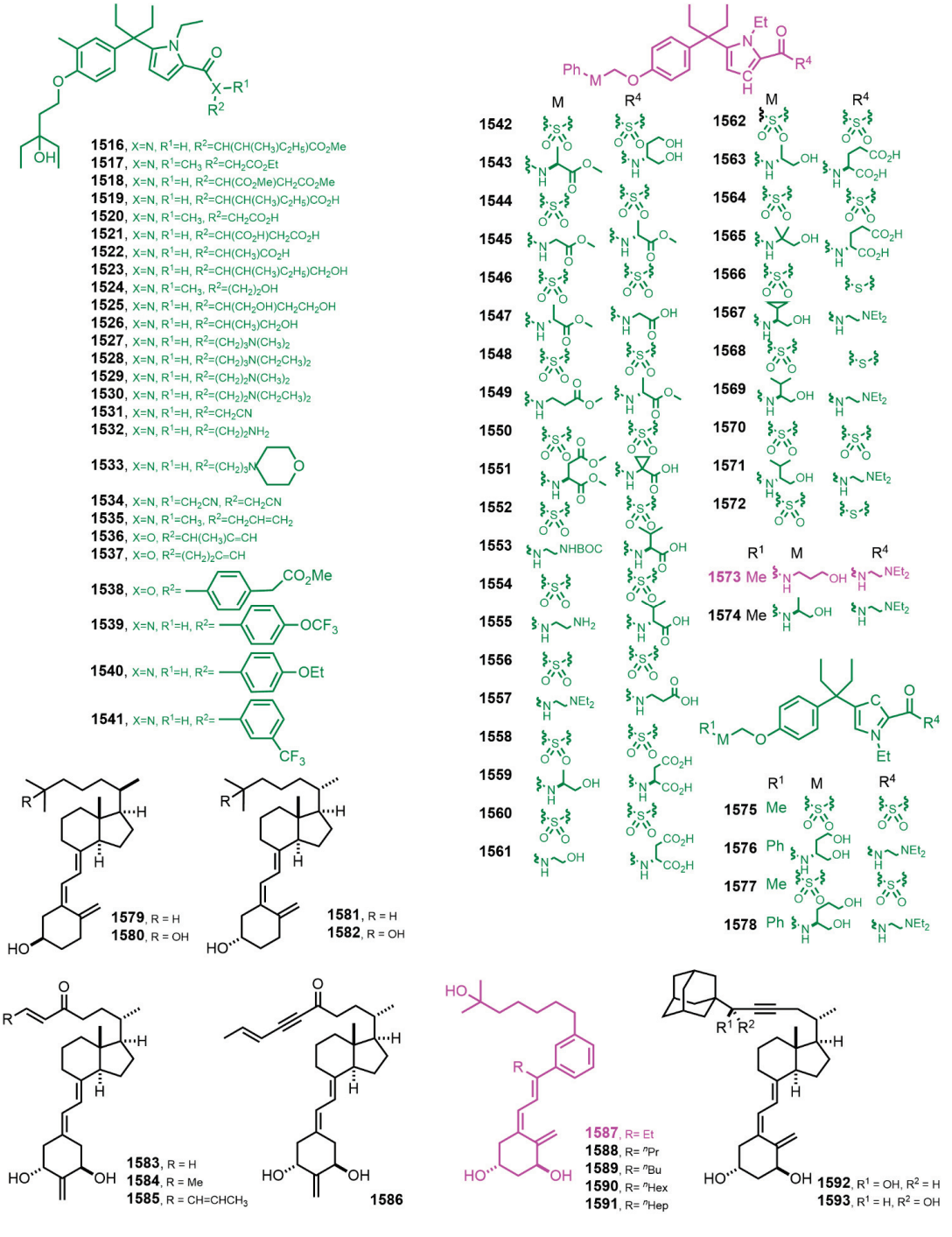

Figure 27. (2018) [359–364].

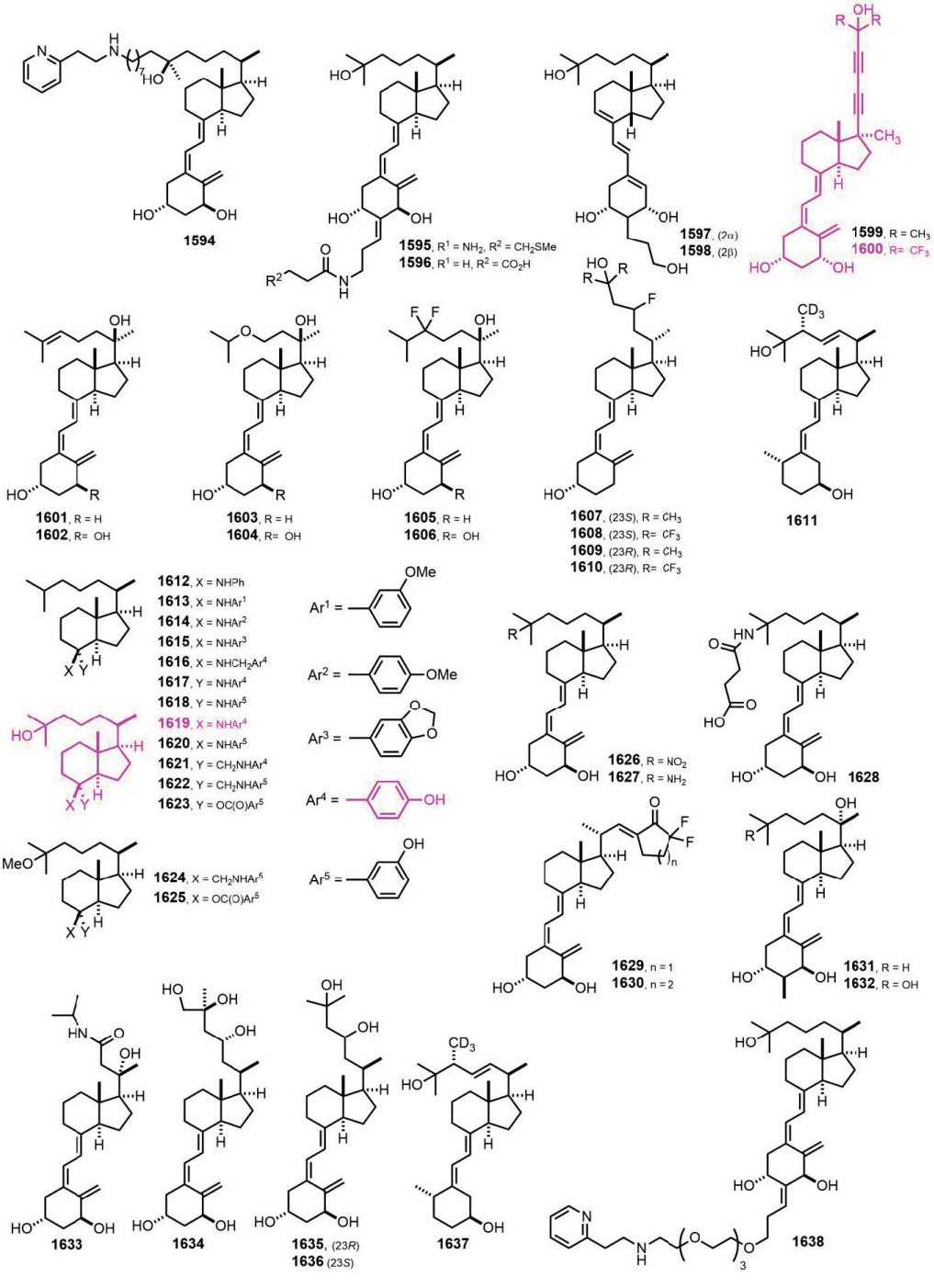

Figure 28. (2018–2019) [365–378].

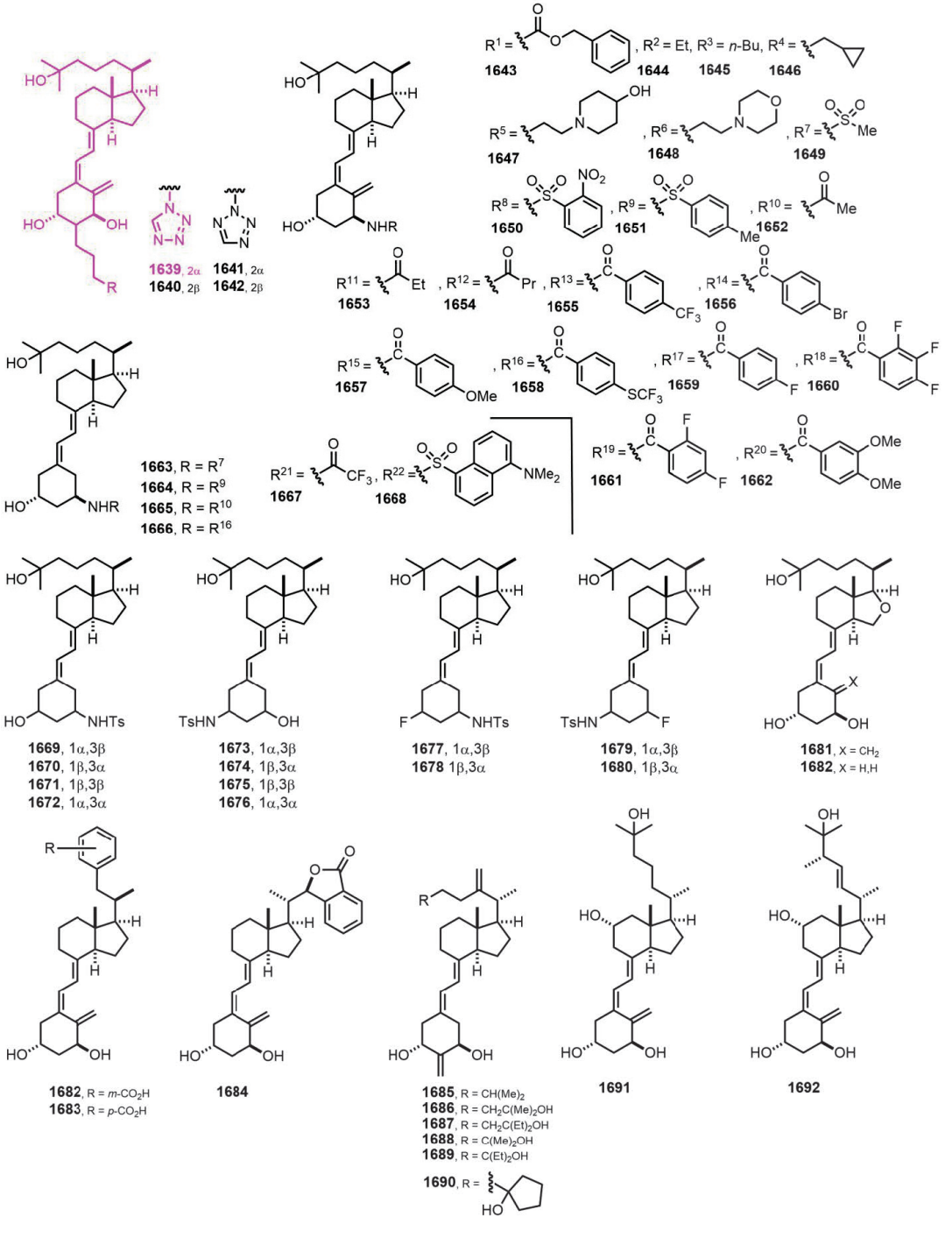

Figure 29. (2019–2020) [379–383].

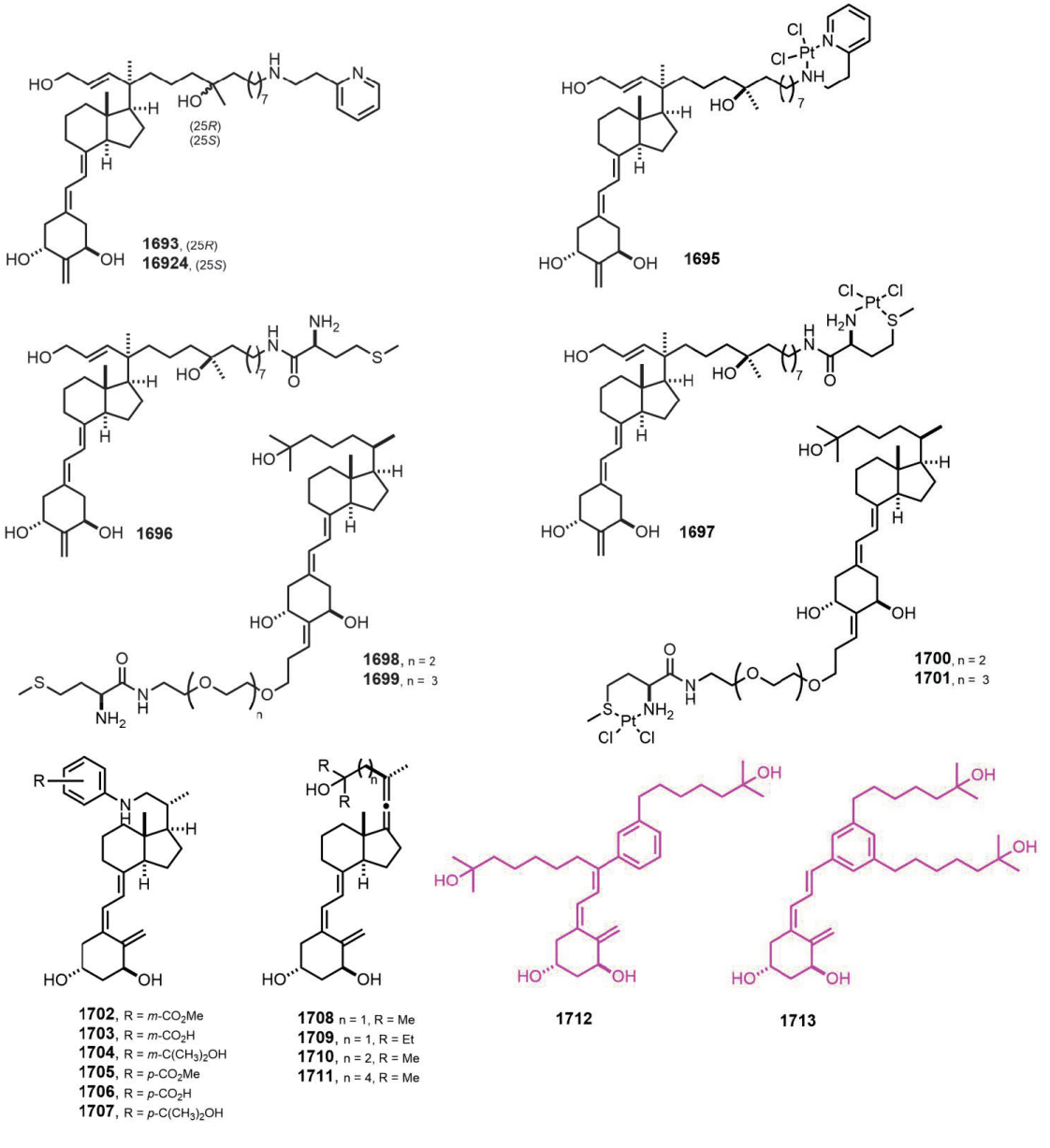

Figure 30. (2020–2022) [384–389].

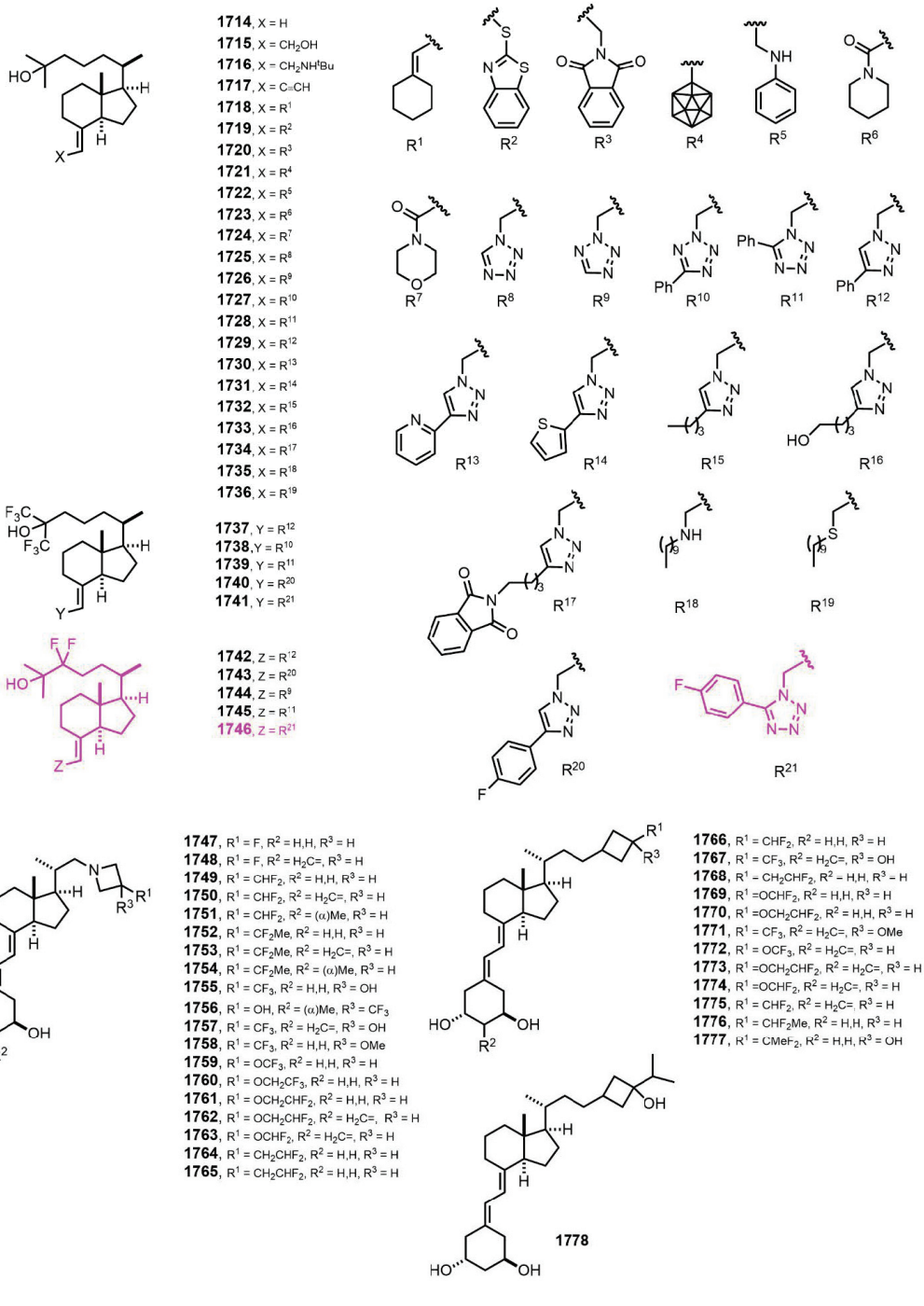

Figure 31. (2021–2022) [390–392].

4. Conclusions

A century has passed since vitamin D was discovered. The structural diversity achieved among vitamin D receptor ligands (1785 ligands involving metabolites, analogs, hybrids, and nonsteroidal ligands). Seeing as vitamin D plays a ubiquitous role in human physiology, VDR ligands have been found to cure or ameliorate the symptoms of various diseases. It is disheartening to note that for more than twenty years no drug based on a VDR ligand (i.e., analogues, hybrids, or nonsteroidal ligands) has been placed on the market because the structural diversity achieved in the VDR ligands might encode new therapies for other illness different than the calcium–phosphorous homeostasis.

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Review

Nongenomic Activities of Vitamin D

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Abstract: Vitamin D shows a variety of pleiotropic activities which cannot be fully explained by the stimulation of classic pathway- and vitamin D receptor (VDR)-dependent transcriptional modulation. Thus, existence of rapid and nongenomic responses to vitamin D was suggested. An active form of vitamin D (calcitriol, 1,25$(OH)_2D_3$) is an essential regulator of calcium–phosphate homeostasis, and this process is tightly regulated by VDR genomic activity. However, it seems that early in evolution, the production of secosteroids (vitamin-D-like steroids) and their subsequent photodegradation served as a protective mechanism against ultraviolet radiation and oxidative stress. Consequently, direct cell-protective activities of vitamin D were proven. Furthermore, calcitriol triggers rapid calcium influx through epithelia and its uptake by a variety of cells. Subsequently, protein disulfide-isomerase A3 (PDIA3) was described as a membrane vitamin D receptor responsible for rapid nongenomic responses. Vitamin D was also found to stimulate a release of secondary massagers and modulate several intracellular processes—including cell cycle, proliferation, or immune responses—through wingless (WNT), sonic hedgehog (SSH), STAT1-3, or NF-kappaB pathways. Megalin and its coreceptor, cubilin, facilitate the import of vitamin D complex with vitamin-D-binding protein (DBP), and its involvement in rapid membrane responses was suggested. Vitamin D also directly and indirectly influences mitochondrial function, including fusion–fission, energy production, mitochondrial membrane potential, activity of ion channels, and apoptosis. Although mechanisms of the nongenomic responses to vitamin D are still not fully understood, in this review, their impact on physiology, pathology, and potential clinical applications will be discussed.

Keywords: vitamin D; VDR; nongenomic response; PDIA3; ultraviolet radiation; megalin; mitochondria; vitamin D analogs

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1. Vitamin D—Overview

Vitamin D is present in almost forms of life, from phytoplankton to humans, and is considered one of the oldest hormones on Earth [1–3]. In humans, the classic endocrine function of this powerful secosteroid is regulation of calcium–phosphorus homeostasis in order to maintain proper function of the body [4]. Initially, vitamin D has been described as a vitamin because it can be acquired from the diet, mainly from fatty fish, fish oil, milk products, and some mushrooms. However, for humans, the major and a natural source of vitamin D is its photochemical production by 7-dehydrocholesterol (7-DHC) [5]. This process is stimulated by ultraviolet type B (UVB) radiation (280–320 nm) and takes place in the basal layer of epidermis of the skin [6,7]. To be exact, photodegradation of the B-ring of 7-DHC resulted in production of three isomers: previtamin D, tachysterol, and lumisterol. Those byproducts are subjected to time-dependent thermal conversion to vitamin D, which—after release from the cells—enters the circulatory system and is transported to all organs by vitamin-D-binding protein (DBP) [8–10]. Regardless of the source, vitamin D requires two subsequent hydroxylations to achievement its full hormonal activity [3,5,11]. First, 25-hydroxylase (CYP2R1) facilitates the production of 25-hydroxyvitamin D_3 (25$(OH)D_3$) in the liver's hepatocytes. This is the major metabolite of vitamin D, and its serum level is widely used as an indicator of vitamin D status [3,12–15]. The second hydroxylation takes

place in proximal tubules of the kidney and requires activity of 1α-hydroxylase (CYP27B1). The final product is a fully active hormone—$1,25(OH)_2D_3$, calcitriol [16,17]. Serum levels of vitamin D are tightly regulated on two levels. Firstly, an excess of sun (ultraviolet radiation type B) leads to photodegradation of vitamin D and the formation of suprasterols [18]. Secondly, elevated levels of $1,25(OH)_2D_3$ stimulate the expression of CYP24A1, which is 24-hydroxylase and is responsible for the catabolism (deactivation) of both $25(OH)D_3$ and $1,25(OH)_2D_3$ [6]. Several other metabolites of vitamin D have been described in recent years, including 3-epi analogs [19–21], lactones [22], and CYP1A1 (CYP450scc) metabolites [23–26]. However, their biological function still is far from being understood.

$1,25(OH)_2D_3$ regulates a variety of cellular processes thought activation of a nuclear receptor—VDR. It binds to VDR, which forms a complex with its coreceptor RXR and is subsequently translocated into the nucleus. Upon activation, the VDR-RXR complex regulates expression from hundreds to more than 3000 genes in the human genome depending on cell type and physiological conditions [27–29]. Interestingly, recent studies have shown that not only $1,25(OH)_2D_3$ can affect the expression of genes. $25(OH)D_3$ also binds to VDR but with an affinity approximately 1000 times lower compared to $1,25(OH)_2D_3$. Transcriptomic analyses of human peripheral blood mononuclear cells (PBMCs) revealed similar patterns of genes affected by $25(OH)D_3$ treatment at 1000 or 10,000 nM concentrations compared to 10 nM $1,25(OH)_2D_3$ [30,31]. Similar overlapping patterns of expression were also observed in the prostate cancer cell line LNCaP [32]. Intestinally, the metallothionein 2A gene was identified as a unique target for $1,25(OH)_2D_3$ but not for $25(OH)D_3$ [33]. Other nonclassic metabolites of vitamin D, such as $20(OH)D_3$ and $20,23(OH)_2D_3$, not only interact with VDR but also target other transcription factors, including RORα and RORγ [34,35] or AhR [28]. These experiments led to the detection of unique genes regulated by alternative nuclear receptors. Furthermore, it seems that the genes coding transcription factors (TFs) are activated by $1,25(OH)_2D_3$, which in turn regulates the expression of additional sets of genes [36], which could be described as secondary genomic response. Recently, regulatory effects of $1,25(OH)_2D_3$ on the expression of microRNAs and long noncoding RNAs have been uncovered as a potential anticancer mechanism [37,38].

The main target of vitamin D is the regulation of the calcium–phosphate homeostasis, which contributes to maintaining proper bone health [1,11,13,39–41]. Furthermore, the presence of VDR is already well documented in the cells of various organs, and it was established that proper function of musculoskeletal, immune, nervous, and cardiovascular systems as well as regeneration of epithelial barriers strongly depends on vitamin D. Vitamin D deficiency ($25(OH)D_3$ level below 20 ng/mL) was not only described as a risk factor for rickets or osteoporosis [39,42] but also impairs the function of the immune system [43–47] and its response to pathogens, including influenza viruses [48] and coronaviruses (SARS-CoV-2) [49]. Vitamin D supplementation resulting in adequate level of vitamin D (preferably 30 ng/mL of $25(OH)D_3$ in the serum) improves muscle performance and reduces falls in vitamin-D-deficient older adults [50]. It also has a neuroprotective role [51,52] and prevents and reduces outcomes of psoriasis [53]. Furthermore, it was suggested that even higher levels of vitamin D (40–60 ng/mL of $25(OH)D_3$ in the serum) are beneficial, reducing the occurrence and severity of multiple types of cancer [23,54–60] and displaying neuroprotective [61] and cardioprotective properties [62,63]. Consequently, high doses of vitamin D were found to be beneficial in the prevention or treatment of several diseases, including cancer [23,54,56,58,59,64], neurodegenerative disorders, autoimmune diseases [43,45,65,66], psoriasis [67,68], and preeclampsia [69]. This must be of special interest because vitamin D deficiency is still a global problem [21,39,55,70–72]. Furthermore, seasonal changes in vitamin D levels that were observed pointed to the necessity of its proper supplementation, especially in the winter season [12,13,72].

In spite of the well-established role of VDR in the biological activity of vitamin D, not all effects of this powerful hormone could be liked to VDR-driven regulation of the expression of the genes. Firstly, some proven physiological effects can be observed within seconds or minutes after stimulation with vitamin D. These do not include activation of gene

expression and subsequent protein synthesis, which take at least a few. Thus, the so-called rapid nongenomic responses to vitamin D have been described. The presence of membrane receptor for vitamin D has been suggested as an analogy to other steroid hormones [73]. Interestingly, in spite of the fact that VDR is a nuclear receptor, its involvement was not fully excluded. However, rapid responses do not rely on its transcriptional activity [74]. In this review, I will discuss different aspects of alternatives to the genomic activities of vitamin D. Figure 1 visualizes our current understanding of the variety of cellular processes activated by vitamin D and its metabolites.

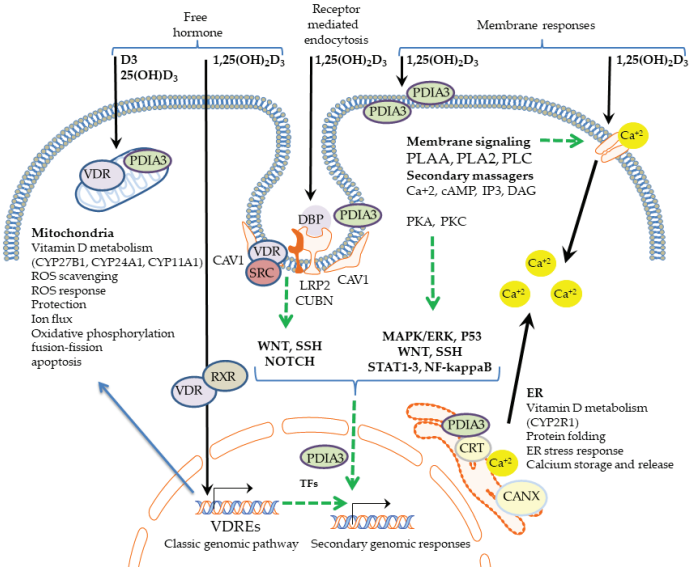

Figure 1. Vitamin-D-activated pathways. All secosteroids, including vitamin D_3, $25(OH)D_3$, $1,25(OH)_2D_3$, and other derivatives, enter the cell directly though the plasma membrane according to the free hormone hypothesis. Alternatively, $1,25(OH)_2D_3$ bound to DBP may be imported by the megalin/cubulin complex (LRP2-CUBN complex) via receptor-mediated endocytosis. Inside the cell, $1,25(OH)_2D_3$ binds the VDR-RXR complex, which is translocated into the nucleus and modulates the expression of targeted genes. Vitamin D metabolism takes place in the mitochondria, including activation of $25(OH)D_3$ by CYP27B1, resulting in formation of $1,25(OH)_2D_3$ and inactivation of both metabolites by CYP24A1. In mitochondria, vitamin D may act as ROS scavengers, thus protecting the mitochondria from damage. Vitamin D metabolites may also directly influence ion transport, oxidative phosphorylation, and ROS response or may do so indirectly through the genomic pathway (blue arrow). PDIA3 mediates $1,25(OH)_2D_3$-dependent membrane-signaling cascades. PDIA3 was detected on both sides of the cell membrane, on the endoplasmic reticulum, and in the cytoplasm, and its translocation to the nucleus was also postulated. PDIA3 mediates 1,25(OH)2D3-dependent activation of PLAA, PLA2, and PLC and the opening of Ca^{+2} and Pi (NaPi II a,c) channels. These result in the rapid accumulation of secondary messengers, including DAG, IP3, cAMP, and Ca^{+2}, followed by PKA, PKC, or CAMK2G activation and the alteration of several downstream targets, including MAPK pathways. VDR was found also to be colocalized with CAV1 and SRC in caveolae. Its membrane activity was linked with the downregulation of WNT, SSH, and NOTCH signaling pathways. Alone or together with VDR after stimulation with $1,25(OH)_2D_3$, PDIA3 modulates the transcription factors NF-kappaB, STAT3, and P53. At the endoplasmic reticulum, PDIA3—together with calreticulin (CRT) and calnexin (CANX)—facilitates protein folding. $1,25(OH)_2D_3$, through VDR transcriptional activity, induces several transcription factors (TFs) which are involved in secondary genomic responses to this multipotent hormone.

2. Direct Effects of Vitamin D on Calcium Transport

The idea of alternative pathways activated by vitamin D arose from an observation of rapid (1–10 min) influx of calcium induced by calcitriol in osteogenic sarcoma cell line ROS 17/2.8 [75] or myocytes isolated from chicken embryonic heart [76]. Consequently, transcaltachia was described as a rapid—measured in seconds to minutes—resorption of calcium by enterocytes in response to calcitriol [77]. Interestingly, the rapid influx of calcium requires as low as picomolar concentrations of calcitriol (0.13 nM [78] or 0.3 nM [79]). Later, activation of rapid nongenomic responses to calcitriol was confirmed in vivo in chicken [80], in cultured mice chondrocytes with VDR knockout, and in osteoblastic cell line ROS 24/1 deprived of VDR expression [81]. The time frame (seconds to minutes) of observed calcium transport excludes involvement of the genomic pathway of response to vitamin D. Thus, the idea of membrane response was suggested.

3. Transmembrane Transport of Vitamin D

It is still presumed, according to free hormone hypothesis, that vitamin D can freely diffuse through the membranes and does not require transporter or channel to enter the cells [82], as can other lipophilic steroids. However, only ~0.04% of $1,25(OH)_2D_3$ is considered to be a free hormone in circulation. Furthermore, the major circulating metabolite of vitamin D–$25(OH)D_3$ is mainly carried by vitamin-D-binding protein (DBP; ~85%) and albumin (~15%). Thus, only a fraction of $25(OH)D_3$ is free (~0.03%) and can be directly absorbed by the target cells. In an addition, the affinity of $1,25(OH)_2D_3$ to DBP is 10 to 100 times lower in comparison to $25(OH)D_3$ but is sufficient to be its main transporter [8]. This also raises an important question: How do vitamin D metabolites bound with high affinity to DBP become freed and enter into cells? It is well established that other hormones, including steroids and thyroid hormones are bound with corresponding globulins in circulation and require carrier proteins for efficient cellular uptake of their complexes [83]. Surprisingly, early studies revealed that deletion of DBP did not result in vitamin D deficiency under normal vitamin D supplementation, which strongly supported the free hormone hypothesis [84]. On the other hand, a deletion of megalin (LRP2) 600-kDa transmembrane glycoprotein, which functions as an endocytic receptor, results in vitamin D deficiency. Later studies reviled that megalin and two additional proteins—cubilin (CUBN) and disabled-2 (DAB2)—are required for effective endocytosis of DBP/vitamin D complexes. It was shown that deletion or deactivation of any of those proteins results in vitamin D deficiency, hypocalcaemia, and subsequent deterioration of bone structure and function [85–87].

Although expression of megalin, as well as megalin-mediated endocytosis of DBP protein, was shown in several tissues, including muscle [88], mammary gland [89], placenta, prostate, colon epithelium [90], and skin tissues, the best-studied model is epithelium of the proximal convoluted tubules of the kidney [91]. It was shown that the megalin complex is responsible for rapid and efficient resorption of small proteins, including DBP binding vitamin D, and this mechanism is necessary to maintain an optimal level of vitamin D in circulation. Interestingly, epithelial cells of proximal convoluted tubules express CYP27B1 and CYP24A1. Thus, the kidney is the primary location of both activation and deactivation of vitamin D [85,86,92]. Recent studies also postulated the role of megalin in membrane to mitochondria trafficking of various proteins (angiotensin II, stanniocalcin-1, and TGF-β) [93,94], which raises the question of whether the same mechanism could be utilized by vitamin D in complex with DBP. In summary, the role of the megalin-mediated transport of DBP-bound vitamin D metabolite in a rapid membrane response to this secosteroid and its mitochondrial targeting is currently under discussion and intensive investigation.

4. Membrane-Bound Receptor(s) and Targets for Vitamin D

As opposed to its genomic counterparts, nongenomic vitamin D responses appear rapidly (within a range of seconds or minutes) and are not susceptible to actinomycin D or cycloheximide. Therefore, they do not require expression of genes or protein synthesis [95].

Thus, a direct interaction of this secosteroid with membrane-bound or intracellular macromolecules was suggested as an acceptable explanation [96,97]. In fact, several steroids and other small regulatory molecules bind to membrane receptors in addition to their nuclear receptors, activating rapid responses [73,97]. Thus, in an analogy, an involvement of a G-coupled membrane receptor (GPCR) in vitamin D signaling was suggested, and its presence initially confirmed in membranous cell extracts [76,98]. Furthermore, the existence of membrane-associated activities of vitamin D was supported by observation of a rapid and efficient mobilization of the secondary messengers, including cAMP and calcium, triggered by $1,25(OH)_2D_3$ [75,79]. Other studies documented fast (15–300 s) activation of phospholipase C (PLC) and calcium influx in response to the stimulus in the ROS 24/1 osteoblastic cell line [98]. It must be underscored that VDR involvement should be excluded because ROS 24/1 cells lack VDR expression. Similar studies on cultured chondrocytes derived from VDR knockout mice confirmed this observation [81]. Unfortunately, no receptor of the GPCR or receptor tyrosine kinase families dedicated to vitamin D or its metabolites has been identified so far. Nevertheless, the early studies of Nemere et al. on transcaltachia [99] resulted not only in the discovery of rapid response to vitamin D and its metabolites but also in the detection of a membrane-associated protein that binds to radiolabeled $1,25(OH)_2D_3$ (KD-value 0.72 nM) [100]. The protein was initially described as 1,25D3-MARRS (membrane-associated rapid response to steroid), but it is also known as PDIA3 (protein disulfide isomerase family A member 3), GRP58, and ERp57 [101–108]. PDIA3 interacts with calreticulin (CALR) and calnexin (CANX) and plays a crucial role in the folding and export of proteins from the endoplasmic reticulum [80,109]. PDIA3 activity is essential for the proper function of the immune [74,110] and musculoskeletal systems [102,111] as well as for mammary gland growth and development [112]. Several groups have reported colocalization of PDIA3 with the cell membrane, cytoplasm, mitochondria, and even the nucleus [113,114]. Most importantly, PDIA3 was found to be involved in a rapid intestinal uptake of calcium and phosphate. Thus, its involvement in the classic function of $1,25(OH)_2D_3$ was postulated [79,80,100,105,107]. It was also confirmed that $1,25(OH)_2D_3$ binds to the a' domain of PDIA3, with an estimated KD of 1 nM [115]. Site-directed mutagenesis studies revealed that the catalytic side of PDIA3 (C406) and its calreticulin (CALR) interaction site (K214 and R282) are required for the rapid response to $1,25(OH)_2D_3$ [116]. Unfortunately, the nature of interaction of PDIA3 with vitamin D metabolites is not fully understood. The binding side for $1,25(OH)_2D_3$ was not fully characterized because only a partial crystal structure of PDIA3 is available [117]. However, several studies have strongly supported involvement of PDIA3 in vitamin D signaling. For example, although a deletion of PDIA3 (PDIA3+/-) is lethal in mice [108,110], its partial silencing ($PDIA3^{+/-}$) resulted in aberration in skeletal development [118], thus at least indirectly linking PDIA3 activity to calcium homoeostasis and vitamin D. Furthermore, targeted disruption of the PDIA3 gene results in an inhibition of rapid calcium transport and attenuates PKA or PKC signaling, thus impairing rapid nongenomic responses to $1,25(OH)_2D_3$ [107,108]. In an addition, PDIA3 was also shown to interact with PLA2-activating protein (PLAA), which led to activation of phospholipaseA2 (PLA2), while targeted disruption of the PDIA3 gene attenuates cellular Ca^{+2} influx through L-type Ca^{+2} channels [119]. Other studies pointed out that uptake of calcium by the ER through the Ca^{+2} pump SERCA2b is regulated by PDIA3 and vitamin D [120]. It should be recalled, however, that the deletion of VDR also impairs bone formation [118], but it seems that PDIA3 but not VDR is essential for fast response to vitamin D, including activation of protein kinase C (PKC) signaling pathway [121] or a protection against UV-induced thymine dimer formation and DNA damage [122]. Furthermore, chondrocytes with a VDR knockdown but expressing PDIA3 showed a rapid increase in PKC levels and accelerated proteoglycan production in response to $1,25(OH)_2D_3$. Thus, a distinct role for VDR and PDIA3 in $1,25(OH)_2D_3$-mediated proliferation control of rat growth plate chondrocytes was postulated [81].

Association of PDIA3 with cell membrane signaling is strongly supported by observation of its interaction within caveolin-1 [123], which is the main scaffolding protein of caveolae (small invaginations of the plasma membrane which play important role in membrane singling and transmembrane trafficking). This observation at least partially explains the nature of PDIA3-driven rapid response to vitamin D through the association of PDIA3 with cell membrane caveolae and the activation of downstream signaling, including calcium mobilization, IP3, DAG, and cAMP production. PDIA3 was also found to regulate other intracellular pathways. For example, it was shown that $1,25(OH)_2D_3$ triggers PDIA3-mediated rapid signaling cascade via CAMK2G (calcium/calmodulin dependent protein kinase II gamma), PLA2, PLC, and PKC, followed by an activation of mitogen-activated protein kinases (MAPK1 and MAPK2) [111,124]. Similarly, the PDIA3 membrane complex takes part in the rapid activation of WNT5A (Wnt family member 5A) by $1,25(OH)_2D_3$-stimulated calcium influx and generation of secondary messengers, such as cAMP and/or IP3 [101]. Finally, some reports suggested nuclear localization of PDIA3 [114,125]. It is well established that PDIA3 has a noncanonic ER retention signal, Q/KEDL, but the presence of a Lys-rich nuclear localization signal was also suggested [111]. However, other studies suggested that TNFα, but not $1,25(OH)_2D3$, triggers fast translocation of PDIA3 to the nucleus [126]. In an addition, an active form of vitamin D was shown to trigger translocation of the complexes of PDIA3, with other transcription factors including STAT3 [127–129] and NF-κB [130]. Furthermore, binding of PDIA3 to DNA in close proximity to the STAT3 binding site was shown, and its regulatory role in the expression of at least a subset of STAT3-dependent genes was postulated [129]. Interestingly, other groups also showed that PDIA3 binds to specific fragments of DNA [104,131] and is involved in the regulation and expression of genes, including MSH6, TMEM126A, LRBA, and ETS1 [104]. Finally, PDIA3 was also found to support trafficking of retinoic acid receptor alpha (RARA) into the nucleus and subsequent degradation in Sertoli cells [132]. However, the effect of vitamin D on this process still requires verification.

In summary, the nature of direct or indirect interactions of $1,25(OH)_2D_3$ with PDIA3 associated with membranes is still not fully understood since the biding side was not fully characterized. However, there is growing evidence that PDIA3 modulates the response to vitamin D. Furthermore, it is still under debate whether $1,25(OH)_2D_3$ triggers translocation of PDIA3 into the nucleus and whether PDIA3 can act as a transcription factor or if PDIA3 only facilitates the trafficking of other transcription factors [133].

5. Direct or Indirect Regulation of Ion Transport by Vitamin D Metabolites

Keeping in mind that one of the best-described fast nongenomic targets of vitamin D is calcium influx, it could be speculated that vitamin D and its derivatives bind directly to ion channels or proteins affecting ion transport across the membranes. For example, the binding of sulfated and glucoronated derivatives of $25(OH)D_3$ to the multidrug resistance proteins SLCO1B1 (OATP1B1) and SLCO1B3 (OATP1B3) was described [134]. On the other hand, it is well established that $1,25(OH)_2D_3$, through the genomic pathway, regulates expression of cell membrane transient receptor potential cation channels (TRPV1, 5, and 6), which at least in part explains the impact of vitamin D on calcium influx and cell proliferation [95,135–137]. Furthermore, $1,25(OH)_2D_3$ increases expression of K^+ two-pore domain channel subfamily K member 3 (KCNK3 expression) [138] but decreases the level of mRNA for potassium channels KCNH1 (Kv10.1) [139,140] and TASK-1 [138]. The electrophysiological studies on conductivity of ion channels showed that $1,25(OH)_2D_3$, at 100 nM concentration, acts as a mild agonist of the TRPV1 channel. In an addition, 25OHD and $1,25(OH)_2D$ can also act as inhibitors of capsaicin-induced TRPV1 activity [136,141]. Molecular docking studies suggested that $1,25(OH)_2D_3$ shared the capsaicin binding site at the vanilloid binding pocket of the TRPV1 [136]. Other studies suggested that the fast effect of $1,25(OH)_2D_3$ on an ion transport may be mediated through activation of L-type calcium channels. Calcitriol triggered an increase in the L-type calcium current and the fast transient outward K^+ current in myocytes. Furthermore, fast response to

vitamin D depended on protein kinase A (PKA) and was absent in myocytes derived from mice with VDR knockout. Subsequent, intracellular Ca^{2+} mobilization resulted in ventricular myocytes' contractility [142,143]. Finally, activation of chloride channels by vitamin D was found to be involved in the protection of human skin (ex vivo) from UV irradiation [144]. Interestingly, the modulation of mitochondrial potassium channels by $1,25(OH)_2D_3$ has been shown recently [145] (the effects of vitamin D on mitochondria are discussed in Chapter 8). These intriguing electrophysiological observations require further investigations in order to provide more mechanistic details and the physiological significance of a direct impact of vitamin D on ion transport.

6. Nongenomic Activity of VDR

It must be underscored that the time frame (seconds to minutes) of rapid membrane responses to vitamin D virtually excludes the involvement of the transcriptional activity of VDR. Furthermore, multiple studies on the cell lines or model organisms with deletion of VDR have strongly suggested that rapid responses to vitamin D do not require the presence of vitamin D receptor (VDR). Thus, it seems that PDIA3, but not VDR, regulates the nongenomic activities of vitamin D [119,121,122]. However, the requirement of VDR in nongenomic activities of vitamin D may be strongly affected by the experimental model used in the study (e.g., cell type specific, type of assay). Furthermore, direct or indirect interaction between VDR and PDIA3 should also be considered. Indeed, several studies reported a membrane localization of VDR as well as colocalization with PDAI3 and caveolin 1 (CAV1) [146]. Interestingly, some reports have suggested that PDIA3 may serve as molecular chaperone for VDR [115,116]. However, as it was postulated above, at least some effects of $1,25(OH)_2D_3$, such as rapid activation of PKC, are also observed in cells lacking VDR and thus are VDR-independent. On the other hand, a mutation within the isomerase catalytic side and removal of the KDEL motive (ER retention signal) of PDIA3 abolish this activity [116].

VDR was also detected in mitochondrial membranes [147–149] or even lipid droplets [150]. Interestingly, the existence of a second ligand-binding domain of VDR responsible for membrane signaling was postulated [27,151]. Although rapid activation of PLA2 was attributed to PDIA3, some other effects—such as $1,25(OH)_2D_3$ activation of the SRC (SRC proto-oncogene, nonreceptor tyrosine kinase) WNT pathway [152–155], sonic hedgehog signaling molecule (SHH) [156–160], and NOTCH [161,162] signaling pathways [155]—were found to be VDR-dependent. Interestingly, VDR and NF-kB share DNA binding sites that may explain the influence of vitamin D on immune response [163]. Taken together, VDR may be involved in the activation of at least some of the nongenomic activities triggered by vitamin D, most probably in cooperation with PDIA3 [101,102,111,116,124,132,146,164,165].

7. Is Vitamin D as a Scavenger of Free Radicals or Their Source?

It is established that UVB is a major factor contributing to skin malignancy, but at the same time, it is essential for epidermal vitamin D production [60]. It could be hypothesized that during evolution, vitamin D may be adopted in direct or indirect response to radiation. Alternatively, UVB-driven vitamin D production may represent its primary function, which protected primitive aquatic organisms from UV or oxidative stress long before development of its endocrine function as a calcium–phosphate regulator.

The concept of a direct involvement of vitamin D in response to UV originated from a simple question: Can one overdose on vitamin D by sun tanning? This issue was addressed more than 40 years ago by Micheal Holick et al. in the classic paper [18]. It was shown that prolonged exposure to UVB did not lead to excessive accumulation of vitamin D. In fact, surplus of vitamin D was fast and efficiently removed by further structural rearrangements involving production of 5,6-transvitamin D_3, suprasterols I and II in the skin [18]. Further studies revealed that irradiation of 5,7-dienes lead also to the formation of 5,7,9(11)-trienes with probable generation of a singlet oxygen, which may act as a pho-

tosensitizer [166,167]. Finally, it was shown that extensive irradiation of vitamin D and its analogs (such as isotachysterol) resulted in the formation of large groups of hydroxyl-, peroxy derivatives [18,168,169]. Detection of nonclassical products of UVB triggered photolysis of 5,7-dienes (7DHC and its natural or synthetic derivatives) might represent a natural mechanism of regulation of vitamin D levels as well as a cell protective mechanism. However, it is still not fully understood whether those generally unstable byproducts may be acceptors or also donors of reactive oxygen species (ROS). For example, accumulation of 5,7-dienes and 5,7,9(11)-trienes is important in the etiology of a 7Δ-reductase deficiency syndrome (Smith-Lemli-Opitz syndrome—SLOS) [166,167,170]. In this syndrome, the lack of the key enzyme converting 7DHC to cholesterol results in multiple metabolic defects with accumulation of 7DHC and its steroidal derivatives, including cholesta-5,7,9(11)-trien-3β-ol (9DDHC) [167] and pregna-5,7-diene-3b,17a,20-triol [170]. It was postulated that oxidation of 7DHC and 9DDHC leads to severe photosensitivity in SLOS patients with potential production of highly reactive singlet oxygen [166,171]. Nevertheless, it is still not known whether the potential phototoxicity of 7DHC derivatives is unique to SLOS patients or if it could be a general property.

Solar UV radiation reaching human skin damages cells and tissues directly or indirectly through the production of ROS, which are exemplified by superoxide and hydrogen peroxide [60,172,173]. UVB also induces DNA lesions, including formation of cyclobutane pyrimidine dimers (CPDs) [174], while UVB-generated ROS target guanine, leading to the subsequent generation of 8-oxo-7,8-dihydro-20-deoxyguanosine (8-OHdG) [55,175]. Unrepaired UV-induced DNA damage results in the accumulation of mutations, which contribute strongly to the formation of skin neoplasms [1,55,60]. On the other hand, it is well established that vitamin D protects skin cells from UVB-induced damage (including DNA damage and induction of inflammation), as was shown on various models, including in vitro, mice models, and human subjects [175–178]. Thus, it was postulated that cutaneous production of vitamin D protects skin cells from UV-driven carcinogenesis. The mechanism requires vitamin-D-induced upregulation of the genes involved in ROS response through VDR-mediated genomic pathways with additional activation of NRF2 [179]. However, PDIA3 was also shown to participate in cells' protection against the formation of thymine dimers after irradiation with UV [122]. The effect was associated with activation of PKC signaling and calcium influx [180]. In addition, $1,25(OH)_2D_3$ protected cells from UVR-induced thymine dimers via stimulation of chloride currents [144]. Photoprotective effects of $1,25(OH)_2D_3$ were also associated with an increase in p53 tumor-suppressor protein translocation to the nucleus and a decrease in the level of nitric oxide species (NOS). Vitamin D can also affect endothelial function through the nongenomic pathway through the induction of PDIA3-mediated calcium, cAMP, Akt, and PKC downstream signaling, which in turn affect endothelial NO synthesis (eNOS) activity [181]. Finally, the potential UV-protective effect of $1,25(OH)_2D_3$ and other vitamin D derivatives and analogues could be attributed to direct impact on mitochondria bioenergetics. It was postulated that the process of DNA repair induced by UV requires substantial energy expenditure, and $1,25(OH)_2D_3$ and its analogs may directly affect mitochondrial activities (discussed below).

In summary, although vitamin D photoproduction may represent the primary and still-existing mechanism of cell protection against UVB and ROS/RNS, it must be underscored that vitamin D hormonal activity, namely VDR-mediated induction of the genes involved in UV and ROS/RNS, responses is also crucial for cell survival. On the other hand, potential involvement of 7DHC derivatives with triene moiety, such as 9DDHC in ROS generation (pro-oxidative), may be unique to SLOS.

8. Mitochondria as a Target for Vitamin D

Mitochondria are not only the powerhouse of the cell but also a key organelle to vitamin D metabolism, including activation, modification, and inactivation by the cytochromes of the P450 family (CYP27A1, CYP27B1, CYP24A1, and CYP11A1) [6,182,183]. All of those enzymes are located in the inner mitochondrial membrane. Thus, at least theoretically, all

steps of the activation of vitamin D including hydroxylation in position 25 (CYP27A1) and position 1 (CYP27B1), as well as deactivation by hydroxylation at C-24, could be conducted within mitochondria. Although it must be acknowledged that CYP2R1 located in the endoplasmic reticulum is the major enzyme responsible for 25-hydoxylation, CYP27A1 also shows such activity [182,184]. Interestingly, CYP11A1 (CYP450scc—the major enzyme of steroidogenesis responsible for conversion of cholesterol to pregnenolone) was shown to also metabolize vitamin D, tachysterol, and lumisterol, which opens a new route of secosteroidogenesis [185–187]. There is no doubt that mitochondria are the direct target for vitamin D and its metabolites, but the mechanism of an entry, a regulation of vitamin D metabolism, or its direct impact on mitochondrial biogenesis or oxidative stress still requires an in-depth investigation. As was discussed previously, megalin may be responsible for vitamin D import to the cell. Interestingly, recent studies have suggested that megalin may also be involved in the translocation of some molecules directly to the mitochondria. Furthermore, mitochondrial megalin was found to be involved in the defense against ROS, and its knockout impairs mitochondrial respiration and glycolysis [93]. Megalin itself was colocalised with mitochondria in association with stanniocalcin-1 and SIRT3, which are involved in a defense against ROS [93]. Interestingly, it was shown that megalin is involved in the intracellular traffic and mitochondrial import of angiotensin II, stanniocalcin-1, and TGF-β [93], which raises the question of whether vitamin D and its metabolites, in concert with DBP, could be acquired and target mitochondria accordingly.

An active form of vitamin D also has indirect impact on mitochondria function through the VDR-dependent regulation of expression of genes involved in the oxidative stress response [145,183,188]. By using transcriptomic analyses focused on mitochondrial genes, it was discovered that $1,25(OH)_2D_3$ inhibits expression of the several genes involved in oxidative phosphorylation and fusion/fission and upregulates genes involved in mitophagy and ROS defense [188]. In seems that the activation of selected genes' coding proteins in response to ROS by $1,25(OH)_2D_3$ is mediated by nuclear-factor-erythroid 2-related factor 2 (NRF2) [179,188]. NRF2 is a key transcription factor that can bind to antioxidant response elements (AREs) on DNA and initiate transcription of the genes responsible for the protection of cells against oxidative stress associated with diabetic neuropathy [188].

An involvement of VDR in mitochondrial physiology was confirmed by observation; deletion of VDR affects mitochondrial membrane potential, enhances ROS production [147], and results in subsequent mitochondrial damage [189]. Removal of VDR also resulted in an increased expression of elements of the respiratory chain, such as cyclooxygenase 2 and 4 (COX2 and 4), the ATP synthase subunits (6MT-ATP6 and ATP5B) [147], and subunits II and IV of cytochrome c oxidase [134]. Interestingly, VDR was also found in mitochondria, but in contrast to its nuclear translocation, its import to mitochondria is most probably ligand-independent [148]. VDR was found to interact with the mitochondrial permeability transition pore (PTP), the voltage-dependent anion-selective channel (VDAC). Recently, it was shown that vitamin D regulates the activity of mitochondrial calcium channels. Furthermore, VDR may also directly impact cholesterol and vitamin D transport through the interaction with the steroidogenic acute regulatory (StAR) protein [148]. The mitochondrial localization of VDR was linked to the redirection tricarboxylic acid cycle (TCA) towards biosynthesis, which is the common feature of neoplastic rather than fully differentiated cells [189]. Thus, it seems that VDR may affect mitochondrial function both through the activation of classic genomic pathways and also directly inside of mitochondria, including interaction with the mitochondrial genome. Interestingly, the protective anti-ROS properties of $1,25(OH)_2D_3$ were shown on isolated mitochondria. Thus, the genomic effects of VDR activation may not be fully required [190]. It must also be underscored that $1,25(OH)_2D_3$ may protect mitochondria from potential toxic insults, as it was shown that it can attenuate the cytotoxicity induced by aluminum phosphide via inhibiting mitochondrial dysfunction and oxidative stress in isolated rat cardiomyocytes [191].

9. Clinical Implications of Nongenomic Pathways Activated by Vitamin D

It is well established that 20 ng/mL of 25(OH)D_3 in the serum is an adequate concentration for bone health. However, several studies and recommendations have suggested a concentration of vitamin D >30 ng/mL as optimal in order to provide extraskeletal benefits. Recently, it was postulated that even higher serum concentrations of vitamin D should be considered. For example, it was calculated that 37 ng/mL during pregnancy decreases the chance of complications in patients with risk of preeclampsia to values characteristic for the entire population [192]. Even higher concentrations (40–50 ng/mL) were proposed to be beneficial for extraskeletal outcomes of vitamin D, including its anticancer properties [193]. This leads to the significant change in recommendation concerning vitamin supplementation (800 IU vs. 4000 IU) and very high doses (50,000–100,000 IU) are recommended for patients with a severe vitamin D deficiency. However, potential hypercalcemia and hypercaluria must be considered as a potential side-effect of such as supplementation. However, several studies revealed that vitamin-D-induced hypercalcemia is observed usually in patients with defects in vitamin D metabolism (e.g., patients with *CYP24A1* mutation) [194]. This raises the very interesting question of whether an activation of the alternative nongenomic pathways is responsible for the additional phenotypical effects of vitamin D. It was shown that 1,25$(OH)_2D_3$ binds to VDR with an affinity of 0.1 nmol/L and to PDIA3 with an estimated Kd of 1 nmol/L [195]. Thus, it could be speculated that higher doses of vitamin D supplementation and higher (30 ng/mL or higher) serum concentrations of 25(OH)D_3 are essential for an effective activation of nongenomic pathways. Nevertheless, this speculation requires in-depth laboratory and clinical investigations.

The description of transcaltachia was historically the first indication of existence of nongenomic pathways activated by 1,25$(OH)_2D_3$ [100]. This rapid vitamin-D-stimulated transport of calcium through intestinal endothelium is an important factor regulating the level of calcium in the body. Another clinical implication of nongenomic pathways is direct scavenging of UV-generated ROS and RNS observed as early as 15–30 min after treatment of irradiated keratinocytes with 1,25$(OH)_2D_3$ (please see [73] for discussion). In an addition to the clear UV-protective effects of vitamin D (see also chapter 7), it was postulated that vitamin D protects against skin photocarcinogenesis [57]. 1,25$(OH)_2D_3$ also affects ERK, PARP-1, and p53 activities and takes part in DNA protection and repair [73]. It is well established that increased production of ROS and deregulation of cellular energetics is a characteristic feature of cancer progression and drug resistance [196]. Thus, vitamin D may directly inhibit carcinogenesis through ROS scavenging. In fact, is also know that activation of VDR-dependent genomic pathway protects cells by activation of transcription of the genes involved in the response to ROS and DNA-repair [197]. Involvement of genomic pathways in the anticancer activities of vitamin D and its derives is also supported by observation that expression of VDR expression decreases with cancer progression [24]. However, it was also postulated that the extraskeletal benefits of vitamin D, including effects on cardiovascular and autoimmune diseases and cancer, are observed at relatively high concentrations [198]. Indeed, it was recently estimated that an increase in the serum concentration of 25(OH)D from 10 to 80 ng/mL would decrease cancer incidence rates by 70 $\pm$ 10% [60]. Thus, it is tempting to suggest that higher doses of vitamin D are required for the activation of alternative pathways, but this still remains to be confirmed.

Another, striking nongenomic activity of 1,25$(OH)_2D_3$ was shown on models of collagen or ADP-induced platelet aggregation. Interestingly, the inhibitory effect of 1,25$(OH)_2D_3$ on platelet aggregation varied depending on the state of diabetes [199]. Although VDR was detected in platelets and associated with mitochondria, because platelets naturally lack cell nuclei [149], only activation of nongenomic pathways should be considered. In an addition, it was also shown that glycemic control was inversely associated with high platelet aggregation and low levels of 25$(OH)_2D$ in diabetic patients [199]. These findings provide important clinical implication of alternative pathways activated by vitamin D.

The impact of PDIA3 and alternative pathways activated by vitamin D was also shown on an experimental model of cholangiopathy induced by Abcb4 knockout in mice. Firstly,

additional silencing of VDR (VDR and Abcb4 double knockout) promotes a proinflammatory phenotype in cholangiocytes. Interestingly, vitamin D—or its analog, calcipotriol—treatment resulted in PDIA3-dependent reduction of inflammatory response [200]. Interestingly, PDIA3 was also found to be essential for the autolysosomal degradation of *Helicobacter pylori* [201]. Thus, activation of membrane response with potential involvement of PDIA3 may play a role in the regulation of immune response, including response against pathogens.

In melanoma, it was found that decreased expression of VDR correlates with poor prognosis. On the other hand, overexpression of PDIA3 was shown to be a poor survival factor in patients with clear cell renal cell carcinoma—ccRCC [127]. Thus, it seems that proper functioning of classic VDR-dependent pathways as well as activation of alternative pathways is essential in order to maintain body homeostasis as well as to protect from potential harmful insults, such as UVB or pathogen infection.

In summary, although genomic pathways activated by VDR still remain the major targets for vitamin D, with clear clinical implications (e.g., rickets), the existence of alternative pathways explains fast responses to vitamin D, and its direct impact on mitochondria and other intracellular processes. Unfortunately, the pleiotropic effects of vitamin D genomic activities, and the potential involvement of VDR in nongenomic regulation, hinder the studies of vitamin D membrane signaling, its effects on mitochondria, and the precise determination of the role of PDIA3 (Table 1).

Table 1. Genomic and membrane pathways activated by vitamin D.

	Genomic	**Membrane**
Time course	Delayed response (hours–days)	Fast response (minutes–hours)
Primary Mechanisms	$1,25(OH)_2D_3$ binds to VDR, which is translocated to the nucleus together with RXR, where the complex binds vitamin-D-responding elements (VDRE) of DNA.	$1,25(OH)_2D_3$ affect activity of membrane proteins including PDIA3 and direct or indirect activity of ion channels. ROS scavenging.
Primary effects	Transcriptional regulation of up to 3000 genes resulting in inhibition of cell proliferation, induction of cell differentiation, immunomodulation, UVB response, ROS response, and alteration of mitochondrial function.	Membrane responses with activation of secondary messengers (calcium, cAMP, IP3, DAG). Transcaltachia, modulation of mitochondrial bioenergetics. Direct cell protection against UV, ROS, and pathogens.
Secondary mechanisms	Alteration of the expression of several TFs results in activation of secondary genomic responses.	Activation of secondary messages may activate MAPK/ERK, P53, WNT, SSH, STAT1-3, and NF-kappaB genomic activities. PDIA3 was found in the nucleus. Thus, its function as transcriptional regulator is considered.
Secondary effects	A dissection of primary from secondary genomic effects still requires careful investigation. Potential secondary impact of membrane signaling on genes expression adds an additional complexity to vitamin D response.	Modulation of activity of signaling pathways MAPK/ERK, P53, WNT, SSH, STAT1-3, and NF-kappaB results in modulation of cell physiology as well as activation of secondary genomic responses.
Role of VDR	VDR act as a transcription factor.	VDR may be engaged in membrane signaling.
Role of PDIA3	Potential secondary genomic effect is considered as PDIA3 was found in the nucleus.	PDIA3 is involved in initiation of membrane signaling.
Role in calcium regulation	Change in the expression of the genes responsible for calcium homeostasis	Rapid direct, or indirect effect on calcium transport (transcaltachia)
Effect on mitochondria	Indirect though alteration of the expression of mitochondria related genes	Potential direct effect on mitochondrial proteins (including cytochromes P450) and potassium ion channels from the inner mitochondrial membrane
UVB protection	Indirect through activation of stress response genes and DNA repair mechanisms	Potential direct ROS scavenging
Immunomodulation	Inhibition of B cell differentiation and immunoglobulin secretion, shift from a Th1 to a Th2 response, induction of T regulatory cells, decrease of expression of proinflammatory cytokines, and stimulation of expression of antimicrobial peptides.	Regulation of calcium influx may affect immune cell physiology.

10. Beyond 1,25(OH)$_2$D$_3$

Although it is not the major scope of this review, potential biological activities of vitamin D metabolites or analogs must be acknowledged. For years, it has been believed that calcitriol is the only active metabolite of vitamin D. In fact, the binding of 25(OH)D$_3$ or 24,25(OH)$_2$D$_3$ to VDR is at least 100–1000 times weaker in comparison to 1,25(OH)$_2$D$_3$. Relatively recently, some alternative metabolic routes for vitamin D have been described, and some unique biological futures of those secosteroids have been proposed. In silico docking experiments showed the variable binding affinities to VDR of various alternative vitamin D metabolites or analogs. However, 1,25(OH)2D3 remained its main unquestioned ligand responsible for activation of the classic genomic response. It seems that alternative metabolites may alter or tune up or down transcription response to vitamin D. For example, 20(OH)D$_3$ and 20,23(OH)$_2$D$_3$ generated by CYP450scc (CYP11A1) inhibit the activity of key factor in immune response NF-kB [202] or stimulate ROS response through NRF2 [179,187] similarly to 1,25(OH)2D3. However, it is believed that in addition to VDR, they may also target other transcription factors, such as RORα and RORγ [34,35] or AhR [28]. For many years, lumisterol and tachysterol we considered nonfunctional isomers of vitamin D, but recent studies have proposed their biological activity and further metabolism, which opens a new chapter in the field of secostroids [187,203]. Thus, it seems that 1,25(OH)$_2$D$_3$ is not the only functional metabolite of vitamin D and that other secosteroids may help in the fine-tuning of vitamin D response or even replace 1,25(OH)$_2$D$_3$, but it seems that higher concentrations are required.

11. Conclusions

Keeping the pluripotent properties of vitamin D in mind, the presence of alternative intracellular pathways may help to explain a variety of responses [204]. VDR genomic activity of course plays the central role in 1,25(OH)$_2$D$_3$ signaling. However, it must be noted that from the evolutionary perspective, some primal functions, such as protection from ultraviolet radiation, should be considered. On the other hand, membrane response, including calcium mobilization, may provide a fast route of action for this powerful secosteroid. The detailed mechanisms underlying membrane nongenomic responses still remain to be elucidated. However, the role of PDIA3, as well as that of membrane-associated VDR, should be considered. Furthermore, direct or indirect interaction of vitamin D metabolites with other proteins including megalin, ion channels, and as their impact on mitochondria may help us to understand the versatility of its phenotypic effects [64,186,205]. However, further in vivo studies and randomized controlled trials [206] are required to confirm physiological and clinical importance of alternative pathways of vitamin D signaling.

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Review

Vitamin D-Mediated Regulation of Intestinal Calcium Absorption

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Abstract: Vitamin D is a critical regulator of calcium and bone homeostasis. While vitamin D has multiple effects on bone and calcium metabolism, the regulation of intestinal calcium (Ca) absorption efficiency is a critical function for vitamin D. This is necessary for optimal bone mineralization during growth, the protection of bone in adults, and the prevention of osteoporosis. Intestinal Ca absorption is regulated by 1,25 dihydroxyvitamin D ($1,25(OH)_2$ D), a hormone that activates gene transcription following binding to the intestinal vitamin D receptor (VDR). When dietary Ca intake is low, Ca absorption follows a vitamin-D-regulated, saturable pathway, but when dietary Ca intake is high, Ca absorption is predominately through a paracellular diffusion pathway. Deletion of genes that mediate vitamin D action (i.e., VDR) or production (CYP27B1) eliminates basal Ca absorption and prevents the adaptation of mice to low-Ca diets. Various physiologic or disease states modify vitamin-D-regulated intestinal absorption of Ca (enhanced during late pregnancy, reduced due to menopause and aging).

Keywords: diet; transcellular; absorption; diffusion; intestine; homeostasis; parathyroid hormone

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1. Introduction

It has now been 100 years since E.V. McCollum first identified a fat-soluble compound in food that supported bone growth and prevented rickets; he called this compound vitamin D. In the intervening century, many scientists have contributed to our understanding for how vitamin D regulates the physiology of calcium (Ca) metabolism. For example, in 1937 Nicolaysen showed that vitamin D is critical for intestinal Ca absorption [1] while later studies by Pansu et al. [2] and Sheikh et al. [3] showed that vitamin D deficiency significantly reduces intestinal Ca absorption. The critical breakthrough defining the mechanism used by vitamin D to regulate Ca metabolism came in the early 1970's when research by Holick et al. [4] and Norman et al. [5] isolated the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D$), from the intestine. Shortly thereafter, Brumbaugh and Haussler [6] discovered the nuclear receptor for $1,25(OH)_2D$, the vitamin D receptor (VDR), in intestinal mucosa as well. Since then, we've learned the detailed mechanism used by $1,25(OH)_2D$ to regulate gene expression [7] while global and conditional VDR knockout mice have allow us to study the function of vitamin D in Ca/bone metabolism and in other physiologic systems. As part of this effort, my research group has shown that the single most important role for vitamin D during growth is the regulation of intestinal Ca absorption [8] but that $1,25(OH)_2D$ signaling through the VDR has a broad array of target genes in the intestine [9]. Because of the central role that vitamin D plays in the regulation of intestinal Ca absorption, this review provides a critical starting point for anyone who wants to understand the physiologic importance of vitamin D.

2. Vitamin D Has a Critical Physiologic Role for Protecting Bone through the Regulation of Intestinal Ca Absorption

Bone mass is lost when dietary Ca intake is inadequate and so one usually thinks of bone when the term "Ca homeostasis" is used. However, Ca homeostasis is not regulated to maintain bone integrity. Instead, bone, the parathyroid gland, intestine, and kidney

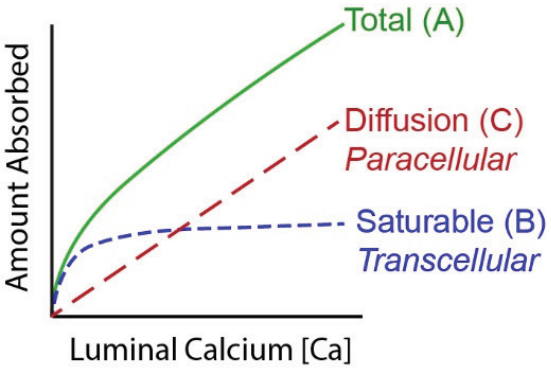
make up a multi-tissue axis that work together to maintain serum Ca within a narrow range (8.9–10.2 mg/dL). As a result, after a meal intestinal Ca absorption is a signal that disturbs and elevates serum Ca while bone formation and resorption, along with renal Ca excretion, respond to fluxes in serum Ca in an attempt to limit perturbations in serum Ca. This coordination was shown clearly by Bronner and Aubert who used Ca kinetics in rat models to show how the body adapts to habitual low Ca intake by increasing Ca absorption efficiency, reducing renal Ca loss, and mobilizing Ca from the bone (i.e., resorption) [10]. Pansu et al. [11] showed the impact of dietary Ca intake on intestinal Ca absorption directly when they found that feeding rats a 0.17%, low Ca diet for 5 weeks increased duodenal Ca absorption efficiency by increasing the saturable component of transport (Vmax increased 55%). Similarly, Norman et al. [12] found that in adult humans, feeding a diet with 300 mg of Ca/day for 8 weeks increased Ca absorption efficiency by 43% compared to subjects consuming 1600 mg Ca/day. These studies, and others like them, led to the identification of 1,25 dihydroxyvitamin D ($1,25(OH)_2$ D) and parathyroid hormone (PTH) as the major hormonal regulators of Ca homeostasis.

When dietary Ca is habitually low, there is a transient reduction in serum Ca that is sensed at the parathyroid gland through the Ca sensing receptor (CaSR). This cell surface receptor mediates signals into the parathyroid gland to increase the production and release of PTH into the circulation—a condition called nutritional secondary hyperparathyroidism. PTH has several important functions in Ca metabolism. First, it regulates renal production of $1,25(OH)_2$ D by inducing the CYP27B1 gene that encodes the enzyme 25 hydroxyvitamin D-1α hydroxylase [13] and it suppresses expression of the CYP24A1 gene that encodes the 25 hydroxyvitamin D-24 hydroxylase [14,15]. $1,25(OH)_2$ D released from the kidney is the most important regulator of increased intestinal Ca absorption. Of course, this physiologic adaptation has limits so that if the degree of dietary Ca deprivation is too great, the adaptation of intestinal Ca absorption will not be sufficient to compensate. This case, $1,25(OH)_2$ D and PTH will both promote bone resorption by stimulating osteoclastic activity while also enhancing renal Ca reabsorption in the proximal renal tubule. Collectively, this physiological adaptation can protect serum Ca but at the expense of bone mass.

The central role for vitamin D as a regulator of intestinal Ca absorption has been known for more than 80 years [1,16]. In vitamin D deficient animals intestinal Ca absorption efficiency falls by >75% [2]. Similarly, dialysis patients with low circulating $1,25(OH)_2$ D levels also have low intestinal Ca absorption [3]. Finally, in elderly adults, secondary hyperparathyroidism can maintain serum $1,25(OH)_2$ D (and Ca absorption) until serum 25-hydroxyvitamin D (25(OH)D) levels fall to $\leq$10 nmol/L, at which point there is not enough substrate to convert to $1,25(OH)_2$ D [17].

A challenging concept for many people examining Ca absorption is that it is not a single process but the sum of events that occur through two routes, a transcellular, saturable pathway and non-saturable, paracellular diffusion pathway [11,18–20] (see Figure 1). The relative importance of these two routes depends upon a person's habitual Ca intake. When Ca intake is low, like most adult American women [21], the saturable pathways predominates while when Ca intake is high the bulk of absorption occurs through the diffusional route. Absorption through these two routes can be modeled mathematically using a Michaelis–Menten-like equation that contains a linear component that models diffusion (Figure 1). The saturable transport pathway comprises three parts, apical membrane Ca entry, transcellular diffusion, and basolateral membrane extrusion. The apical membrane transport occurs down a concentration gradient while basolateral membrane extrusion is against a concentration gradient and requires energy [22]. The saturable pathway is present in the proximal small intestine (duodenum and jejunum), cecum, and colon [23–27] but is absent in the ileum [2]. Studies in rat duodenum [2] and in differentiated monolayers of the human intestinal cell line Caco-2 [28], show that $1,25(OH)_2$ D acts on the saturable transport component where it increases the Vmax (maximal absorptive capacity) but not Km (the affinity of the process for Ca). This suggests that $1,25(OH)_2$ D increases the production of intestinal Ca transporters (which we'll discuss below) but that this increase has limits.

In contrast, the passive Ca movement across the intestinal barrier occurs at ~13% of the luminal Ca level per hour in humans [20]) and is seen in all segments of the intestine. There is some evidence that the non-saturable portion of Ca absorption in the human ileum is also vitamin D sensitive; the slope of the non-saturable transport pathway is reduced in chronic renal disease patients, and it returns to normal after 1,25$(OH)_2$ D injection [20].



$$A = B + C$$

$$A = \frac{(Vmax * [Ca])}{(Km + [Ca])} + S * [Ca]$$

Figure 1. A Mathematical model of intestinal calcium (Ca) absorption. By studying Ca absorption over a range of luminal Ca levels it has been shown that the total amount of Ca absorbed across the intestinal barrier can be described as a curvilinear function. Total transport (A) is the sum of a saturable component (likely transcellular, B) that can be defined by the Michaelis–Menten equation and a diffusional process (C) that is defined by a straight line. [Ca] = luminal Ca concentration; S = the slope of the diffusional component; Vmax = the maximum transport rate seen for the saturable transport component; Km = the luminal concentration of the mineral at $\frac{1}{2}$ the Vmax.

In normal healthy adults, the Km for the saturable component of Ca absorption from the small intestine of adults is 3.3 mM, a concentration met by 265 mg Ca in a meal (calculated from data in [19,20]). As a result, when a person eats a meal with ~400 mg (1/3 the RDA for Ca), saturable Ca transport is about 60% of total Ca absorption. However, the apparent efficiency of total Ca absorption falls as the meal Ca intake level is increased and the diffusional component of absorption takes a larger role. Normally, the amount of Ca absorbed in each intestinal segment is determined by: (a) the presence of the saturable and non-saturable pathways, (b) the residence time in the segment, and (c) the solubility of Ca within the segment (e.g., lower Ca solubility at higher pH [29–31]) (Figure 2). Although 1,25$(OH)_2$ D clearly increases intestinal Ca absorption efficiency [2,32,33], some have argued that elevated serum 25(OH)D levels might also regulate intestinal Ca absorption. This is not supported by several large, well-designed studies that show no benefit of increasing serum 25(OH)D levels beyond 50 nmol/L [34–37].

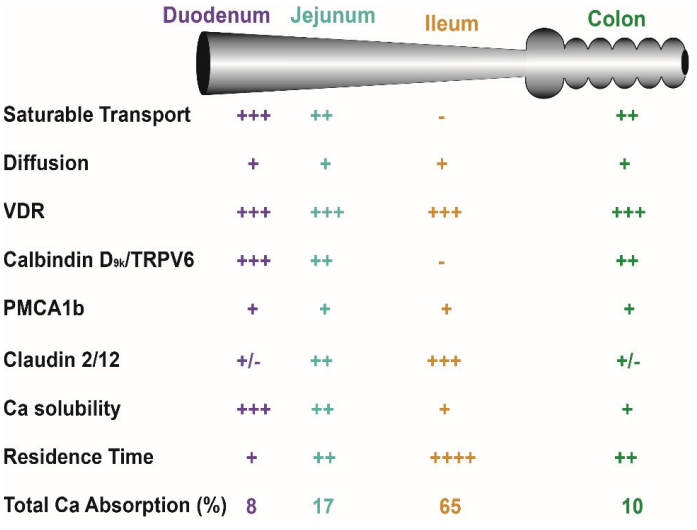


Figure 2. Critical factors that influence intestinal calcium (Ca) absorption. Many factors influence net Ca absorption and distinct Ca absorption mechanisms used in various intestinal segments. See Bronner and Pansu [38] for a discussion of solubility and transit time as factors affecting Ca absorption. The number of "+" signs reflects the magnitude of the parameter across tissues while a "-" sign indicates that the parameter is absent in a segment.

There are many studies that show adequate dietary Ca and vitamin D, and therefore total intestinal Ca absorption, is necessary for adequate bone growth. Deficiency of either Ca or vitamin D in growing children or animals causes nutritional rickets characterized by under-mineralized bone and low bone mass [39,40]. This is consistent with the concept that bone matrix cannot mineralize in the absence of mineral. However, net Ca absorption (which reflects both transport routes) is positively correlated to Ca balance in children, reflecting the critical role for Ca absorption in optimizing peak bone mass [41]. Consistent with this idea, we have shown that efficiency of Ca absorption through the saturable, vitamin-D-regulated pathway is significantly positively correlated with femoral trabecular bone volume/total volume in a genetically diverse population of 11 inbred mouse lines [42]. In addition, Patel et al. [43] reported that femur neck BMD was significantly positively correlated with Ca absorption efficiency in adult men. Several studies also indicate the high intestinal Ca absorption efficiency can protect against femoral bone loss in mice fed low Ca diets [44] or reduce the risk of osteoporotic hip fracture in women with low dietary Ca intake [45]. Collectively, these data show that both adequate Ca intake and genetically programed high intestinal Ca absorption efficiency are necessary to build and protect strong bones.

3. Vitamin D Effects on Intestinal Ca Absorption Are Mediated through the VDR

$1,25(OH)_2$ D regulates Ca metabolism and intestinal Ca absorption by regulating gene transcription, a process that requires binding of the hormone to the Vitamin D Receptor (VDR), a nuclear receptor that is a ligand-activated transcription factor [7,46]. A number of studies have shown the critical importance of VDR for the regulation of Ca and bone metabolism. For example, children with inactivating mutations in the VDR gene (i.e., type II genetic rickets) have defects in Ca metabolism that include lower intestinal Ca absorption efficiency [47]. Similarly, VDR knockout mice have severe defects in bone growth and mineralization as well as a >70% reduction in Ca absorption efficiency [48,49]. While the gross phenotype of VDR knockout mice is abnormal bone, several lines of evidence indicate that the most important role for VDR in Ca/bone metabolism during growth is the control

of intestinal Ca absorption. First, the phenotype of the intestine-specific VDR knockout mouse is identical to dietary Ca deficiency (osteomalacia, reduced serum Ca, elevated serum 1,25$(OH)_2$ D levels) [50]. Conversely, feeding high Ca/high phosphate/high lactose "rescue" diets that promote passive/diffusional intestinal Ca absorption can prevent the abnormal bone and Ca metabolism phenotype of VDR knockout mice [51]. Finally, experiments from my lab showed that the VDR knockout mouse phenotype (e.g., hypocalcemia, elevated serum PTH, low bone mineral density) can be completely prevented by intestine epithelial cell-specific, transgenic expression of VDR that normalizes intestinal Ca absorption [8].

Several studies show that lower intestinal VDR levels disrupt the physiologic response to 1,25$(OH)_2$D. In VDR KO mice, low level, intestine-specific transgenic VDR expression (10% of wild-type values) was insufficient to maintain normal intestinal Ca absorption [52]. Meanwhile, my lab has shown that a 50% reduction in intestinal VDR levels blunts 1,25$(OH)_2$ D-regulated intestinal Ca absorption efficiency [53]. Consistent with the idea that reduced VDR function impairs intestinal Ca absorption, several studies have shown that Ca absorption efficiency is reduced in people with the longer, less transcriptionally active "f" allele of the Fok I restriction fragment length polymorphism [54–56]. Collectively these data support the hypothesis that variations in VDR level or function can influence vitamin-D-regulated intestinal Ca absorption as well as optimal intestinal responses to the increased serum 1,25$(OH)_2$ D levels that accompany dietary Ca restriction.

4. Molecular Models of Ca Absorption

Ion microscopy reveals that Ca can enter at the apical membrane and flow through the absorptive epithelial cell in 20 min [57]. However, during vitamin D deficiency Ca becomes trapped in the region just below the microvilli. Treating vitamin D deficient chicks with 1,25$(OH)_2$ D reverses this effect starting 2–4 h after treatment [58], consistent with the induction of gene expression mediated through the VDR. In 1986 Bronner et al. [59] critically reviewed transport data from a wide variety of well-controlled mechanistic studies, and from this analysis built the facilitated diffusion model (Figure 3, with protein distributions across the intestinal tract in Figure 2). In the first step of this model, brush border membrane uptake of Ca is mediated by an apical membrane Ca channel, which was later identified as the transient receptor potential cation channel vanilloid family member 6 (TRPV6, originally called CaT1 or ECAC2) [60]. TRPV6 gene expression is strongly regulated by 1,25$(OH)_2$ D in the duodenum of mice [33,61] and in Caco-2 cells [62] and this induction is mediated by VDR binding enhancers upstream from the transcription start site [63,64]. Induction of TRPV6 mRNA precedes the increase in duodenal Ca absorption that occurs following 1,25$(OH)_2$ D injection [33]. While initial studies suggested that 1,25$(OH)_2$ D-mediated Ca absorption was not reduced in TRPV6 knockout mice [65,66], later studies showed that TRPV6 knockout mice [66], and mice with a D541A variant TRPV6 that inactivates Ca movement through the channel [67], had a blunted ability to increase Ca absorption in response to feeding a low Ca diet. In addition, my lab has shown that intestine-specific transgenic expression of TRPV6 increases Ca absorption efficiency and that this prevents the abnormalities in bone/Ca metabolism of VDR knockout mice [68].

An alternative model for apical membrane Ca uptake during Ca absorption is that Ca flows through the L-type Ca channel $Ca_v1.3$ (Figure 3), a transporter activated by glucose-induced membrane depolarization following a meal (reviewed in [69]). However, several studies do not support a physiologic role of $Ca_v1.3$ for vitamin-D-regulated Ca absorption in growing mice [70,71]. In contrast, other studies suggest that $Ca_v1.3$ may have a prolactin-regulated role in transcellular Ca transport during lactation [72] and contributes to Ca absorption prior to the development of vitamin-D-regulated Ca absorption in the neonatal mouse [73].

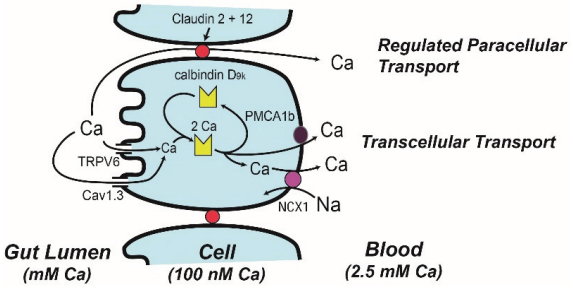


Figure 3. A Model describing intestinal calcium (Ca) absorption. The transcellular absorption pathway is described by the facilitated diffusion model while a regulated paracellular transport mechanisms mediated by claudin 2 and claudin 12 provides selectivity for Ca movement through the tight junction complex. For details of how vitamin D regulates various aspects of these models refer to the text.

The central player in the facilitated diffusion model is calbindin-D, a cytoplasmic Ca binding protein [59] found in intestine (the 9 kd form, calbindin D_{9k}) and the kidney (the 28 kd form, calbindin D_{28k}) [74] (Figures 2 and 3). This was based on studies that show: (a) intestinal calbindin D_{9k} protein levels are positively correlated to Ca absorption [59], (b) intestinal calbindin D_{9k} levels are significantly reduced in vitamin D deficient animals and in VDR knockout mice [48,75], (c) $1,25(OH)_2$ D injections increase intestinal calbindin D_{9k} levels [76], and (d) theophylline-mediated inhibition of Ca binding to calbindin D_{9k} disrupts intestinal Ca absorption [77]. These observations led to the hypothesis that calbindin D acts as a ferry for intracellular Ca movement during Ca absorption [57,78]. In contrast, other studies indicate that calbindin D_{9k} is not essential for intestinal Ca absorption but may instead act as an intracellular Ca buffer that protects cells from increases in intracellular Ca during Ca absorption. For example, neither basal nor $1,25(OH)_2$ D-induced Ca absorption are reduced in calbindin-D_{9k} null mice [66,79]. In contrast, $1,25(OH)_2$ D-induced Ca absorption is reduced by 60% in calbindin-D_{9k}/TRPV6 double knockout mice [66], suggesting the interaction of TRPV6 and calbindin-D_{9k} has a special role in Ca absorption. Another observation that suggests elevated calbindin levels alone are not sufficient to drive intestinal Ca absorption is that calbindin-D protein remains elevated in the intestine even after $1,25(OH)_2$ D-induced Ca absorption returns to normal in chicks [80] and mice [33]. Finally, we have observed that intestinal calbindin-D_{9k} levels increase in intestine-specific TRPV6 transgenic mice with elevated intestinal Ca absorption efficiency even in VDR knockout mice [68]. This suggests that calbindin-D_{9k} is an intracellular Ca buffer that increases in response to elevated transcellular Ca absorption and that it is not induced to act as a facilitator of transcellular Ca movement.

The final step in the facilitated diffusion model is the extrusion of Ca from the cell. This is an energy dependent process [22] mediated by the plasma membrane Ca ATPase 1b (PMCA1b) [81,82] (Figures 2 and 3). Deletion of the PMCA1b gene (Atp2b1), or the 4.1R protein that stabilizes PMCA1b in the basolateral membrane, reduces basal and $1,25(OH)_2$ D-induced intestinal Ca absorption [83,84]. While some suggest that the basolateral extrusion of Ca is also be mediated by a sodium-Ca exchanger [85], disrupting the sodium gradient necessary for sodium-Ca exchange did not block duodenal Ca transport [22].

Most intestinal Ca absorption research has focused on vitamin-D-regulated saturable Ca transport but several studies have shown that vitamin D signaling can increase Ca diffusion across the jejunum and ileum [20,86]. Tudpor et al. [87] found that $1,25(OH)_2$ D induced Ca ion movement across the intestinal barrier by a solvent drag mechanism that may involve charge selectivity of the tight junction. This is similar to the role of paracellin 1 (aka claudin 16), a tight junction protein that regulates ion-specific movement of magnesium and Ca in the kidney [88]. Consistent with this idea, $1,25(OH)_2$ D treatment significantly increased claudin-2 and 12 mRNA levels in Caco-2 cells and siRNA against these claudins

reduced Ca permeability across Caco-2 cell monolayers [89]. In vivo, claudin 2/claudin 12 double knockout mice (but not single KO mice) have reduced Ca absorption across the colon but not small intestine [90]. Claudin-2 and -12 expression is highest in the distal small intestine [91] but essentially absent from the duodenum. A role for claudins in paracellular Ca transport may explain why the non-saturable component of ileal Ca absorption was reduced in chronic kidney disease patients with low serum $1,25(OH)_2$ D levels [20].

Two other models have been presented to explain vitamin D-mediated, transcellular intestinal Ca absorption: vesicular transport and transcaltachia. The vesicular transport model is an alternative to the role proposed for calbindin-D as a Ca ferry during transcellular intestinal Ca absorption. This is based on the observation that $1,25(OH)_2$ D treatment increased the activity and cycling of lysosomes [92,93], that Ca accumulates within brush border membrane endosomes [94] and in lysosomes [95] during Ca absorption, and that disrupting lysosomal pH prevents lysosomal Ca accumulation and blocks Ca absorption [95,96]. While these data suggest that vesicular movement may be a legitimate pathway for uptake and movement of Ca through the intestinal epithelial cell, it is not clear what makes the vesicular transport pathway specific for Ca. Transcaltachia is a mode of Ca transport that occurs within minutes of exposing the basolateral side of enterocytes to physiologic levels of $1,25(OH)_2$ D. Transcaltachia has been directly demonstrated in the perfused chick duodenum [97]. Some data suggests transcaltachia results from $1,25(OH)_2$ D binding to a unique, alternative ligand binding pocket [98,99] in VDR within caveolae [100], i.e., a novel non-nuclear role for the receptor. Other data suggest the basolateral membrane protein mediating transcaltachia is a multi-functional Membrane Associated Rapid Response Steroid receptor (MARRS). However, while intestine-specific deletion of MARRS in mice reduced cellular $1,25(OH)_2$ D binding, disrupted $1,25(OH)_2$ D regulated Ca and phosphate uptake into enterocytes [101,102] and reduced basal Ca absorption in by 30% [103], these reports have not reported the physiological impact of MARRS deletion on bone density. Additionally, the rapid fluxes in serum $1,25(OH)_2$ D needed for transcaltachia have not been reported during the consumption of Ca-rich meals when transcaltachia would have to occur for the physiologic benefit of the process to be realized. As such, transcaltachia is not a generally accepted mechanism for vitamin-D-regulated intestinal Ca absorption.

5. Physiologic Regulation of Vitamin D-Mediated Intestinal Ca Absorption

As it has been described above, the major physiologic condition where vitamin D signaling is engaged to regulate intestinal Ca absorption is the habitual consumption of a low Ca diet. However, there are a number of other physiologic states that affect vitamin D metabolism or action to influence intestinal Ca absorption, i.e., growth and development, pregnancy/lactation, and aging.

The bulk of mechanistic studies on intestinal Ca absorption have been conducted in growing 2–3-month-old rodents, but recent studies in mice indicate that vitamin D-mediated Ca absorption is not important prior to weaning [73]. This is not completely surprising as VDR is not expressed prior to 14d postnatally in rodents [104,105]. Ca absorption studies in premature infants also suggests that Ca absorption during late development is vitamin D independent [106]. Research suggests that during childhood and adolescence, growth hormone (GH) and its physiologic mediator insulin-like growth factor I (IGF-1) promote intestinal Ca absorption in two ways. The first effect is through activation of renal CYP27B1 and the elevation of serum $1,25(OH)_2$ D levels [107]. However, in adult animals, GH treatment increases intestinal Ca absorption without significantly increasing serum $1,25(OH)_2$ D levels [108]. It does so by modulating intestinal VDR levels and increasing cell sensitivity to $1,25(OH)_2$ D [109]. The effect of GH on Ca absorption is likely mediated through IGF-1 but the intestinal actions of IGF-1 may also be independent of vitamin D signaling [49,110].

Dietary Ca requirements increase significantly during the third trimester of pregnancy and during lactation to meet the needs of the fetus and term infant. While pregnancy

causes a vitamin D-independent increase in Ca absorption whose mechanism is not clearly understood [111–114], during late pregnancy serum $1,25(OH)_2$ D levels and intestinal Ca absorption are both elevated [115]. This is because of PTH-independent $1,25(OH)_2$ D production by the placenta [116]. Ca absorption is also regulated during lactation in rodents (but not humans) but this is due to a prolactin-dependent mechanism [117,118]. However, prolactin cooperates with $1,25(OH)_2D_3$ to regulate intestinal Ca transport and the expression of TRPV6 and calbindin-D_{9k} in rats [119], suggesting prolactin acts together with $1,25(OH)_2D_3$ to increase active intestinal Ca absorption.

Aging reduces Ca absorption efficiency [120–125]. Yet, despite the fact that age-associated Ca malabsorption was discovered 50 ago, we still do not know the molecular mechanism underlying this phenomenon. Lower serum $1,25(OH)_2D$ levels in the elderly has been reported in some studies [126,127] but not others [128]. In fact, some research indicates that serum $1,25(OH)_2$ D is higher in older subjects even though fractional Ca absorption is not changed [128–130]. Similar age-associated intestinal resistance to $1,25(OH)_2$ D signaling has been formally demonstrated in rats [124] and humans [125]. Some evidence suggests that this phenomenon may be caused by lower intestinal VDR levels [130–132] but after adulthood is reached, age-related declines in intestinal VDR content are modest (−20%) [130,131] or non-existent [124]. Consistent with the lack of an impact of age on VDR expression, we recently reported that the open chromatin regions that control the expression of the intestinal VDR gene are not different between 3- and 21-mo-old mice, [104]. Thus, while my research group [53] has shown that a 50% reduction in intestinal VDR level blunts the intestinal response to elevated serum $1,25(OH)_2D$ levels, the inconsistency in the reports on the impact of age on intestinal VDR levels suggests that other mechanisms may contribute to age-associated intestinal resistance to vitamin D.

Another aspect of aging that could negatively impact vitamin D metabolism or intestinal regulation of Ca absorption is the decline in sex hormone levels. Consistent with this, estrogen loss severely disrupts Ca metabolism in post-menopausal women, including reducing Ca absorption [133,134]. While estrogen signaling directly regulates intestinal Ca absorption [135–137], it also enhances the intestinal responsiveness to $1,25(OH)_2$ D [138]. Some [109,139,140], but not all [141], studies report that low estrogen levels reduce intestinal VDR levels and that this is responsible for intestinal vitamin D-resistance following estrogen loss. In prepubertal boys, testosterone therapy increased intestinal Ca absorption by 61% [142] and this was accompanied higher serum IGF-1 levels that might influence vitamin D metabolism. As men age, both Ca absorption efficiency and serum levels of the sulfated form of the testosterone prohormone DHEA, dehydroepiandrosterone sulphate (DHEAS), fall significantly [143]. However, the change in Ca absorption was independent of changes in serum $1,25(OH)_2$ D, suggesting changes in androgen signaling do not alter vitamin D metabolism. It is not known if testosterone regulates intestinal VDR levels.

6. Conclusions

A large amount of data supports the conclusion that adequate vitamin D status and adequate production of the metabolite $1,25(OH)_2$ D are needed to support transcellular, saturable intestinal Ca absorption. $1,25(OH)_2$ D regulates intestinal biology by activating the VDR to stimulate gene expression. This increases the maximum capacity of the Ca transport system by increasing levels of a transporter that mediates saturable, transcellular Ca transport. The saturable, vitamin-D-regulated component of intestinal Ca absorption plays a significant role in maintaining Ca absorption efficiency because in most individuals, serum $1,25(OH)_2$ D levels are elevated by habitually low dietary Ca intake that is common in the general population. The exact mechanism that describes Ca movement through the enterocyte is still in question. The facilitated diffusion model is the best characterized mechanism but there are some inconsistencies in the model that must be resolved through additional research. In contrast, research supports a model for regulated paracellular Ca movement through tight junctions that predominates across the ileum and in the early

postnatal period. Overall, the data suggest that vitamin D signaling regulates intestinal Ca absorption by different mechanisms that are segment specific.

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Vitamin D and Diseases of Mineral Homeostasis: A *Cyp24a1* R396W Humanized Preclinical Model of Infantile Hypercalcemia Type 1

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Abstract: Infantile hypercalcemia type 1 (HCINF1), previously known as idiopathic infantile hypercalcemia, is caused by mutations in the 25-hydroxyvitamin D 24-hydroxylase gene, *CYP24A1*. The R396W loss-of-function mutation in *CYP24A1* is the second most frequent mutated allele observed in affected HCINF1 patients. We have introduced the site-specific R396W mutation within the murine *Cyp24a1* gene in knock-in mice to generate a humanized model of HCINF1. On the C57Bl6 inbred background, homozygous mutant mice exhibited high perinatal lethality with 17% survival past weaning. This was corrected by crossbreeding to the CD1 outbred background. Mutant animals had hypercalcemia in the first week of life, developed nephrolithiasis, and had a very high 25(OH)D_3 to 24,25$(OH)_2D_3$ ratio which is a diagnostic hallmark of the HCINF1 condition. Expression of the mutant *Cyp24a1* allele was highly elevated while *Cyp27b1* expression was abrogated. Impaired bone fracture healing was detected in CD1-R396$^{w/w}$ mutant animals. The augmented lethality of the C57Bl6-R396W strain suggests an influence of distinct genetic backgrounds. Our data point to the utility of unique knock-in mice to probe the physiological ramifications of CYP24A1 variants in isolation from other biological and environmental factors.

Keywords: infantile hypercalcemia type 1; idiopathic infantile hypercalcemia; CYP24A1; 25-hydroxyvitamin D 24-hydroxylase; hypercalcemia; 24,25-dihydroxyvitamin D_3; fracture repair

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1. Introduction

Idiopathic infantile hypercalcemia (IIH) was first reported by Lightwood in the 1950s in the United Kingdom. An elevation in the number of IIH cases was observed following the introduction of an increased prophylactic dose of vitamin D in infant formula and milk products [1,2]. The infants were found to have high levels of serum and urinary excretion of calcium, physiological levels of serum phosphate and magnesium, and occasionally low serum alkaline phosphatase (ALP). Reducing the vitamin D dose by half lowered the observed incidence. Because some infants were not affected by treatment, Lightwood later hypothesized that the disease was caused by fluctuation in infants' sensitivity to vitamin D.

The general manifestations of this rare disease are high serum calcium and high urinary calcium loss, failure to thrive, vomiting, constipation, polyuria with dehydration, and Ca deposits in the kidney, with susceptibility to renal complications such as nephrolithiasis or nephrocalcinosis. Affected infants may also manifest muscular hypotonia and lethargy, which leads to growth impairment, with delays in mental and mobility development [3].

Affected adults can also manifest some extra-renal pathologies, such as Ca deposits in the joints or the cornea, and low bone mineral density with osteoporosis, mainly due to enhanced osteoclastic activity [4,5]. The genetic cause of IIH remained unknown until Schlingmann and coworkers identified it in 2011. They found inactivating mutations in *CYP24A1* that drive the disease evolution [6].

Since mutations in the *SLC34A1* (solute carrier family 34 member 1, a type II sodium-phosphate cotransporter) gene cause a similar clinical presentation [7], the nomenclature has been changed to infantile hypercalcemia type 1 for *CYP24A1* mutations (HCINF1, OMIM 143880) and infantile hypercalcemia type 2 for *SLC34A1* affected patients (HCINF2, OMIM 616963).

To date, more than 50 disease-causing mutations of *CYP24A1* have been described in the literature [8–13]. Alterations of the *CYP24A1* sequence can impact CYP24A1 structure and activity in different ways. Mutations are predicted to alter the substrate or heme binding site, the binding of adrenodoxin, access of the substrate or exit of the product, or generally to affect protein folding [6,14–17].

The currently available *Cyp24a1* knockout strain [18] developed more than 20 years ago exhibits hypercalcemia, hypercalciuria, and nephrocalcinosis and could represent a model of HCINF1. Supporting this view, it was shown that surviving *Cyp24a1*-null animals possess a much-reduced ability to clear a bolus dose of [1β-^{3}H]1α,25-$(OH)_2D_3$ compared with wild-type littermates [19]. However, it should be noted that the *Cyp24a1*-deficient strain was generated with a selection cassette inserted in the opposite orientation to replace the heme-binding exon [18]. The *Cyp24a1*-deficient mice from this strain does not express any *Cyp24a1* message [18]. This contrasts with the situation in patients who, in many cases, express the mutated *CYP24A1* mRNA and protein [16]. Moreover, some of the *CYP24A1* mutations identified in HCINF1 patients are hypomorphic mutations, demonstrating residual or altered activity [6]. There is thus a need to generate improved preclinical animal models of infantile hypercalcemia type 1 based on human mutations to better understand the effects of dysfunctional vitamin D metabolism in HCINF1 patients.

We have introduced the site-specific R396W mutation within the murine *Cyp24a1* gene in knock-in mice. The R396W loss-of-function mutation is the second most frequent mutated allele observed in affected HCINF1 patients [8]. Profiling vitamin D metabolites using a sensitive LC-MS/MS-based assay demonstrated that the R396W strain is a valid preclinical model of HCINF1.

2. Materials and Methods

2.1. Generation of the R396W Knock-in Strain

All animal procedures were reviewed and approved under Animal Use Protocol number 7470 by the Shriners Hospitals for Children—Canada Institutional Animal Care and Use Committee and followed the guidelines of the Canadian Council on Animal Care. Mice were maintained in an environmentally controlled barrier animal facility with a 12 h light, 12 h dark cycle, and had access to mouse chow and water ad libitum.

The R396W knock-in embryonic stem cells were generated by Cyagen Biosciences (Santa Clara, CA, USA) under contract. A confirmed targeted clone was injected into C57Bl6/N blastocysts. Chimeras produced by blastocyst injection of targeted embryonic stem cells transmitted the knock-in mutation to their progeny and heterozygous mice were interbred to obtain all three genotypes (wild-type: C57Bl6-R396$^{+/+}$; heterozygous: C57Bl6-R396$^{+/w}$; homozygous mutant: C57Bl6-R396$^{w/w}$). For phenotyping, mice were sacrificed at postnatal Day 7 (P7) and at 4 months of age.

The C57Bl6-R396W strain was also outbred to CD1 mice to generate the CD1-R396W strain. The phenotype of these mice was analyzed at 3 months of age.

2.2. Genotyping

All mice were genotyped using genomic DNA isolated from carcasses found in cages prior to weaning or from ear punches at 3 weeks of age. For the R396W strains, the different

genotypes were determined using a custom TaqMan single-nucleotide polymorphism (SNP) genotyping assay from Applied Biosystems (Life Technologies, Foster City, CA, USA) with *Cyp24a1* primers (forward: 5′-GCTTACCCCAAGTGTGCCATT-3′ and reverse: 5′-CCAGAACGGTTGGCTTGTC-3′) flanking the single-point mutation site. The 396R SNP primer (VIC-conjugated) was 5′-AAGGGTCCGAGTTGTG-3′ and the 396W SNP primer (FAM-conjugated) was 5′-AAGGGTCCAAGTTGTG-3′ (mutated nucleotide underlined).

2.3. Survival Analysis

Cages were monitored daily until weaning (day 21) and when found, cadavers/remains were collected for genotyping. The percent survival curves per genotype were graphed using GraphPad Prism (San Diego, CA, USA) version 7.04.

2.4. Serum Biochemistry

Total serum calcium was measured using an automated analyzer. Serum levels of vitamin D metabolites were assayed by LC-MS/MS following 4-(2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl)ethyl)-1,2,4-triazoline-3,5-dione (DMEQ-TAD) derivatization as described previously [20]. Briefly, serum aliquots and calibrators were diluted 1:3 with water and spiked with internal standards. Proteins were precipitated by sequentially adding 0.1 M HCl, 0.2 M zinc sulfate, and methanol, with vortexing after the addition of each component. Tubes were centrifuged 10 min at 12,000× *g* and supernatants were transferred to borosilicate glass tubes. Organic extraction was carried out by adding equal volumes of hexane and methyl tertiary butyl ether with vortexing after the addition of each component. The upper organic phase was transferred into LC-MS/MS sample vials and evaporated under nitrogen flow. Dried residues were derivatized by addition of 0.1 mg/mL DMEQ-TAD dissolved in ethyl acetate for 30 min at room temperature in the dark, then a second time for 60 min. The reaction was stopped by addition of ethanol, samples were dried and redissolved in 60:40 methanol:water running buffer. LC-MS/MS analysis was performed using an Acquity UPLC connected in line with a Xevo TQ-S mass spectrometer in electrospray positive mode (Waters). Chromatographic separations were achieved using a BEH-Phenyl UPLC column (1.7 µm, 2.1 × 50 mm) (Waters) and methanol/water-based gradient solvent system. Simultaneous assay of $1,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$ was possible based on cross-reactivity of an anti-$1,25(OH)_2D_3$ antibody slurry (Immundiagnostik, Manchester, NH) with $1,24,25(OH)_3D_3$. The serum was incubated with 100 µL of anti-$1,25(OH)_2D_3$ antibody slurry [21] for 2h at room temperature with orbital shaking at 1200 rpm. The slurry was isolated by vacuum filtration and vitamin D metabolites were eluted, derivatized with DMEQ-TAD, and separated using a longer LC step as previously described [22,23].

2.5. Microcomputed Tomography of Whole Kidneys

Kidneys were harvested from mice at P7 and fixed in 4% paraformaldehyde diluted in PBS for 24 h, washed two to three times, and then stored in 70% ethanol. Kidneys were scanned with high-resolution microcomputed tomography using a SkyScan model 1272 scanner (Bruker, Kontich, Belgium).

2.6. Gene Expression Monitoring

Kidneys were harvested at P7 and 4 months, dissected free of surrounding tissue, immersed in RNA later (Ambion, Austin, TX, USA), and stored at −80 °C until ready for testing. Quantitative gene expression was assessed by real-time reverse transcriptase polymerase chain reaction (RT-qPCR). Briefly, kidneys were homogenized in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was isolated according to the manufacturer's protocol. One (1) µg of RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies Applied Biosystems, Waltham, MA, USA). Real-time qPCR using TaqMan universal PCR master mix and gene-specific Taqman assays for *Cyp24a1* (Mm00487244_m1), *Cyp27b1* (Mm01165918_m1), vitamin D receptor (VDR, Mm01309608_m1), *Casr* (Mm00443375_m1),

Calbindin D9k (*S100g*, Mm00486654_m1), Calbindin D28k (*Calb1*, Mm00486645_m1), *Trpv5* (Mm01166030_m1), *Trpv6* (Mm00499069_m1), *Npt2a* (*Slc34a1*, Mm00441450_m1), *Npt2b* (*Slc34a2*, Mm01215846_m1), *Npt2c* (*Slc34a3*, Mm00551746_m1), *Npt3* (*Slc17a2*, Mm00522866_m1), *Nkcc2* (*Slc12a1*, Mm01275821_m1), and *Nhe3* (*Slc9a3*, Mm01352473_m1) was performed using a QuantStudio 7 real-time PCR system (Life Technologies Applied Biosystems). The assay was performed in triplicate. Relative quantification of mRNA was performed according to the comparative C_t method and normalized to housekeeping genes.

2.7. Intramedullary Rodded Tibial Osteotomy

The surgical procedure was performed on mice under general isoflurane anesthesia as described in Martineau et al. [24]. Briefly, an incision was performed above the right knee to free the patellar ligament from lateral tissue. A 25G spinal needle wire guide was inserted down the medullary canal through a 26G needle pushed through the tibial plateau. The wire guide was bent at a right angle, cut at the tibial plateau, and secured by a mattress suture on either side of the patellar ligament after the needle was pulled out. The tibial shaft was cut using micro scissors about 2–3 mm above the tibiofibular junction. Topical analgesics were applied at the wound site, then the skin was sutured. Carprofen was provided at the time of surgery and for the following 48 h post-osteotomy. The mice were sacrificed using isoflurane and CO_2 at day 10 (D10) post-surgery.

2.8. Three-Point Bending Assays

For three-point bending assays, callus tibiae were thawed overnight at room temperature and tested for mechanical properties using an Instron model 5943 single-column table frame machine (Instron, Norwood, MA, USA). The fractured bones rested on two fulcra set 6 mm apart. Load-sensing cell was applied to the widest part of the callus. Raw output used for comparison was strength (load at break, in N).

2.9. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 7.04. Statistical tests involved 2-tailed *t*-tests for gene expression monitoring, 1-way ANOVA followed by Dunnett's or Tukey's post hoc test for calcemia and three-point bending assays, respectively, or 2-way ANOVA with Sidak's post hoc test for vitamin D metabolites measurements. The statistical significance threshold was set at a *p*-value of less than 0.05.

3. Results

3.1. Severe Postnatal Lethality in C57Bl6-R396$^{w/w}$ Mutant Animals

The R396W knock-in targeting construct was electroporated into C57Bl6/N embryonic stem cells. The targeting event was confirmed using both PCR screening and Southern blotting. One (1) correctly targeted clone was injected into C57Bl6/N blastocysts, which allowed to derive germline-transmitting chimeras on the homogeneous C57Bl6/N genetic background. Heterozygous progeny was mated inter se to obtain all three genotypes (wild-type: C57Bl6-R396$^{+/+}$; heterozygous: C57Bl6-R396$^{+/w}$; homozygous mutant: C57Bl6-R396$^{w/w}$).

The mutant allele was transmitted at the expected Mendelian frequency, but we detected severe postnatal lethality in the homozygous mutant animals. We observed a significant number of dead pups of the C57Bl6-R396$^{w/w}$ genotype from birth, which culminated in a 17% survival rate of homozygous mutant pups from postnatal day 14 onwards (Figure 1). This is a striking difference from the 50% postnatal lethality measured in the global *Cyp24a1*-deficient strain maintained on a mixed genetic background for more than 20 years [18].

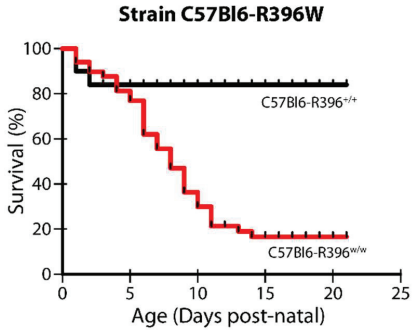

Figure 1. Postnatal lethality in C57Bl6-R396$^{w/w}$ mutant pups. Cages were monitored daily until weaning (day 21) and cadavers were genotyped. Percent survival for wild-type and homozygous mutants is shown.

3.2. Early Postnatal Hypercalcemia in C57Bl6-R396$^{w/w}$ Mutant Animals

We measured serum calcium levels in wild-type, heterozygous and homozygous mutant pups during the second postnatal week (P7 to P10). C57Bl6-R396$^{w/w}$ mutant animals had significantly elevated circulating calcium levels at P7 compared to wild-type and heterozygous control littermates (Figure 3.3). This was accompanied by the marked formation of kidney stones (nephrolithiasis) (Figure 3). We did not investigate for signs of extra renal calcification. Circulating serum levels in surviving mutant pups past P7 were within the normal range (Supplementary Figure S1).

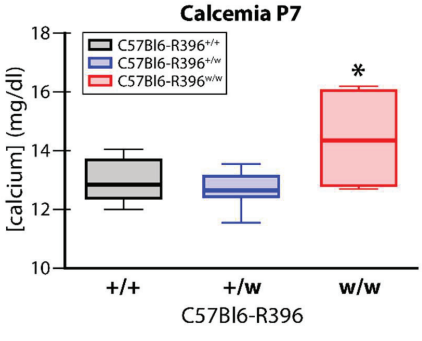

Figure 2. Serum calcium levels in C57Bl6-R396 littermates at postnatal day 7. Total serum calcium was measured using an automated analyzer. +/+, C57Bl6-R396$^{+/+}$; +/w, C57Bl6-R396$^{+/w}$; w/w, C57Bl6-R396$^{w/w}$. *, $p < 0.05$ by one-way ANOVA and Dunnett's post hoc test.

3.3. Gene Expression Monitoring

We compared kidney tissue gene expression in C57Bl6-R396$^{w/w}$ mutants and wild-type littermates at P7 and 4 months. The R396W mutant *Cyp24a1* allele was highly overexpressed (30-fold increase) in pups (Figure 4A); this was reduced in surviving adult mutant animals but still remained almost 10-fold higher as compared to wild-type littermates (Figure 4B). As observed in the global *Cyp24a1*-deficient animals [18,19], *Cyp27b1* expression was markedly inhibited at all ages (Figure 4A,B). Vitamin D receptor expression was moderately but significantly overexpressed (Figure 4A,B).

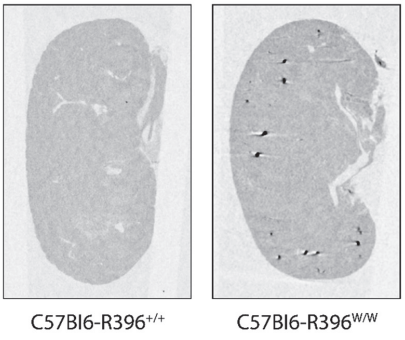

Figure 3. Nephrolithiasis in C57Bl6-R396$^{w/w}$ mutant pups. Kidneys were harvested at P7 and imaged by microCT.

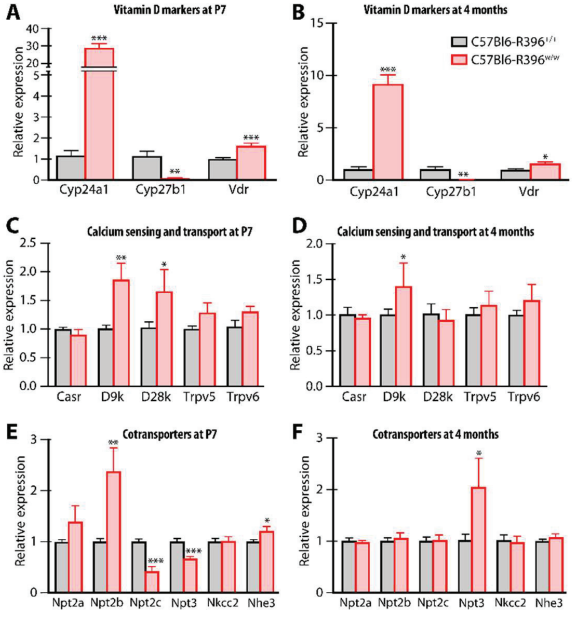

Figure 4. Gene expression monitoring in newborn and adult C57Bl6-R396 littermates. Quantitative gene expression was assessed by RT-qPCR with TaqMan probes on mRNA extracted from kidneys. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, double-sided *t*-tests for each gene. (**A**) Vitamin D markers at P7. (**B**) Vitamin D markers at 4 months. (**C**) Calcium sensing and transport at P7. (**D**) Calcium sensing and transport at 4 months. (**E**) Cotransporters at P7. (**F**) Cotransportersat 4 months.

The calcium transporters calbindin D9k and calbindin D28k were significantly overexpressed in mutant pups and this normalized for D28k in adults (Figure 4C,D). There were no differences in the expression levels of the calcium-sensing receptor (*Casr*) or the calcium channels *Trpv5* or *6* between genotypes (Figure 4C,D).

In newborn C57Bl6-R396$^{w/w}$ mutants, we measured the increased expression of the sodium-dependent phosphate transport protein 2b (Npt2b, *Slc34a2*) while related transporters Npt2c (*Slc34a3*) and Npt3 (*Slc17a2*) were expressed at lower levels in mutant animals compared to wild-type littermates (Figure 4E). With the exception of Npt3, which was over-

expressed in adult homozygous mutant mice, the expression of co-transporters normalized as animals aged to adulthood (Figure 4F).

3.4. CD1-R396W Strain

We crossed the C57Bl6-R396W strain to CD1 outbred mice in an attempt to decrease the extreme perinatal lethality associated with carrying the R396W mutant allele on a homogeneous genetic background. In this mixed genetic background, survival of the CD1-R396$^{w/w}$ mutants was improved to 86% (Figure 5). This allowed us to compare vitamin D metabolites between the strains and examine additional phenotypic manifestations.

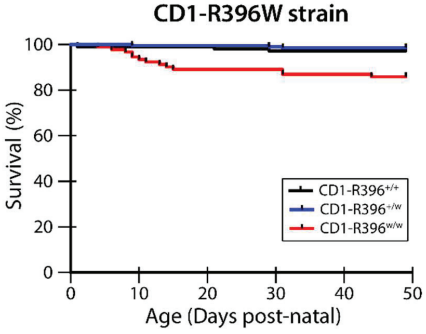

Figure 5. Survival of CD1-R396$^{w/w}$ mutant pups. Cages were monitored daily until postnatal day 49 and cadavers found in cages were genotyped. Percent survival for all 3 genotypes is shown.

3.5. Vitamin D Metabolites

We used a sensitive LC-MS/MS-based assay to profile multiple serum vitamin D metabolites [20] in the global *Cyp24a1*-null mice, the C57Bl6-R396W strain, and the CD1-R396W line. The mutant genotypes of all three strains exhibited a five- to six-fold increase in circulating 25(OH)D_3 compared to control genotypes (Table 1). Surprisingly, levels of 24,25$(OH)_2D_3$ remained detectable but were reduced by 3- to 5.4-fold in mutant mice. These changes led to a very high 25(OH)D_3 to 24,25$(OH)_2D_3$ ratio in homozygote mutants which is a diagnostic hallmark of the infantile hypercalcemia type 1 condition [20]. Concentrations of 25(OH)D_3-26,23-lactone were at the lower limit of detection of the assay, in accord with the demonstrated 23-hydroxylation activity of CYP24A1 [25]. Levels of 1,24,25$(OH)_3D_3$ were also significantly reduced in homozygous mutant animals sporting the R396W mutation (Table 1). Despite the much reduced CYP24A1 catabolic enzyme activity in mutant mice, the serum concentration of the hormone 1,25$(OH)_2D_3$ remained normal (Table 1) presumably due to much reduced *Cyp27b1* expression noted in Figure 4A,B. This adaptation is presumably necessary to permit the viability of the surviving animals.

3.6. Impaired Bone Fracture Healing in CD1-R396$^{w/w}$ Mice

The improved survival of CD1-R396$^{w/w}$ mutants permitted their skeletal analysis. There were no detectable steady-state skeletal phenotypic manifestations in the mutant animals as shown by normal trabecular bone volume, trabecular separation, thickness, and separation (Supplementary Figure S2A–D). Since we have observed impaired bone fracture repair in global *Cyp24a1*-deficient mice [24], we challenged the CD1-R396$^{w/w}$ mutant animals to heal surgical osteotomies as a model of bone fracture recovery. The intramedullary rodded immobilized fracture surgery was performed on the left tibia at 3 months of age. The analyses of the fractured bones were performed on post-surgery day 10 (D10). The tibiae with calluses were dissected and the intramedullary nail was carefully removed. The biomechanical properties of the repaired calluses were evaluated using the three-point bending test.

At D10 post-surgery, the analysis of the callus showed a significant decrease in load at break (strength) in heterozygous and homozygous mutant mice compared to wild-type littermates (Figure 6).

Table 1. Vitamin D metabolites in preclinical models of infantile hypercalcemia type 1.

Strain/Genotype	Sex	25(OH)D_3 (ng/mL)	24,25$(OH)_2D_3$ (ng/mL)	Ratio 25:24,25	1,24,25$(OH)_3D_3$ (pg/mL)	25(OH)D_3-26,23-lactone (ng/mL)	1,25$(OH)_2D_3$ (pg/mL)
Cyp24a1$^{+/-}$	m	–	–	–	–	–	-
	f	17.21 ± 2.36	6.68 ± 1.05	2.60 ± 0.10	72.80 ± 4.20	3.84 ± 0.72	27.80 ± 4.20
Cyp24a1$^{-/-}$	m	–	–	–	–	–	–
	f	101.29 ± 20.61 ***	1.45 ± 0.11 ***	70.60 ± 15.7 #	n.d.	0.06 ± 0.02 #	34.00 ± 6.20
C57bl6-R396$^{+/+}$	m	19.59 ± 2.90	6.71 ± 0.76	2.91 ± 0.33	57.77 ± 10.88	2.76 ± 0.32	27.39 ± 3.35
	f	18.59 ± 2.50	7.38 ± 0.96	2.53 ± 0.25	66.53 ± 13.18	3.16 ± 0.89	21.28 ± 5.96
C57bl6-R396$^{w/w}$	m	110.73 ± 15.40 #	2.59 ± 0.44 #	43.10 ± 4.08 #	17.81 ± 3.53 #	0.07 ± 0.03 #	37.06 ± 8.85
	f	106.16 ± 26.73 #	2.12 ± 0.44 #	49.71 ± 4.37 #	11.09 ± 5.35 #	0.05 ± 0.02 #	37.17 ± 12.12 **
CD1-R396$^{+/+}$	m	17.23 ± 3.77	6.31 ± 1.89	2.94 ± 1.07	74.66 ± 34.78	2.24 ± 0.70	45.83 ± 22.74
	f	18.24 ± 4.14	8.80 ± 2.14	2.08 ± 0.19	55.19 ± 17.50	2.56 ± 0.67	27.43 ± 9.77
CD1-R396$^{w/w}$	m	81.90 ± 19.39 #	2.26 ± 0.53 #	36.58 ± 5.35 #	15.18 ± 5.84 #	0.06 ± 0.05 #	45.12 ± 16.57
	f	99.16 ± 13.59 #	2.23 ± 0.39 #	44.90 ± 5.53 #	7.49 ± 1.95 #	0.03 ± 0.01 #	29.24 ± 7.98

Results are means ± S.D. Ratio 25:24,25, ratio of 25$(OH)_2D_3$ concentrations over 24,25$(OH)_2D_3$ concentrations. n.d., non-detectable. **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.0001$ vs. corresponding wild-type sex within strain by 2-way ANOVA and Sidak's post hoc test.

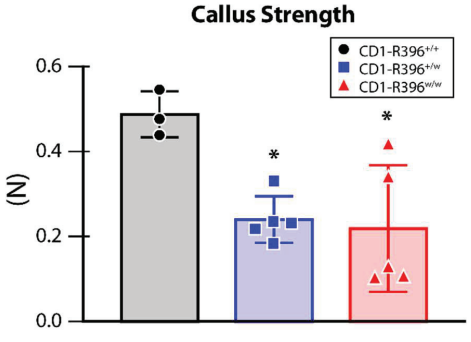

Figure 6. Impaired bone fracture healing in CD1-R396$^{w/w}$ mutant animals. Callus strength (load at break) was calculated from the three-point bending test. *, $p < 0.05$ by one-way ANOVA with Tukey's post hoc test.

4. Discussion

The global inactivation of *Cyp24a1* in mice confirmed the physiological importance of CYP24A1 in the catabolism of 1,25$(OH)_2D_3$ [18,19] and elucidated the mechanism of action of 24,25$(OH)_2D_3$ in bone fracture healing [24]. As a preclinical model of infantile hypercalcemia type 1, however, it left to be desired, since the mutation involved replacing the heme-binding exon of *Cyp24a1* by the PGK-neo selection cassette [26] in the opposite transcriptional orientation [18], a genetic change that is significantly different from the mutated alleles identified in patients suffering from the disease (reviewed in [8]). We set out to generate a mouse strain expressing a mutated allele of *Cyp24a1* that corresponds to what has been described in patients and decided on one of the first identified mutations [6] which turns out to be the second most frequent mutated allele observed in affected HCINF1 patients, R396W [8].

We first established the mutation on a homogeneous genetic background, and this resulted in a dramatic rate of perinatal lethality. The elevated calcium concentrations in the first week of life may have contributed to the mortality rate. Despite significant changes in

the expression of sodium-phosphate cotransporters, circulating phosphate concentrations were not affected by the mutation (Supplementary Figure S3), similar to what is observed in patients [27,28]. The differences in the expression of calcium and phosphate transporters observed soon after birth normalized in the surviving population that reached adulthood.

As reported for the global *Cyp24a1*-deficient mice [19], the expression of the vitamin D 1α-hydroxylase gene, *Cyp27b1*, was completely inhibited in young and older C57Bl6-R396$^{w/w}$ mutants, most likely as a protection mechanism to avoid hypervitaminosis. Such an explanation is consistent with the fact that young and older C57Bl6-R396$^{w/w}$ mutants have serum 1,25$(OH)_2D_3$ levels within the normal range. Of note was the very high expression of the mutated *Cyp24a1* allele in homozygous mutants. This contrasts with the measurable but diminished expression of *CYP24A1* in affected patients [16].

The R396W mutation is a loss-of-function mutation [6], yet we measured circulating levels of 24,25$(OH)_2D_3$ in both the inbred and outbred R396W mutated strains. These measurable levels of dihydroxylated metabolite could represent the activity of other cytochrome P450s or be due to the migration of interfering dihydroxyvitamin D metabolites with similar retention time and mass spectral characteristics in LC-MS/MS. We estimate this latter probability as very low. On the other hand, it has been reported that cytochrome P450s distinct from CYP24A1 can synthesize 24,25$(OH)_2D_3$, but it remains unclear if this activity, measured in vitro [29], has relevance in vivo. At any rate, the ability to detect circulating levels of 24,25$(OH)_2D_3$ in CYP24A1-deficient mice or patients allows for calculating the ratio of the metabolite to its precursor 25$(OH)D_3$, namely serum 25$(OH)D_3$:24,25$(OH)_2D_3$ [20,30–32]. Clinically, this ratio is a more reliable indicator of HCINF1 due to *CYP24A1* mutations than serum 24,25$(OH)_2D_3$ alone because it eliminates the possibility that the patient might have a low serum 24,25$(OH)_2D_3$ level due to vitamin D deficiency. The high 25$(OH)D_3$:24,25$(OH)_2D_3$ ratio measured in R396W homozygote mutants on both inbred and outbred backgrounds aligns with the reports from over 100 HCINF1 patients [20,32]. We thus posit that the R396W knock-in strains are valid preclinical models of HCINF1.

Another phenotypic manifestation of the R396W mouse mutation that mimics what is observed in patients is the presence of kidney stones. Indeed, hypercalciuria and the risk of renal stones continue in most subjects with *CYP24A1* mutations throughout adult life [28,33,34]. In patients, the disorder may in fact first manifest as hypercalciuria and painful kidney stones in adulthood, without childhood symptoms [16,35–38]. Based on gene frequency studies, Nesterova and colleagues estimate that the frequency of kidney stones in the general population due to *CYP24A1* mutations might be as high as 4–20% [16].

The perinatal lethality phenotype of *Cyp24a1* homozygous mutants exhibits significant variable penetrance depending on the genetic background upon which the mutation is established. Crossbreeding the R396W mutation on an outbred CD1 background most adequately represents the human condition with no obvious complications unless challenged. Exploiting the differential penetrance of the perinatal lethality between the 3 strains of *Cyp24a1* deficiency available could allow for the identification of modifier gene(s) [39] impacting vitamin D and mineral ion homeostasis.

The *Cyp24a1*-deficient mouse strain [18] was an invaluable tool to study the physiological role of 24,25$(OH)_2D_3$ in mammalian fracture repair. *Cyp24a1*$^{-/-}$ mice show suboptimal endochondral ossification during fracture repair with smaller calluses that exhibit inferior biomechanical properties [24]. The strength of the repairing callus was reduced in heterozygous and homozygous CD1-R396$^{w/w}$ mutant mice compared to wild-type littermates. This change was the only phenotypic manifestation that we observed in heterozygous carriers of the mutation. The impaired bone fracture healing observed in CD1-R396$^{w/w}$ mutant animals establishes that two distinct preclinical models of *Cyp24a1* deficiency exhibit bone fracture repair defects. These results support the need to study bone fracture healing in patients with HCINF1.

5. Conclusions

Introducing the site-specific R396W mutation within the murine *Cyp24a1* gene in knock-in mice generated a valid preclinical model of infantile hypercalcemia type 1 with the characteristic high $25(OH)D_3$:$24,25(OH)_2D_3$ ratio that is a diagnostic hallmark of the condition.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14153221/s1, Supplementary Figure S1. Serum calcium levels in C57Bl6-R396W littermates, Supplementary Figure S2. Steady-state trabecular parameters in CD1-R396W knock-in strain, Supplementary Figure S3. Serum phosphate levels in surviving wild-type and mutant C57Bl6-R396W mice at 3 months of age.

Author Contributions: A.A., D.K. and M.K. generated data. M.K., G.J. and R.S.-A. participated in data analysis and interpretation. G.J. and R.S.-A. obtained the funding and participated in the conception and design of the study. R.S.-A. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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nutrients

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Review

Vitamin D and Bone: A Story of Endocrine and Auto/Paracrine Action in Osteoblasts

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Abstract: Despite its rigid structure, the bone is a dynamic organ, and is highly regulated by endocrine factors. One of the major bone regulatory hormones is vitamin D. Its renal metabolite 1α,25-OH2D3 has both direct and indirect effects on the maintenance of bone structure in health and disease. In this review, we describe the underlying processes that are directed by bone-forming cells, the osteoblasts. During the bone formation process, osteoblasts undergo different stages which play a central role in the signaling pathways that are activated via the vitamin D receptor. Vitamin D is involved in directing the osteoblasts towards proliferation or apoptosis, regulates their differentiation to bone matrix producing cells, and controls the subsequent mineralization of the bone matrix. The stage of differentiation/mineralization in osteoblasts is important for the vitamin D effect on gene transcription and the cellular response, and many genes are uniquely regulated either before or during mineralization. Moreover, osteoblasts contain the complete machinery to metabolize active 1α,25-OH2D3 to ensure a direct local effect. The enzyme 1α-hydroxylase (*CYP27B1*) that synthesizes the active 1α,25-OH2D3 metabolite is functional in osteoblasts, as well as the enzyme 24-hydroxylase (*CYP24A1*) that degrades 1α,25-OH2D3. This shows that in the past 100 years of vitamin D research, 1α,25-OH2D3 has evolved from an endocrine regulator into an autocrine/paracrine regulator of osteoblasts and bone formation.

Keywords: vitamin D metabolism; vitamin D receptor; bone; osteoblasts; differentiation and mineralization

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1. Introduction

The skeleton plays a fundamental role in the human body by providing structural support and allowing movement. Moreover, it has a protective role for vital internal organs and stem cells, is a source for mineral and growth factors, and is the center of regulatory pathways. Bone is highly dynamic and undergoes continuous remodeling throughout life; it can repair itself. To illustrate this, damaged or (micro)fractured areas are removed by osteoclastic bone resorption, which is followed by new bone formation by osteoblasts (bone remodeling). Bone formation is characterized by secretion of an extracellular proteinaceous matrix, which is subsequently mineralized. Bone remodeling is tightly controlled by an interplay of local, bone and bone marrow-derived factors (e.g., cytokines, growth factors, chemokines) and endocrine factors. One of these endocrine factors is the seco-steroid 1α,25-dihydroxyvitamin D_3 (1α,25-OH_2D_3). 1α,25-OH_2D_3 can affect bone in a direct as well as an indirect manner [1–3]. The indirect effect occurs via control of calcium reabsorption in the kidney and absorption in the intestine, as well as via control of parathyroid hormone production. Although rickets and osteomalacia were prevented in vitamin D receptor (*VDR*) knockout mice fed with a rescue diet that contained high levels of calcium and phosphorus, not all bone changes were rescued, indicating the importance of a direct role for 1α,25-OH_2D_3 in bone metabolism [4–6]. The presence of VDRs in cells of the osteoblast lineage [7,8] enables direct effects of 1α,25-OH_2D_3 on bone metabolism. *VDR* expression in osteoblasts can be regulated by 1α,25-OH_2D_3 itself, as well

as by other factors including parathyroid hormone, glucocorticoids, transforming growth factor-β, and epidermal growth factor [9–13]. Transgenic mice specifically overexpressing the *VDR* in osteoblasts have increased trabecular bone volume and increased bone strength, supporting an anabolic effect of 1α,25-OH_2D_3 [14]. This observation was confirmed in a study using mice with a different genetic background [15]. Interestingly, a study with global *VDR* knockout mice [5] knockout mice reported a similar phenotype, with increased trabecular thickness and increased osteoid volume and osteoblast numbers, suggesting an inhibitory effect of 1α,25-OH_2D_3 on bone formation. This was supported by data from an osteoblast-specific *VDR* knockout mouse study [16]. In this latter study, the bone effect appeared to be via reduced bone resorption. The effects on bone may be related to overall levels of calcium intake [17], but whether this explains the apparent opposite effects in murine studies remains to be established. Nevertheless, these observations support a direct effect of 1α,25-OH_2D_3 on bone metabolism via osteoblasts. There is less consensus on VDR expression in osteoclasts. Genomic deletion of the *VDR* in osteoclasts did not impact the positive effect of a 1α,25-OH_2D_3 analog (eldecalcitol) on bone mass [7]. This is supported by Verlinden et al., who reported that *VDR*s in osteoclast precursors are not essential to maintain bone homeostasis [18]. It was concluded that 1α,25-OH_2D_3 regulates osteoclasts indirectly via cells of the osteoblast lineage. In the current review, we will focus on 1α,25-OH_2D_3 in osteoblast function and bone metabolism.

2. Literature Search Strategy

We built on our pre-existing literature database and expanded this with a new search from 2016 until October 2022. With the support of the Erasmus MC Medical Library Literature Search Service, the search strategy was developed and executed. Supplemental Figure S1 shows in detail the search strings used. In this way, we obtained a list of 2713 publications on vitamin D. From this dataset, we excluded 2583 clinical and (genetic) epidemiological association studies and focused on 128 bone-related molecular and cellular studies. Two publications appeared to be retracted after the search was performed.

3. Osteoblasts

Osteoblasts originate from mesenchymal stromal cells via a tightly controlled differentiation process. The eventual fate of osteoblasts is three-fold, either to become lining cells that cover the bone surface, or to become embedded in the extracellular matrix as osteocytes, or to die via apoptosis.

3.1. Proliferation and Apoptosis

The data on 1α,25-OH_2D_3 effects on osteoblast proliferation are variable. Inhibition [19–27], as well as stimulation [20,28] or no effect [29,30] on the proliferation of osteoblasts of mouse, rat, and human origins have been reported. Effects on cell viability [31] and apoptosis [32,33] have also been documented. Although different directions in effect have been observed, these data demonstrate direct effects of 1α,25-OH_2D_3 on osteoblast proliferation and survival. The direction of effect may depend on the timing of treatment, dosage, origin, and environment of the osteoblasts [27,34–36].

3.2. Differentiation

Immature mesenchymal stromal cells differentiate into osteoblasts that produce extracellular matrix proteins, enzymes, and matrix vesicles involved in the mineralization of the extracellular matrix produced (Figure 1). It has been demonstrated that 1α,25-OH_2D_3 impacts all of these processes [3,37,38]. 1α,25-OH_2D_3 stimulation of differentiation has been shown in all in vitro studies using human osteoblasts, human mesenchymal stem cells, and osteogenic-induced pluripotent stem cells [30,39–46]. Most studies with rat osteoblasts resemble these studies using human osteoblasts and show increased differentiation [29,47,48]. Studies with mouse osteoblasts are more diverse. These studies show inhibition [49,50], as well as stimulation of osteoblast differentiation by 1α,25-OH_2D_3 [51]. The definitive

explanation for the discrepancies in 1α,25-OH_2D_3 effects between, on the one hand, mouse osteoblast cultures, and on the other hand, between mouse and human/rat osteoblast cultures, is absent; however, several explanations can be put forward. The source of osteoblasts may play a role. Different sites of the skeleton differ in origin and bone formation, such as enchondral (long bones) and intramembranous (calvaria) sites. 1α,25-OH_2D_3 did not affect osteoblasts from cortical bone, and inhibited differentiation of calvaria-derived cells [52,53]. Furthermore, within one skeletal element, differences in osteoblast regulation are observed. A recent study reported differences between periosteal- and bone-marrow-derived osteoblasts in cortical bone [54]. Whether this fully explains the diverse effects observed is not clear, but it shows the importance of origin for the eventual activity and regulation. This may also relate to stage of osteoblast differentiation, donor age, culture conditions, etc., which have been shown to relate to 1α,25-OH_2D_3 action [17,47,55,56]. Furthermore, differences may be species-intrinsic, and may have a genomic explanation. 1α,25-OH_2D_3 increases *RUNX2* and *BGLAP* (osteocalcin) gene expressions in human osteoblasts, while in murine osteoblasts, 1α,25-OH_2D_3 treatment inhibits the gene expressions of *RUNX2* and *BGLAP* [43,57–61].

A picture that emerges from all in vitro osteoblast data is that the osteoblast (micro)environment is a determinant of the eventual outcome of 1α,25-OH_2D_3 action. The extracellular milieu (growth factors, cytokines, matrix proteins, ions (calcium/phosphate), and other signaling molecules) and the intracellular milieu (e.g., the insulin-like growth factor binding protein-6) are important for the eventual effect of 1α,25-OH_2D_3 [62,63]. For example, interactions with transforming growth factor-β, insulin-like growth factor, bone morphogenetic proteins, and interferon have been demonstrated [64–69]. Consequently, the absence or presence of these, but potentially other factors as well, can modulate 1α,25-OH_2D_3 action and determine the eventual response. An example of interaction with other intracellular regulatory pathways is Wnt signaling. Wnt signaling plays an important role in osteoblast differentiation and bone formation. An interplay between 1α,25-OH_2D_3 and Wnt signaling has been described [70–74].

Osteoblast differentiation, bone matrix production, and mineralization, as part of bone formation, are high energy-demanding processes [75–77]. Regulation of energy metabolism impacts osteoblast differentiation and bone formation [78–80]. Vitamin D and energy metabolism have been discussed in relation to obesity and metabolic syndrome [81] and to cancer [82–84], but data on vitamin D and energy metabolism in the context of osteoblast differentiation remain limited. Forkhead Box O (*FoxO*) transcription factors are regulated by 1α,25-OH_2D_3 in murine MC3T3 osteoblasts. *FoxO3a* is upregulated, *FoxO1* is downregulated, and *FoxO4* is unchanged after 1α,25-OH_2D_3 treatment. si-RNA knockdown of the *FoxO*s did not change 1α,25-OH_2D_3 inhibition of proliferation [85]. Unfortunately, the effect on differentiation was not reported. Changes in *FoxO* expression were coupled to increase in reactive oxygen species accumulation, which may be linked to cellular metabolism and bone formation [75,80,86]. Glucose, insulin, and 1α,25-OH_2D_3 regulation of osteoblast proliferation, alkaline phosphatase activity, and production of (uncarboxylated) osteocalcin have been studied in isolated rat osteoblasts, but unfortunately, no coupling to mineralization was made [87]. Nevertheless, these data, together with those on interactions between vitamin D and *PPARγ* signaling in osteoblast differentiation [88], support that control of energy metabolism can be a vitamin D target in bone formation and mineralization.

3.3. Mineralization

Mineralization can be divided into two phases. In the first phase, formation of hydroxyapatite (HA) crystals takes place in nano-sized extracellular matrix vesicles produced by osteoblasts. In the second phase, HA is propagated outside these vesicles, with a resulting buildup of mineral in the extracellular matrix [89,90]. Calcium and phosphate concentrations increase inside matrix vesicles via involvement of specific proteins, and when the solubility product of calcium and phosphate is exceeded, mineral deposits are

formed inside the extracellular vesicles and the second phase of mineralization starts with the release of the preformed HA crystals [90,91]. Proteomic analyses of extracellular matrix vesicles revealed many proteins with a potential role in mineralization [92,93]. Gene profiling studies also identified novel regulators of osteoblast matrix mineralization [94].

Mineralization is controlled by a balanced action of promoters and inhibitors. Alkaline phosphatase and bone sialoprotein are important promoters [95,96]. Alkaline phosphatase increases the phosphate concentration in matrix vesicles by hydrolyzing inorganic pyrophosphate. Pyrophosphate is an inhibitor of mineralization; consequently, alkaline phosphatase also decreases the level of this inhibitor. Pyrophosphatase phosphodiesterase 1 (NPP1, encoded by the gene *ENPP1*) and ankylosis protein (ANK) are involved in inhibiting mineralization. NPP1 generates pyrophosphate, and the transmembrane protein ANK allows pyrophosphate to pass through the plasma membrane to the extracellular matrix; thus, HA formation is inhibited in the extracellular vesicles [97,98]. 1α,25-OH_2D_3 stimulates mineralization via direct action on osteoblasts [68,88,99]. 1α,25-OH_2D_3 can influence the mineralization process via gene expression and matrix vesicle production. Gene expression profiling studies demonstrated that the 1α,25-OH_2D_3 effect is not likely primarily due to changes in the expression of extracellular matrix genes, and thereby to changes in composition of the extracellular matrix [99]. Studies on the expression and production of procollagen type I by human osteoblasts showed stimulation [100,101] as well as no effect [101–104], or inhibition [105].

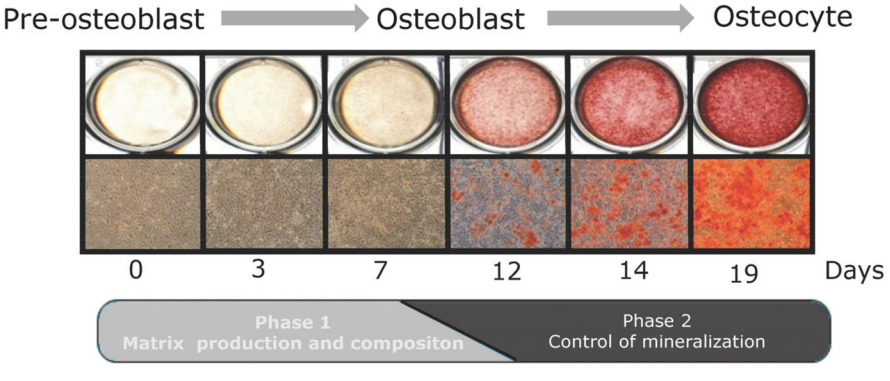

Figure 1. Alizarin red staining of osteoblast culture exemplifying the pre-mineralization and mineralization phases. Red staining shows mineralization. Details on cell culture and Alizarin red staining procedures can be found in Woeckel et al. [99]. Adapted with permission from Eijken, M., Koedam, M., van Driel, M., Buurman, C.J., Pols, H.A.P., van Leeuwen J.P.T.M. The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization. Mol Cell Endocrinol 2006, 248(1–2):87–93. https://doi.org/10.1016/j.mce.2005.11.034. 2006, J.P.T.M. van Leeuwen.

It is postulated that vitamin D may enhance mineralization by stimulating both NPP1, generating pyrophosphate, and alkaline phosphatase, generating phosphate from pyrophosphate [106]. This involves acceleration of the production of alkaline phosphatase-positive matrix vesicles, leading to enhanced formation and deposition of HA crystals, and eventually mineralization. This direct effect of vitamin D occurred in the period prior to the onset of mineralization, and also involved accelerated extracellular matrix maturation [99]. Interestingly, treatment with vitamin D after initiation of mineralization did not affect mineralization. This supports the above-described osteoblast differentiation stage dependency of the 1α,25-OH_2D_3 effect. A study by Yajima et al. described the significance of 1α,25-OH_2D_3 for osteocytic perilacunar mineralization [107].

1α,25-OH_2D_3 also directly stimulates the production of inhibitors of mineralization. VDR-dependent 1α,25-OH_2D_3 expression of *ENPP1* and *ANK* in murine osteoblasts led to an increase in the mineralization inhibitor pyrophosphate [108]. 1α,25-OH_2D_3 also

stimulates activin A expression in human osteoblasts. Treatment with the activin A blocker follistatin enhanced vitamin-D-induced mineralization of human osteoblasts [109]. 1α,25-OH_2D_3 also increases the expression of osteopontin, which is shown to inhibit mineralization. These observations may provide a fine-tuning mechanism to prevent excessive mineralization of bone. 1α,25-OH_2D_3 induction of carboxylated osteocalcin may be in line with this. 1α,25-OH_2D_3-stimulated mineralization is enhanced by blocking osteocalcin carboxylation by warfarin [109]. The interaction of 1α,25-OH_2D_3 with other factors, as described above, also holds for mineralization, for example, the interaction with DKK1, the inhibitor of Wnt signaling [74].

The counterbalance of bone formation and mineralization by osteoblasts is bone resorption by osteoclasts. In the healthy skeleton, these processes are in balance, securing healthy and strong bones. The osteoblasts/osteocytes are the major regulators of osteoclast formation and action via production of the stimulating factor RANKL, and the RANKL inhibitor, osteoprotegerin (OPG). 1α,25-OH_2D_3 influences the RANKL/OPG ratio, and thereby also impacts bone resorption [110–113]. 1α,25-OH_2D_3 is involved at both the bone formation and the bone resorption sides of the balance, and is an important player in maintaining healthy bones via direct effects on bone, in addition to indirect effects via calcium and phosphate homeostasis [114].

3.4. Gene Expression

The basis of all cellular effects of 1α,25-OH_2D_3 involves VDR-mediated transcriptional regulation. The VDR is a member of the nuclear receptor family. Upon binding to 1α,25-OH_2D_3, the VDR heterodimerizes with the retinoic X receptor (RXR), and binds as a dimer to the vitamin D response element (VDRE) in the DNA to regulate gene expression [115]. Over the years, many studies have unraveled the molecular fundamentals of 1α,25-OH_2D_3 transcriptional regulation. Examples and information can be found in these publications and references therein [116–118]. In a previous publication, we discussed 1α,25-OH_2D_3 and gene transcription in osteoblasts [38]. This will not be repeated or discussed in detail in this review.

A factor that may determine the transcriptional effect of 1α,25-OH_2D_3 effect is not only the basal level of gene expression [51,119], but also the stage of osteoblast differentiation [99]. Studies with rat osteoblasts in the early 1990s showed already that effects of 1α,25-OH_2D_3 on osteoblasts may depend on the osteoblast differentiation phase [119]. An example is the 1α,25-OH_2D_3 stimulation of phosphaturic hormone fibroblast growth factor 23 (FGF23) only in late-stage differentiation osteoblasts and osteocytes [120,121]. FGF23 is a hormone that acts in the kidney to enhance phosphate excretion, and suppresses 1α,25-OH_2D_3 synthesis by inhibiting 1α-hydroxylase (CYP27B1), forming an important loop in the regulation of mineralization [122,123]. Vitamin D signaling in osteocytes [124] is further supported by the 1α,25-OH_2D_3 regulation of *PHEX* (phosphate-regulating neutral endopeptidase, X linked), which suppresses FGF23 transcription [125].

The various osteoblast differentiation stages actually reflect different functional stages of the osteoblast, e.g., proliferation, extracellular matrix production, mineralizing and mechanosensing. It is important to keep in mind the osteoblast differentiation stage when studying 1α,25-OH_2D_3 effects, as this may be an important determinant of the eventual effect (e.g., stimulation or inhibition) on gene transcription and subsequent cellular responses and bone formation. The relationship between the osteoblast differentiation stage and 1α,25-OH_2D_3 gene expression control was shown by Woeckel et al. [99]. 1α,25-OH_2D_3 changed the expression of different sets of genes in the phase before the onset of mineralization, and during the mineralization. For this review, we performed a reanalysis of this gene profiling study [99] with the 2022 updated annotation. Comparison of transcripts regulated (i.e., two-fold up or down) in the phase before and after the start of mineralization (Figure 1) demonstrated that only 2.5% (18 out of the 721 regulated transcripts) were regulated in both phases (Table 1). The gene symbols of the transcripts regulated in both phases are shown in Table 2. To focus in more detail on phase-specific gene expression, we

next selected the transcripts that were uniquely regulated in either the pre-mineralization or in the mineralization phase [99]. In this regard, the transcripts should be at least two-fold up- or downregulated in one phase (either pre-mineralization or mineralization phase), and not regulated (fold change on average between 0.8 and 1.2) in the other phase (either the mineralization or pre-mineralization phase). Table 3 shows the number of transcripts uniquely regulated in either of these phases, and Table 4 reports the gene symbols belonging to these transcripts. This binary comparison of pre-mineralization and mineralization phases is not absolute and does not mean that further zooming in on specific phases of osteoblast differentiation will not reveal other sets of vitamin-D-regulated genes. However, it does support the notion that vitamin D gene regulation during osteoblast differentiation and mineralization displays temporal dynamics, and it does show that for proper interpretation of vitamin D effects, knowledge on the differentiation and functional stage of cells and tissues is important. This knowledge can explain the apparent differences in 1α,25-OH_2D_3 effects that have been reported.

Table 1. Number of transcripts on average that are 2-fold up- or downregulated in the pre-mineralization or mineralization phase of human osteoblasts *.

Condition	# of Genes UP	# of Genes DOWN
Pre-mineralization phase	155	164
Mineralization phase	166	236
In both phases	10	8

* Experimental procedures and culture conditions of human osteoblasts (SV-HFO) are described in Woeckel et al. [99]. Two-fold change is based on the average expression at the timepoints in the pre-mineralization or mineralization period.

Table 2. Gene symbols of transcripts that are 2-fold upregulated or downregulated in both the pre-mineralization and mineralization phases of human osteoblasts (i.e., 10 and 8 in both phases in Table 1) *.

Upregulated	Downregulated
ABCC3	*AGAP10*
CYP24A1	*CCL20*
MAGEE1	*DDIT3*
RARRES2	*GRK4*
RICH2	*LOC727869*
SLC25A45	*NFE2L2*
SULT1C2	*ODF1*
THBD	*TSC22D2*
TMEM180	
TOX3	

* Experimental procedures and culture conditions of human osteoblasts (SV-HFO) are described in Woeckel et al. [99]. Two-fold change is based on the average expression at the timepoints in the pre-mineralization or mineralization period.

Table 3. Number of transcripts uniquely 2-fold up- or downregulated in either the pre-mineralization or in the mineralization phase of human osteoblasts *.

Condition	# of Genes UP	# of Genes DOWN
Pre-mineralization phase	65	66
Mineralization phase	77	100

* Experimental procedures and culture conditions of human osteoblasts (SV-HFO) are described in Woeckel et al. [99]. The 2-fold and 0.8–1.2-fold change is based on the average expression at the timepoints in the pre-mineralization or mineralization period.

Table 4. Overview of transcript gene names that are uniquely 2-fold up- or downregulated in either the pre-mineralization or mineralization phase of human osteoblasts *.

Pre-Mineralization Phase				Mineralization Phase			
Upregulated		Downregulated		Upregulated		Downregulated	
AQR	RAB9BP1	ADAM22	RARA	ABCD4	MYH11	AASDH	MOSPD1
ARHGEF7	RLTPR	ADORA1	RBM	AKAP13	NFIX	ABCD3	MRPS23
ATAT1	SARDH	ATF7IP2	RIMKLB	ANKRD11	ORC5L	ABT1	MS4A1
ATG16L1	SHISA8	BAGE	SLC19A1	APIP	PCDHB3	ACTR3C	MTUS2
ATP1A4	SLC38A11	BRS3	SLC26A7	ARHGDIB	PDLIM5	ANUBL1	NCRNA00188
BCL11A	SZT2	BRWD1	SLC3A1	ASH1L	PDZRN4	AP5S1	NDRG2
BMF	TEX9	BST2	SNRPN	ATM	PGAP1	B4GALNT2	NDUFB7
BMP15	TMEM120B	C1orf68	TBK1	BNC2	PLEKHG2	C11orf65	NRAP
C15orf48	TMEM33	CACNA1A	TFAP4	BPTF	PPP4R4	C14orf156	NUDT14
C2orf27A	UBE2G2	CCDC144C	THPO	BRD4	PRPF18	C14orf2	OGFR
C3orf20	UBXN10	CSF2RA	TMPRSS15	CAP1	PTGES	C17orf104	PANK2
C8orf34	UNC13C	CTNS	TRIB3	CCDC67	PTGS1	C4orf36	PAPPA
CCDC124	ZC3H12A-DT	DEFB132	TRMT2A	CCDC76	RAB3IP	CCL5	PAX8
COL24A1	ZNF668	EDA	TTBK2	CD14	RASAL2	CCT2	PIP5K1A
CTU2	ZNF703	ERCC6L2	ZNF396	CLCN4	RG9MTD2	CNOT2	PLCH1
DCTN2		FAM219A	ZNF93	CROCCL1	SERTAD4	COX7C	PMCH
DOCK6		FCGR2C		DCLK3	SMARCA4	CSRP2BP	PML
DST		FLJ10213		DPP4	SRGAP1	DAZL	POLE4
DUSP28		FSD1L		EGFR	SRRM2	DBI	POLR2K
EPG5		GAS2		EP300	SULF1	DCUN1D1	PTPRA
EYA2		GLIPR1		FAM102A	TBC1D13	DNAH1	RHEB
GABRB3		GPR155		FAM186A	TBL1X	DUSP16	RPAIN
GNRHR		HM13		FAM20C	UGGT2	EEF1D	RPL13
HCRTR2		ICA1		FGF7	VCAN	EGFL8	RPL14
HIST1H4C		KLHL36		FLJ11292	ZNF397	EHD1	RPL34
HSPB7		KLK7		FLJ13773	ZNF462	ELP6	RPS11
IL1RN		LEKR1		FOXP2	ZSWIM1	ESPNL	SEMA6D
KCNJ15		LELP1		GABRA5		EXOG	SHLD1
LOC100131283		LIN28B		H2AFY		EXOSC2	SLC10A7
LOC148987		LOC100286895		HMCN1		FABP4	SLC9A5
LOC149351		LOC100287114		HOXA6		FAM126A	SNAP23
LOC285205		LOC283854		HSPA12A		FAM27A	SNCAIP
LOC645591		LOC285692		IL17C		FAXC	SNTG1
LOC728903		LOC390595		INTS4		FUT7	STEEP1
LOC780529		LOC440944		KCNAB1		GOSR1	STK32A
LRRC46		MAN1A2		KCNG3		GPR39	STMN3
LYZL6		MAPRE3		KRTAP3-3		GSN	SUPT16H
MGC42157		MGC12916		LOC100127980		HCG4P6	TAL1
MRS2		MRPL19		LOC100128640		IRGQ	TBC1D8
NCOR2		MSR1		LOC100131993		KCNIP3	TEN1
NOX4		MYO10		LOC283682		KY	TLK1
NTRK2		NR2E3		LOC285500		LOC100133109	TWF1

Table 4. *Cont.*

Pre-Mineralization Phase		Mineralization Phase	
Upregulated	**Downregulated**	**Upregulated**	**Downregulated**
OR1J4	NUP210L	LOC388210	LOC100287911 TXNIP
PDE1A	OTX2	LOC441461	LOC100289246 UHRF1BP1L
PENK	PCLO	MAGEB18	LOC338862 UQCRB
PGM2L1	PKP2	MARK2	LOC643749 UQCRQ
PHC3	PLXNA2	MEGF10	LPAR5 VMA21
POU2F1	POU2F2	MGAT5B	MATR3 WFDC21P
PRRG2	PRLR	MLXIP	MMP16 XAF1
PTCD3	RAD54L2	MS4A6A	MMP17 ZNF880

* Experimental procedures and culture conditions of human osteoblasts (SV-HFO) are described in Woeckel et al. [99].

4. Vitamin D Metabolism

Metabolism, synthesis of the active form of 1α,25-OH_2D_3 as well as its inactivation, has been an important research topic since the identification of vitamin D. This has contributed to the understanding of the initiation and termination actions of vitamin D and its endocrine function. Figure 2 shows the classical vitamin D metabolism pathway. Serum levels of 1α,25-OH_2D_3 are determined by the activity of the renal enzyme 1α-hydroxylase (CYP27B1). 24-Hydroxylase (CYP24A1) is the first step of a 1α,25-OH_2D_3 inactivation cascade present in all target tissues. In the next sections, we discuss CYP27B1 and CYP24A1 in osteoblasts.

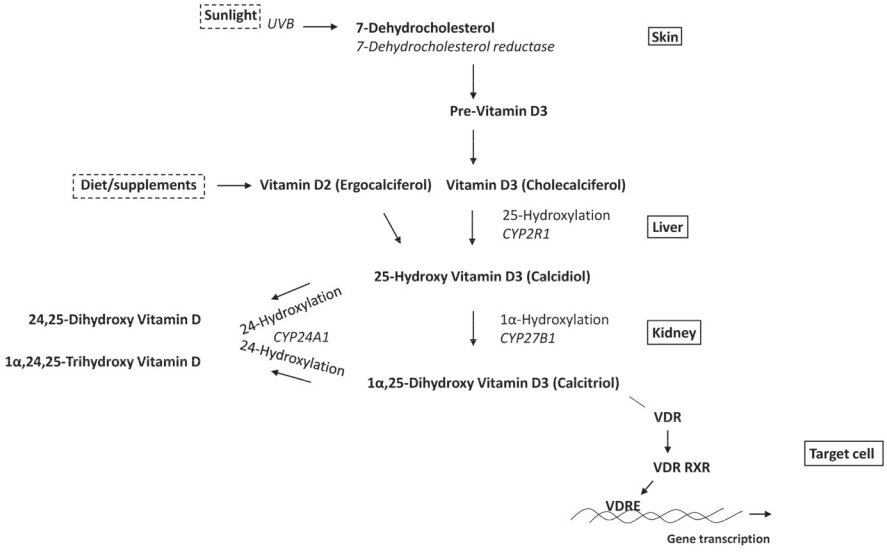

Figure 2. Schematic representation of classic vitamin D metabolism and signaling pathway. Either from sunlight or food, vitamin D is converted via enzymatic reactions in the liver and kidney into its active metabolite, 1α,25-OH_2D_3, which binds to the VDR. Gene activation follows after binding of the vitamin D/receptor complex to vitamin D response elements (VDREs) in target genes.

4.1. CYP27B1

In the late 1970s and early 1980s, reports were already coming out that in tissues other than the kidney, 1α,25-OH_2D_3 can be synthesized. Cells isolated from chicken calvaria [126] and human osteosarcoma cells, as well as bone cells isolated from an ileac crest

biopsy [127], can produce 1α,25-OH_2D_3.. Its functional significance in human osteoblasts was shown by the fact that inhibition of 1α-hydroxylase activity by ketoconazole blocked the 25(OH)D_3 induction of *CYP24A1* and osteocalcin expression [30]. This was supported by studies on siRNA silencing in human osteoblasts [19,46]. Additional evidence came from a study showing the importance of CYP27B1 for proliferation and osteogenic differentiation of human mesenchymal stromal cells (MSCs) [128,129]. MSCs of older donors had reduced *CYP27B1* expression and resistance to 25(OH)D_3 regulation of osteoblast differentiation [130]. Broader tissue distribution of extra renal CYP27B1 expression beyond bone was recently summarized by Bikle et al. [131].

However, renal synthesis is still considered the major contributor to circulating 1α,25-OH_2D_3 levels. Only in diseases such as sarcoidosis extra is renal synthesis sufficient to contribute to circulating levels. The presence of 1α,25-OH_2D_3 synthesis within bone provides a means to explain the associations of bone phenotypes and other parameters with circulating 25(OH)D_3 and not with 1α,25-OH_2D_3, as discussed by Anderson and colleagues [132,133]. Pharmacokinetic differences between locally produced 1α,25-OH_2D_3 from 25(OH)D_3 and added 1α,25-OH_2D_3 have been suggested from a cell culture study [134]. Further studies, in particular, in vivo studies, are needed for full appreciation of the impact of an autocrine/paracrine role of 1α,25-OH_2D_3.

Observations that the vitamin-D-binding protein receptors cubulin and megalin, as well as the vitamin D_3 25-hydroxylase genes *CYP2R1* and *CYP3A4*, are also expressed in human osteoblasts, supports an autocrine/paracrine role [19,30,131].

Renal CYP27B1 is tightly controlled by factors such as parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF23), which are involved in calcium and phosphate homeostasis. Extrarenal CYP27B1 expression is differently regulated, and probably involves other factors and tissue specificity [135]. For example, PTH and ambient calcium do not regulate *CYP27B1* in human osteoblasts [30], while 1α,25-OH_2D_3 reduces *CYP27B1* expression in human MSCs similar as in the kidney [136]. Several growth factors and cytokines can regulate CYP27B1 expression. IGF-I increases *CYP27B1* expression in human MSCs [136]. Interleukin-1 stimulates while interferon-β reduces *CYP27B1* expression in human osteoblasts [30,69]. The earlier described impact of the osteoblast differentiation stage on 1α,25-OH_2D_3 action can also be translated to expression of CYP27B1. *CYP27B1* expression is increased by 25(OH)D_3 in human MSCs [136], but not in mature osteoblasts [30].

4.2. CYP24A1

The first step in the degradation cascade of 1α,25-OH_2D_3 is hydroxylation at the C-24 position by 24-hydroxylase (CYP24A1) [137]. CYP24A1 is expressed in all vitamin D target cells, and its expression is very rapidly and strongly increased after 1α,25-OH_2D_3 binding to VDRs [138–141]. The VDR level is tightly linked to the induction of CYP24A1 expression and 24-hydroxylase activity and, consequently, degradation of 1α,25-OH_2D_3. Thus, the homologous upregulation of VDRs concomitantly induces the inactivation of 1α,25-OH_2D_3, and thereby limits its effect [142,143]. Hydroxylation at the C-24 position of 1α,25-OH_2D_3 or 25(OH)D_3 alone does not immediately lead to an inactive vitamin D molecule. Henry and Norman demonstrated in the 1970s the functional significance of 24,25$(OH)_2$D3 for normal chicken egg hatchability and calcium and phosphorus homeostasis [144,145]. The effects of 24,25$(OH)_2D_3$ on bone metabolism were shown in human, chicken, rat, and mouse studies. 24,25$(OH)_2D_3$, synergistically with PTH, directly stimulates mineralization, and 24,25$(OH)_2D_3$ decreases the number and size of resorption sites on the bone surface [146,147]. 24,25$(OH)_2D_3$ restores and accelerates the bone mineral apposition rate in vitamin-D-deficient and in parathryoidectomized rats [147]. 24,25$(OH)_2D_3$ did not change bone histomorphometric parameters in ovariectomized rats [148], but 24,25$(OH)_2D_3$, and not 1α,25-OH_2D_3, increased bone strength [149].

Several studies focused on 24-hydroxylated vitamin D molecules and fracture healing. 24,25$(OH)_2D_3$ binds to fracture calluses [150], and improves fracture healing [151–153]. Serum 24,25$(OH)_2D_3$ levels were found to correlate with fracture healing in chicken [151],

but not in a small human study in 1978 [154]. However, a study on pre-dialysis renal insufficiency patients supported a direct, i.e., PTH-independent, functional role of 24,25$(OH)_2D_3$ in human bone. 24,25$(OH)_2D_3$, together with 1α,25-$(OH)_2D_3$, preserved the osteoblast perimeter and improved mineralization, while 1α,25-$(OH)_2D_3$ alone was ineffective [155]. A direct effect on bone, in particular osteoblasts, is supported by in vitro studies showing that, similarly to 1α,25-OH_2D_3, 24,25$(OH)_2D_3$ has direct effects on human osteoblast differentiation [45]. Knowing that 24-hydroxylation per se does not lead to inactivation of vitamin D molecules, it is important to understand target tissue/target cell dynamics of the next steps in the degradation cascade. Control of the velocity of the subsequent steps in the degradation pathway can be a means to regulate vitamin D action in target tissues/cells. Together, these data on CYP24A1 and the biological activities of 24,25$(OH)_2D_3$ add to the notion of an auto/paracrine vitamin D regulatory system in bone. This system is most likely not restricted to bone and may also be present in other tissues.

5. Conclusions

This review revealed that the central role for vitamin D in bone physiology is directed via osteoblasts and depends on their stage of development. VDRs and the vitamin-D-metabolizing enzymes CYP27B1 and CYP24A1, known from the vitamin D endocrine system, are present and functional in osteoblasts. This uncovers a direct local role for 1α,25-OH_2D_3 vitamin D in osteoblast function, and expands the vitamin D action profile from endocrine regulation of calcium and phosphate homeostasis to an auto/paracrine regulatory network in bone. Several target-tissue-derived factors (growth factors, cytokines), intracellular signaling cascades (Wnt), and functional states of the osteoblast interact with this auto/paracrine network and determine the eventual response. In this way, vitamin D controls the proliferation, apoptosis, differentiation, and mineralization of osteoblasts, as well as their gene profile and interaction with other factors that maintain healthy bone. Moreover, even local degradation products of vitamin D metabolism (24,25$(OH)_2D_3$) have a beneficial contribution to osteoblast function. Together, these observations underscore the importance of contextual knowledge (molecular and cellular) in order to fully understand and appreciate the effects of vitamin D on bone cells.

This warrants research for the next 100 years: future studies may focus on assessing tissue levels of vitamin D metabolites in addition to circulating levels, and study functionality of the complete metabolic profile of vitamin D.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nu15030480/s1, Figure S1: Literature search strategy performed October 2022.

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Review

Interaction of Vitamin D with Peptide Hormones with Emphasis on Parathyroid Hormone, FGF23, and the Renin-Angiotensin-Aldosterone System

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Abstract: The seminal discoveries that parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) are major endocrine regulators of vitamin D metabolism led to a significant improvement in our understanding of the pivotal roles of peptide hormones and small proteohormones in the crosstalk between different organs, regulating vitamin D metabolism. The interaction of vitamin D, FGF23 and PTH in the kidney is essential for maintaining mineral homeostasis. The proteohormone FGF23 is mainly secreted from osteoblasts and osteoclasts in the bone. FGF23 acts on proximal renal tubules to decrease production of the active form of vitamin D ($1,25(OH)_2D$) by downregulating transcription of 1α-hydroxylase (*CYP27B1*), and by activating transcription of the key enzyme responsible for vitamin D degradation, 24-hydroxylase (*CYP24A1*). Conversely, the peptide hormone PTH stimulates $1,25(OH)_2D$ renal production by upregulating the expression of 1α-hydroxylase and downregulating that of 24-hydroxylase. The circulating concentration of $1,25(OH)_2D$ is a positive regulator of FGF23 secretion in the bone, and a negative regulator of PTH secretion from the parathyroid gland, forming feedback loops between kidney and bone, and between kidney and parathyroid gland, respectively. In recent years, it has become clear that vitamin D signaling has important functions beyond mineral metabolism. Observation of seasonal variations in blood pressure and the subsequent identification of vitamin D receptor (VDR) and 1α-hydroxylase in non-renal tissues such as cardiomyocytes, endothelial and smooth muscle cells, suggested that vitamin D may play a role in maintaining cardiovascular health. Indeed, observational studies in humans have found an association between vitamin D deficiency and hypertension, left ventricular hypertrophy and heart failure, and experimental studies provided strong evidence for a role of vitamin D signaling in the regulation of cardiovascular function. One of the proposed mechanisms of action of vitamin D is that it functions as a negative regulator of the renin-angiotensin-aldosterone system (RAAS). This finding established a novel link between vitamin D and RAAS that was unexplored until then. During recent years, major progress has been made towards a more complete understanding of the mechanisms by which FGF23, PTH, and RAAS regulate vitamin D metabolism, especially at the genomic level. However, there are still major gaps in our knowledge that need to be filled by future research. The purpose of this review is to highlight our current understanding of the molecular mechanisms underlying the interaction between vitamin D, FGF23, PTH, and RAAS, and to discuss the role of these mechanisms in physiology and pathophysiology.

Keywords: vitamin D; vitamin D metabolism; fibroblast growth factor-23; klotho; parathyroid hormone; 1α-hydroxylase; RAAS

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1. Introduction

Vitamin D was first described in the 1920s in attempts to find the cause of rickets that had reached epidemic proportions in industrial cities in Middle and Northern Europe at that time [1–4]. However, it would take almost another century after this seminal discovery until the key factors regulating vitamin D metabolism were found, and a more complete

understanding of the vitamin D hormonal system had evolved. Triggered by the finding in the 1970s that parathyroid hormone (PTH) is a major endocrine regulator of vitamin D metabolism [5–8], and by the discovery of fibroblast growth factor-23 (FGF23) in the year 2000, it became clear that peptide hormones and small proteohormones play pivotal roles in the regulation of vitamin D metabolism by participating in the crosstalk between different organs. In addition, it was discovered in the year 2002 that renin secretion in the kidney is regulated by vitamin signaling, establishing a novel link between vitamin D and the renin-angiotensin-aldosterone system (RAAS) [9].

This review aims to summarize what we currently know about the interaction of the vitamin D hormonal system with peptide hormones and small proteohormones, focussing on PTH, FGF23, and the RAAS.

2. Brief Overview of Vitamin D Metabolism

Vitamin D is a secosteroid derived from cholesterol in animals (cholecalciferol or vitamin D_3), or from ergosterol in fungi and protozoa (ergocalciferol or vitamin D_2). Both forms of vitamin D will be referred to as vitamin D in this review. Vitamin D is either taken up from dietary sources, or it is produced in the skin by UVB-mediated photochemical transformation of 7-dehydrocholesterol. Vitamin D itself is biologically inactive and needs to be metabolically activated by two hydroxylation steps occurring in the liver and the kidney. The canonical vitamin D activation pathway involves 25- and subsequent 1α-hydroxylation. The 25-hydroxylation step occurs in the liver, forming the most abundant circulating form of vitamin D, 25-hydroxyvitamin D (25(OH)D). There is only a little endocrine regulation of this step. In contrast, the 1α-hydroxylase (*CYP27B1*)-mediated synthesis of the vitamin D hormone, 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$) in proximal tubules of the kidney is strictly regulated [10]. The kidney is the major source of circulating $1,25(OH)_2D$ under physiological conditions [11]. However, circulating 25(OH)D can also be converted into $1,25(OH)_2D$ locally in cells expressing 1α-hydroxylase [12,13]. $1,25(OH)_2D$ is the biologically active principle in the vitamin D hormonal system, regulating gene transcription through a nuclear receptor protein, the vitamin D receptor (VDR). It is now generally accepted that the VDR mediates all actions of the vitamin D endocrine system [14]. The VDR is ubiquitously expressed, and as a consequence vitamin D signaling has a major impact on gene regulatory networks in many cell types, regulating transcription of about 3% of the human genome [15]. Typically, the VDR-$1,25(OH)_2D$ complex binds to vitamin D response elements (VDREs) in regulatory regions of target genes as a heterodimer with the retinoid X receptor (RXR) [14].

The most important physiological function of $1,25(OH)_2D$ is in the gut, stimulating intestinal absorption of calcium and phosphate. Therefore, intact vitamin D signaling is essential for bone and mineral homeostasis in most vertebrates [16]. To avoid hypercalcemia and hyperphosphatemia as a potential untoward consequence of excessive vitamin D signaling, the catabolism of $1,25(OH)_2D$ is tightly regulated. Catabolism of 25(OH)D and $1,25(OH)_2D$ in the kidney and all vitamin D target cells is mediated by 24-hydroxylase (*CYP24A1*), the key enzyme of the vitamin D inactivation pathway [17]. Several independent lines of evidence have shown that the regulation of *CYP24A1* is an important part of the homeostatic control of circulating and intracellular concentrations of $1,25(OH)_2D$ [18,19]

Although 25- and subsequent 1α-hydroxylation represent the predominant vitamin D activation pathway, other activation pathways exist, at least at a local level. It has been shown that the cytochrome p450 enzyme CYP11A1 is able to hydroxylate vitamin D_3 at the C17, C20, C22, and C23 positions, giving rise to $20(OH)D_3$ and $22(OH)D_3$ as the major metabolites [20,21]. Vitamin D_2 can be hydroxylated by CYP11A1 at the C17, C20, and C24 positions [20,21]. CYP11A1 is mainly found in adrenal glands, skin, and placenta. $20(OH)D_3$ and $22(OH)D_3$ are detectable in human serum, albeit at 30- and 15-fold lower concentrations than $25(OH)D_3$ [20]. However, $1,20(OH)_2D_3$ remained undetectable in human serum, suggesting that circulating $20(OH)D_3$ is not a substrate of renal 1α-hydroxylation [20]. This is consistent with the low affinity of 1α-hydroxylase for 20(OH)D [22]. In contrast,

the trihydroxylated metabolites 1,20,24$(OH)_3D_3$, 1,20,25$(OH)_3D_3$, and 1,20,26$(OH)_3D_3$ are found in adrenal extracts, suggesting that 1α-hydroxylation of CYP11A1-produced 20$(OH)D_3$ may occur locally in tissues also expressing CYP27B1 such as the adrenal glands, possibly after metabolization by CYP24A1 [20].

The main endocrine regulators of 1α– and 24-hydroxylase expression in the kidney are PTH, FGF23, and 1,25$(OH)_2D$. All these endocrine regulatory factors impact on renal 1α– and 24-hydroxylase expression in a reciprocal manner [23]. PTH stimulates the expression of 1α–hydroxylase, and suppresses that of 24-hydroxylase. On the other hand, FGF23 and 1,25$(OH)_2D$ suppress 1α–hydroxylase, while at the same time stimulating 24-hydroxylase expression [17]. Hence, PTH augments renal production of 1,25$(OH)_2D$, whereas FGF23 and 1,25$(OH)_2D$ suppress it.

3. The Parathyroid-Kidney Axis in the Regulation of Vitamin D Metabolism

As mentioned above, it was discovered in the 1970s that 1α,25$(OH)_2D$ production in the kidney is regulated by PTH, establishing a novel feedback loop between kidney and parathyroid gland [5–8]. PTH is an 84-amino acid peptide hormone secreted by chief cells of the parathyroid gland in response to changes in the concentration of ionized blood calcium [24]. PTH acts through the parathyroid receptor 1 (PTHR1), a G-protein coupled receptor. All actions of PTH increase blood calcium: PTH signaling increases osteoclast activity, thereby promoting Ca release from the bone [25], favors calcium retention by enhancing Ca reabsorption in the thick ascending limb and in distal convoluted tubule of the kidney [26], and stimulates 1,25$(OH)_2D$ synthesis in proximal renal tubules, thereby indirectly increasing Ca absorption from the intestines [27]. In turn, normalization of blood calcium levels and increased circulating 1,25$(OH)_2D$ reduce PTH secretion. Hence, 1,25$(OH)_2D$ and PTH form a tightly controlled feedback loop in which PTH stimulates 1,25$(OH)_2D$ synthesis, whereas 1,25$(OH)_2D$ inhibits PTH secretion (Figure 1). Apart from its effects on calcium metabolism, PTH has a phosphaturic action in the kidney, inhibiting phosphate reabsorption in proximal tubules by downregulating the sodium-phosphate cotransporters NaPi2a and NaPi2c [28]. Therefore, PTH also acts as a phosphate-lowering hormone.

PTH enhances renal 1,25$(OH)_2D$ production by stimulating the expression of the CYP27B1 enzyme responsible for biosynthesis of active vitamin D, but also by downregulating the expression of CYP24A1, thereby inhibiting vitamin D catabolism [25]. Meyer and coworkers have provided important insights into the mechanisms of how PTH signaling controls *Cyp27b1* transcription in the kidney, using ChIP-Seq analysis [29,30]. The latter authors identified a kidney-specific regulatory region located in an intron of the adjacent *Mettl1* gene that is crucial for the regulation of *Cyp27b1* expression by PTH [29]. Mice with a deletion of the enhancer in the *Mettl1* gene showed a global *Cyp27b1* knockout phenotype with profoundly reduced basal *Cyp27b1* expression, distinctly reduced circulating 1,25$(OH)_2D$ concentrations, upregulated PTH, and loss of the PTH-mediated induction of *Cyp27b1* [29]. Furthermore, PTH treatment of mice induced a rapid recruitment of the phosphorylated transcription factor CREB to the enhancer region in the *Mettl1* gene in the kidney [30]. ChIP-Seq analysis also revealed that the PTH-induced suppression of the *Cyp24a1* gene in the kidney is mediated through a kidney-specific regulatory region located downstream of the *Cyp24a1* gene [30,31]. Deletion of this regulatory region blunted the suppressive effect of PTH on *Cyp24a1* transcription in vivo [31].

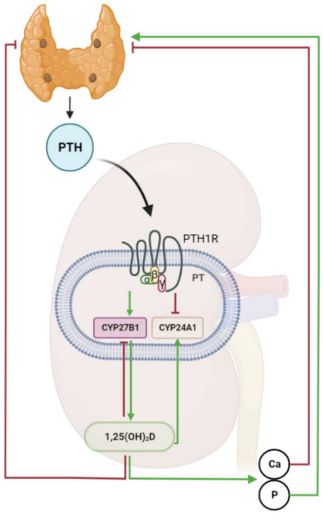

Figure 1. Parathyroid-kidney axis. Parathyroid hormone (PTH) secretion from the parathyroid gland is increased in response to a decrease in ionized plasma calcium (Ca) or to an increase in the blood concentration of inorganic phosphate (P). PTH binds to the parathyroid hormone-1 receptor (PTH1R) in proximal tubules (PT) of the kidney, promoting $1,25(OH)_2D$ production by upregulating 1α-hydroxylase (*Cyp27B1*) and suppressing 24-hydroxylase (*Cyp24A1*) expression. As part of a feedback inhibition, $1,25(OH)_2D$ downregulates its own production by stimulating *Cyp24A1* and inhibiting *Cyp27B1* expression in the kidney. Circulating $1,25(OH)_2D$ signals back to the parathyroid gland, where it inhibits PTH transcription and secretion. Arrows indicate the direction and nature of regulation (green, stimulation; red, inhibition). Created with BioRender.com.(Biorender 2022, Toronto, Ontario, Canada).

As part of a negative feedback mechanism, vitamin D signaling suppresses PTH transcription and secretion as evidenced by a large amount of data from in vivo and in vitro experiments as well as from clinical studies. Incubation of isolated bovine parathyroid cells with $1,25(OH)_2D$ for 48 h caused a reduction of PTH mRNA levels to 50% of control values [32]. This was confirmed in mouse parathyroid explants where treatment with vitamin D metabolites reduced PTH secretion [33]. In rats, injection of $1,25(OH)_2D$ also decreased PTH gene transcription dose- and time-dependently [34]. This reduction was associated with an upregulation of VDR mRNA levels in the parathyroid gland. Studies in mice lacking VDR have shown that $1,25(OH)_2D$ requires the VDR to exert its suppressive effects on *PTH* gene transcription. Administration of $1,25(OH)_2D$ had no effect on *PTH* transcription in global VDR knockout mice, supporting the notion that the VDR is required for modulation of PTH by vitamin D signaling. In addition, global deletion of the VDR results in elevated PTH mRNA abundance and profoundly increased PTH serum levels [35]. However, because of the ubiquitous expression of the VDR and the perturbation of mineral metabolism in these mice, it is difficult to distinguish if these effects are due to severe hypocalcemia or if they are indeed due to lack of the VDR in the parathyroid. More insights into this matter were gained when mice with a specific deletion of the VDR in the parathyroid gland were examined. In contrast to global VDR knockout mice, parathyroid-specific conditional VDR knockout mice did not show elevated *PTH* mRNA levels, but presented with moderately increased serum PTH levels [35]. The latter finding was explained by a downregulation of the calcium-sensing receptor in parathyroid-specific VDR knockout mice [35]. In agreement with the latter findings, VDR mutants on a calcium and phosphorus-enriched rescue diet were protected against secondary hyperparathyroidism (sHPT), and a switch of global VDR mutants on rescue diet to a calcium-reduced challenge

diet led to severe sHPT associated with hypertrophy and hyperplasia of parathyroid glands, together with profound bone loss. Notably, sHPT was fully corrected by switching VDR mutant mice on a challenge diet back to the rescue diet, suggesting that signaling by the calcium-sensing receptor regulates chief cell function in the absence of signaling through the VDR [36].

In conclusion, there is ample evidence that active vitamin D analogs suppress PTH transcription and secretion in a VDR-dependent manner as part of a pharmacological effect. However, the suppressive effect of vitamin D signaling on *PTH* gene transcription in vivo is dispensable under physiological conditions, suggesting that VDR signaling can certainly suppress PTH secretion, but that the main regulation under normal conditions occurs via ionized blood calcium.

The exact mechanisms by which active vitamin D analogs modulate *PTH* gene transcription is still controversial. Different mechanisms have been proposed. Mackey et al. demonstrated that the VDR binds directly to a vitamin D response element in the promoter of the human *PTH* gene, independent of the RXR [37]. In the rat *PTH* gene promoter, it was found that VDR/RXR heterodimers bind to a negative, DR3-type element [38]. Another scenario suggests that the liganded VDR does not bind to the *PTH* gene promoter, but rather binds to and inactivates the vitamin D interacting receptor (VDIR), a positive effector of *PTH* gene transcription [39,40].

Interestingly, the parathyroid gland not only expresses VDR and 24-hydroxylase, but also 1α-hydroxylase [41]. Circulating 25(OH)D can indeed suppress PTH secretion, but the conversion rate is low, and 25(OH)D has much lower potency when compared to $1,25(OH)_2D$, correlating with its much lower affinity for the VDR [25]. The identification of 1α-hydroxylase in the parathyroid gland suggests that local production of $1,25(OH)_2D$ might also influence PTH secretion. However, it remains to be elucidated if local conversion of 25(OH)D into $1,25(OH)_2D$ is able to significantly modulate PTH secretion, and if expression of 1α-hydroxylase in the parathyroid gland undergoes any physiological regulation.

The importance of the vitamin D–PTH axis can be clearly seen in chronic kidney disease (CKD). In patients with CKD, renal $1,25(OH)_2D$ synthesis declines as kidney function deteriorates. As a consequence, hypocalcemia and sHPT develop [42]. Therefore, active vitamin D analogs are administered routinely with the aim to normalize PTH levels. However, although the treatment has been shown to be beneficial in terms of reducing PTH levels by 40 to 60% in different randomized control trials, development of hypercalcemia is a possible adverse effect that needs to be closely monitored [43]. It is important to note in this context that over-suppression of PTH by vitamin D analogs is a risk factor for adynamic bone disease in CKD patients [44]. Another potential pitfall of this treatment strategy is that administration of vitamin D analogs tends to become less effective over time in about 20–30% of individuals [45]. Interestingly, not all vitamin D analogs have the same effect on lowering PTH levels, and their potential to induce hypercalcemia is different [45]. Larger randomized controlled trials are necessary to determine whether one agent is superior to the other in terms of the balance between beneficial and untoward treatment effects, in order to optimize treatment strategies in these patients.

4. The Bone-Kidney Axis in the Regulation of Vitamin D Metabolism

The discovery that putative gain-of-function mutations in the *FGF23* gene are the cause of the inherited renal phosphate-wasting disease autosomal dominant hypophosphatemic rickets in the year 2000 heralded a new era in our understanding of vitamin D metabolism [46]. This discovery led to the subsequent unfolding of a previously unrecognized feedback system between bone and kidney, the bone-kidney axis, in the regulation of vitamin D metabolism. Soon after the initial description of FGF23 as a putative phosphaturic hormone [46] it became evident that FGF23 is also a powerful regulator of vitamin D metabolism. When Shimada and coworkers injected recombinant FGF23 into mice they observed not only hypophosphatemia, but also a distinct suppression of renal 1α-hydroxylase

(*CYP27B1*) mRNA expression [47]. This finding inaugurated the link between FGF23 and vitamin D metabolism.

FGF23 belongs to the group of endocrine fibroblast growth factors (FGFs), and is mainly produced by osteoblasts and osteocytes in bone [48,49]. During the secretion process, a part of the intact protein is cleaved by proteases such as FURIN into N- and C-terminal fragments at a conserved cleavage site [50]. The intact form of FGF23 is a 32 kDa glycoprotein circulating in the bloodstream. Only the intact form of FGF23 can induce signaling through a receptor complex consisting of FGF receptors (FGFR) and the co-receptor αKlotho [51,52]. FGFR1c is the main FGFR mediating FGF23 signaling under normal conditions [51,53]. The c isoform of FGFR1 is generated by alternative splicing of *Fgfr1* mRNA [53,54].

The secretion of bioactive, intact FGF23 in bone is regulated at the transcriptional and posttranscriptional level by factors such as $1,25(OH)_2D$, PTH, phosphate, iron status, and pro-inflammatory cytokines [55]. Among these factors, $1,25(OH)_2D$ is probably the most robust stimulator of FGF23 secretion as evidenced by data from in vivo and in vitro experiments. Treatment of cultured osteoblast-like cells with $1,25(OH)_2D$ results in a dose- and time-dependent increase in *Fgf23* transcription [56,57] Similarly, injection of mice with $1,25(OH)_2D$ causes a VDR-dependent rise in circulating intact Fgf23 [58,59] In addition, the blood levels of intact Fgf23 are lower in *VDR*- and 1α-hydroxylase-deficient mice than those in WT mice, showing that intact vitamin D signaling is a physiological regulator of Fgf23 secretion [58,59]. Genome-wide association studies in a large number of subjects reported that a single nucleotide polymorphism lying upstream of the *CYP24A1* gene was the strongest predictor of the blood concentrations of FGF23, confirming the importance of the vitamin D hormonal system for the regulation of circulating FGF23 levels in humans [60].

Although it is clear that $1,25(OH)_2D$ is a transcriptional regulator of the *Fgf23* gene in a VDR-dependent manner, the exact molecular mechanism of this regulation at the genomic level still remains unclear, and there may be species differences. Whereas several functional VDREs were identified in the promoter of the human *FGF23* gene [61], VDREs are absent in the murine *Fgf23* gene promoter [57], and ChIP-Seq analysis in the murine osteocyte-like cell line IDG-SW3 failed to provide evidence for VDR binding to regulatory regions in the *Fgf23* locus [62]. Therefore, at least in mice, the $1,25(OH)_2D$-induced upregulation of *Fgf23* transcription may be mediated indirectly via DNA binding of other transcription factors. The regulatory region responsible for the $1,25(OH)_2D$-induced upregulation of *Fgf23* transcription in mice has been narrowed down to an enhancer region located directly upstream of the *Fgf23* promoter [63,64]. When this enhancer region was deleted in mice, the $1,25(OH)_2D$-mediated upregulation of *Fgf23* transcription was completely lost [64]. However, the specific transcription factor(s) binding to the enhancer remain unknown.

Secretion of intact FGF23 is also regulated at the posttranslational level in the bone. Intracellular proteolysis of intact FGF23 by FURIN results in the secretion of the biologically inactive N-terminal and C-terminal fragments. Post-translational O-glycosylation at tyrosine 178 by polypeptide N-acetylgalactosaminyltransferase3 (GALNT3) inhibits FURIN-mediated cleavage, thus favoring secretion of intact FGF23. In contrast, phosphorylation at serine 180 (S180) by extracellular kinase family member 20c (FAM20) inhibits glycosylation, thus facilitating FURIN-mediated cleavage [65]. Hence, the posttranslational modification within or near the FURIN-mediated cleavage site is an important determinant of the ratio between intact and cleaved FGF23 secreted by bone cells [50]. It has been shown that PTH injection acutely alters the ratio of intact to cleaved FGF23 in mice [66]. Whether $1,25(OH)_2D$, similar to PTH, may regulate secretion of intact FGF23 not only at the transcriptional, but also at the posttranslational level remains to be elucidated.

The most important target organ of the endocrine actions of FGF23 is the kidney. FGF23 targets proximal and distal renal tubules by binding to basolateral αKlotho/FGFR1c complexes [67,68]. In proximal renal tubules, FGF23 signaling has a dual action: (i) it downregulates the apical membrane abundance of sodium-phosphate co-transporters, causing reduced transcellular phosphate uptake from the urine and, hence, increased urinary phos-

phate excretion [69–71]; (ii) it suppresses the transcription of 1α-hydroxylase and increases transcription of 24-hydroxylase, thereby reducing renal production of $1,25(OH)_2D$ [71]. In distal renal tubules, FGF23 signaling causes enhanced reabsorption of calcium and sodium by upregulating the apical membrane abundance of the epithelial calcium channel TRPV5 and of the sodium-chloride cotransporter NCC [67,72].

There is compelling evidence from animal experiments and rare genetic disorders in humans that intact FGF23 signaling is essential for the endocrine control of vitamin D metabolism in the kidney. In humans, loss-of-function mutations in *FGF23* or *αKLOTHO* are associated with elevated circulating $1,25(OH)_2D$ levels and subsequent hypercalcemia and hyperphosphatemia, leading to progressive soft tissue calcifications [73–75]. Similarly, knockout of the *Fgf23* of *αKlotho* genes in mice leads to early lethality caused by unleashed production of $1,25(OH)_2D$, hypercalcemia, hyperphosphatemia, and ectopic calcifications, a phenotype that can be rescued by concomitant ablation of vitamin D signaling [76–84].

Despite the strong evidence from in vivo studies, the molecular reason for the complete failure of the physiological regulation of renal 1α- and 24-hydroxylases in the absence of FGF23 signaling is still not entirely clear. What do we know about the signaling cascade involved? Based on the similarity of the phenotypes of *αKlotho*$^{-/-}$ and *Fgf23*$^{-/-}$ mice, it is clear that the FGF23-mediated suppression of 1α-hydroxylase transcription in proximal tubular epithelium is an αKlotho-dependent process [51]. In addition, specific deletion of *Fgfr1* in proximal renal tubules has been shown to blunt the FGF23-induced suppression of circulating $1,25(OH)_2D$ levels in vivo [85]. Therefore, at least under physiological conditions, the receptor for FGF23 at the cell membrane of proximal tubules is the FGFR1c/αKlotho receptor complex. At high circulating concentrations of FGF23 such as those found in *Hyp* mice, a model of excessive endogenous FGF23 secretion, FGFR3 and 4 may also play some role in the FGF23-mediated regulation of renal vitamin D metabolism, because ablation of *Fgfr3* and *Fgfr4* increases $1,25(OH)_2D$ levels in *Hyp* mice [86]. FGFRs are tyrosine kinase receptors, and ligand binding results in dimerization and subsequent activation of intracellular phosphorylation cascades [54]. It is well known that the FGF23-mediated regulation of phosphate transport in proximal tubular epithelium involves activation of extracellular signal-regulated kinase-1/2 (ERK1/2) [68]. Activation of ERK1/2 is also one of the first steps in the control by renal 1α- and 24-hydroxylases by FGF23 signaling, as evidenced by the fact that pharmacological ERK1/2 inhibition increases serum $1,25(OH)_2D$ levels in *Hyp* mice [87,88]. However, the signaling pathway downstream of ERK1/2 remains unknown.

At the genomic level, the seminal studies of Meyer and coworkers have shed additional light on the gene regulatory networks involved in the control of 1α- and 24-hydroxylase transcription by FGF23 signaling [29,89]. ChIP-Seq experiments revealed that the kidney-specific regulatory elements leading to suppression of renal 1α-hydroxylase transcription after FGF23 injection in mice are located in introns of a neighboring gene, the *Mettl21b* gene [29]. The functional significance of these kidney specific enhancer sites were demonstrated by the fact that deletion of these sites completely ablated the FGF23-induced suppression of 1α-hydroxylase gene transcription in vivo [29]. However, the specific transcription factors binding to these regulatory regions remain to be elucidated. In addition, it is unclear why simultaneous deletion of all three FGF23-regulated enhancer sites in the *Mettl21b* gene identified by ChIP-Seq experiments did not result in uncontrolled $1,25(OH)_2D$ production and a phenotype similar to *Fgf23* or *αKlotho* deficient mice [29]. A potential explanation for this discrepancy is that FGF23 signaling has reciprocal effects on 1α- and 24-hydroxylase transcription in the kidney, at the same time suppressing 1α-hydroxylase while upregulating 24-hydroxylase transcription [71]. Therefore, the phenotype of *Fgf23* or *αKlotho* deficient mice may only develop in the absence of this dual effect on vitamin D metabolism, affecting both synthesis and degradation of $1,25(OH)_2D$.

It was previously thought that the regulation of 24-hydroxylase transcription by FGF23 is mediated indirectly through the VDR, because treatment of global VDR knockout mice with recombinant FGF23 suppressed 1α-hydroxylase, but did not upregulate 24-hydroxylase transcription [89]. Moreover, injection of recombinant FGF23 failed to induce

24-hydroxylase transcription in kidney-specific 1α-hydroxylase knockout mice, unable to produce $1,25(OH)_2D$ in the kidney [29]. However, it is unclear whether one can infer from the latter experiments that the FGF23-mediated regulation of 24-hydroxylase is VDR-dependent, because both models are characterized by a profound downregulation of renal 24-hydroxylase expression, potentially masking acute effects of FGF23 treatment. Strong evidence in favor of a VDR independent regulation of renal 24-hydroxylase by FGF23 comes from experiments in which an enhancer region downstream of the 24-hydroxylase gene, previously identified by ChIP-Seq experiments, was deleted in mice. In contrast to the $1,25(OH)_2D$-mediated induction of 24-hydroxylase, the FGF23-mediated induction of 24-hydroxylase expression was blunted in these mice, showing that FGF23 and $1,25(OH)_2D$ have independent effects on 24-hydroxylase expression in vivo [31]. However, the exact mechanisms by which FGF23 signaling regulates renal 24-hydroxylase are still unknown.

Taken together, a paradigm has evolved during the past 20 years in which FGF23 and $1,25(OH)_2D$ represent the endocrine signal molecules linking bone and kidney in the regulation of vitamin D metabolism. In this negative feedback system, bone-derived FGF23 suppresses $1,25(OH)_2D$ production in the kidney, whereas $1,25(OH)_2D$ increases FGF23 secretion in bone (Figure 2). The pathophysiological implications of this feedback system are manifold. In diseases characterized by excessive FGF23 secretion such as X-linked hypophosphatemia or chronic kidney disease, enhanced FGF23 signaling suppresses $1,25(OH)_2D$ production in the kidney [90]. However, it is important to consider that treatment with active vitamin D analogs may increase circulating intact FGF23, which in clinical situations with already elevated levels of circulating FGF23 is increasingly recognized as an untoward side effect of the treatment due to the potential negative cardiovascular effects of FGF23 [91].

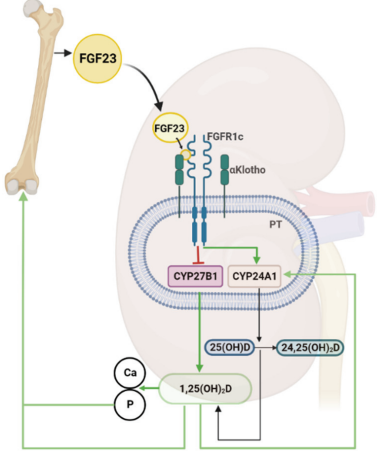

Figure 2. Bone-kidney axis. Fibroblast growth factor 23 (FGF23) is mainly produced in bone under physiological conditions. The active vitamin D hormone ($1,25(OH)_2D$) and phosphate (P) stimulate FGF23 secretion from bone. Circulating FGF23 binds to a receptor complex consisting of FGF receptor-1c (FGFR1c) and of the co-receptor αKlotho in proximal tubules (PT) of the kidney. FGF23 signaling inhibits transcription of 1α-hydroxylase (*CYP27B1*), the key enzyme for vitamin D synthesis, while stimulating transcription of 24-hydroxylase (*CYP24A1*), the main enzyme responsible for vitamin D catabolism. 25-hydroxyvitamin D (25(OH)D) produced in the liver and circulating in the bloodstream is converted in the kidney into $1,25(OH)_2D$ by CYP27B1, or into $24,25(OH)_2D$ by CYP24A1. $1,25(OH)_2D$ stimulates intestinal absorption of calcium (Ca) and P, thereby increasing the blood concentrations of both minerals. Arrows indicate the direction and nature of regulation (green, stimulation; red, inhibition). Created with BioRender.com (Biorender 2022, Toronto, Ontario, Canada).

5. Vitamin D and the Renin-Angiotensin-Aldosterone System (RAAS)

The most important physiological role of vitamin D is its function in the maintenance of mineral homeostasis. However, in the 1980s seasonal variations in the incidence of cardiovascular diseases were for the first time attributed to seasonal variations in vitamin D status [92]. Together with the findings that the VDR is expressed in cardiovascular tissues such as cardiomyocytes, endothelial and vascular smooth muscle cells, this seminal discovery suggested that vitamin D signaling may have a role in the cardiovascular system [93–96]. Indeed, observational studies in humans have found an association between vitamin D deficiency and hypertension, left ventricular hypertrophy (LVH) and heart failure [97–100], and experimental studies in mice and rats provided evidence for a role of vitamin D signaling in the regulation of cardiovascular function. One of the most important regulatory systems of hemodynamics is the RAAS. Juxtaglomerular cells in afferent arterioles in the kidneys store and secrete renin [101]. The main role of renin is to cleave the liver-derived plasma protein angiotensinogen to angiotensin I. Angiotensin I is physiologically inactive and needs to be metabolized to angiotensin II (AngII), which then binds to angiotensin II type I (AT1) and angiotensin II type II (AT2) receptors to exert its effects [101]. The cleavage of angiotensin I to angiotensin II is mediated by angiotensin-converting-enzyme (ACE1) present on vascular endothelial cells mainly in lungs and kidneys. Angiotensin II is a major regulator of peripheral vascular tone, and increases sodium reabsorption from the kidney. Furthermore, it stimulates release of aldosterone from the zona glomerulosa in the adrenal cortex. Aldosterone binds to mineralocorticoid receptors and promotes sodium and water reabsorption in the epithelial cells of the distal convoluted tubule and collecting duct, thereby increasing blood volume and subsequently elevating arterial blood pressure [101,102].

In a seminal study published in 2002, Li and coworkers reported that mice with a global VDR deletion were characterized by increased blood pressure, cardiac hypertrophy, increased renin mRNA and protein levels, as well as elevated plasma angiotensin II production [9]. The angiotensinogen levels in the liver were unchanged when compared to WT mice, suggesting that the increase in plasma AngII levels was due to renin over-activity. Although the latter study for the first time established a potential link between vitamin D signaling and the RAAS, a major caveat in that study was that global VDR knockout mice on a normal diet present with hypocalcemia and secondary hyperparathyroidism, both of which can individually influence the cardiovascular outcomes observed. The authors aimed to address this by feeding a calcium- and phosphorus-enriched rescue diet, and by examining mice at 20 days of age with the rationale that hypocalcemia in VDR knockout mice is not yet developed at this early stage. Interestingly, they found that the dietary treatment for five weeks normalized calcium levels in VDR knockout mice, but the mice still showed increased renin expression. However, the rescue diet did not normalize serum PTH, and PTH itself can stimulate the RAAS [9]. Therefore, based on the study by Li and coworkers [9], it was not entirely clear whether the VDR functions as an endocrine suppressor of renin biosynthesis. A later proposed mechanism by which $1,25(OH)_2D$ could suppress renin gene transcription is by blocking the activity of the cyclic AMP response element in the renin gene promoter [103]. A recent study in VDR knock-out mice fed the rescue diet found an increase of almost 50% in renin mRNA expression in the kidney, as well as elevated serum renin levels when compared to controls on normal or rescue diet. Interestingly, renin activity and thereby the ability to generate angiotensin I was lower in knock-out mice and there were no significant changes in any of the cardiovascular parameters measured [104]. On the other hand, Jia and coworkers found that *VDR* deficiency in 8 week-old mice increased blood pressure by elevating oxidative stress factors and RAAS activity [105]. However, the authors did not report data on mineral metabolism or PTH levels in these mice.

Zhou and coworkers utilized 1α-hydroxylase knock-out mice to investigate if the cardiovascular effect of $1,25(OH)_2D$ is dependent on calcium and/or phosphorus. 1α-hydroxylase knockout mice have a similar phenotype compared with global VDR knockout

mice, making them a useful tool to investigate the role of the VDR in hypertension [106]. Similar to VDR mutant mice, 1α-hydroxylase knockout mice also presented with hypertension and cardiac hypertrophy that was associated with upregulation of the RAAS in cardiac and renal tissue [106]. Treatment of these mice with $1,25(OH)_2D$ suppressed RAAS activity and reversed the phenotype [106]. Similar to the study by Jia and coworkers, the authors did not report PTH levels, making it hard to dismiss the role of PTH in mediating cardiovascular changes.

When we analyzed aged global VDR mutant mice maintained on rescue diet since weaning, we also found increased systolic blood pressure and impaired systolic and diastolic function in 9-month-old VDR knockout mice [107]. However, these changes were due to a lower bioavailability of the vasodilator nitric oxide (NO), leading to endothelial dysfunction, increased arterial stiffness, structural remodeling of the aorta, and impaired systolic and diastolic heart function in 9-month-old mice. Interestingly, kidney renin mRNA levels, urinary aldosterone and plasma renin activity were not different between WT and VDR knockout mice fed the rescue diet, suggesting that the cardiovascular changes induced by the lack of VDR signaling were RAAS-independent. On the other hand, an increase in renin mRNA expression and serum aldosterone levels were observed in global VDR knockout mice fed the normal diet, supporting the notion that secondary hyperparathyroidism might be responsible for RAAS activation and subsequent hemodynamic changes in mice lacking VDR [107].

Mice lacking VDR specifically in the endothelium were able to shed more light on the question whether hypertension in global VDR knockout mice is caused by RAAS activation or is independent of RAAS. These animals had no changes in mineral metabolism, and thereby serve as a better model to test tissue-specific effects of vitamin D [108]. At baseline, the cardiovascular phenotype of 12-week-old endothelium-specific VDR knockout mice was comparable to that of WT mice. However, mice with VDR deletion in the endothelium were more susceptible to treatment with low doses of Ang II as evidenced by increases in systolic, diastolic, and mean arterial pressure when compared to WT mice [108]. Furthermore, in the absence of endothelial VDR, acetylcholine-induced aortic relaxation was significantly impaired, and aortic mRNA and protein abundance of eNOS was reduced, suggesting that the VDR has a protective role in regulating vascular tone and that the changes observed are independent of RAAS [108].

Interestingly, observational studies support an inverse relationship between circulating vitamin D levels and RAAS in hypertensive and non-hypertensive subjects [109–112]. This was confirmed in a small open-label study where daily treatment of vitamin D deficient, hypertensive individuals with 15,000 IU vitamin D for 4 weeks increased 25(OH)D and $1,25(OH)_2D$, decreased PTH levels, reduced supine mean arterial pressure (MAP) and reduced kidney specific RAAS activation [113]. However, randomized controlled trials investigating the interaction between vitamin D and RAAS are scarce. In the study by McMullan and coworkers, supplementation with 50,000 IU vitamin D per week over eight weeks increased 25(OH)D levels, but neither affected blood pressure nor elements of the RAAS [114]. However, the failure to find an effect of vitamin D supplementation on RAAS in the latter study may be due to the fact that normotensive subjects were investigated, and that the subjects were vitamin D sufficient and that the dosage of vitamin D administered was lower [114]. Similarly, in recent large, randomized controlled trials such as VIDA and VITAL with over 5000 and 25,000 vitamin D sufficient subjects, respectively, no beneficial effects of vitamin D supplementation on primary and secondary cardiovascular endpoints were found [115,116]. Unfortunately, the latter trials did not include measurements of RAAS activity as endpoints. Whether the effects would be similar in vitamin D deficient individuals remains to be investigated.

ACE2 enzyme is a more recently discovered homologue of ACE1. In contrast to ACE1, ACE2 is not as ubiquitously expressed, and is found mainly in the kidneys, heart, and testis [117]. While ACE1 produces Ang II, ACE2 converts angiotensin II (Ang II) into angiotensin 1–7 (Ang 1–7), binds to Mas receptors (MasR), and has a powerful vasodilatory,

antifibrotic, antiarrhythmic, and antihypertensive effect [118]. Therefore, ACE2 acts as a negative regulator of the RAAS and together with ACE1 participates in maintaining RAAS homeostasis and blood pressure. It has been reported that vitamin D signaling induces the ACE2/Ang-(1-7)/MasR axis activity, and thereby has a protective role in cardiovascular and pulmonary diseases [119]. Shedding of ACE2 by disintegrin and metalloproteinase domain 17 (ADAM17) from the membrane results in production of soluble ACE2 (sACE2). Interestingly, sACE2 appears to be a biomarker in patients with heart failure, reflecting increased ACE activity [120]. Furthermore, administration of calcitriol to rats with LPS-induced acute lung injury increased ACE2 mRNA expression, and had a positive effect on clinical manifestations and pathological changes in these animals [121,122]. Mice lacking VDR presented with a more severe acute lung injury and higher mortality when compared to WT controls, as a result of excessive induction of angiopoietin-2 and angiotensin II, suggesting that VDR signaling regulates both the angiopoietin-2 and the RAAS pathways [123]. Calcitriol and paracalcitriol decreased ACE1 concentration, ACE1/ACE2 ratio, reduced oxidative stress, and exerted renoprotective effects in diabetic rats, additionally supporting the protective effect of vitamin D [124,125]. It is tempting to speculate that some of the discrepancies observed in animal studies dealing with the effects of vitamin D signaling on the RAAS may be resolved by examining the ACE2/Ang-(1-7)/MasR axis.

While the regulation of RAAS by vitamin D has been extensively studied, the data on modulation of vitamin D metabolism by RAAS is scarce. Currently, there is no evidence that RAAS can directly influence 1α-hydroxylase activity or VDR expression. However, indirect effects of the RAAS on vitamin D metabolism are conceivable, because impacts of RAAS components on αKlotho and FGF23 have been reported. Notably, in several animal models characterized by RAAS activation there is a clear downregulation of αKlotho abundance in the kidney [126–128]. Furthermore, long-term angiotensin II infusion also downregulated renal αKlotho expression at mRNA and protein level [129]. This mechanism is thought to be AT1-dependent, because administration of losartan completely abolished the effect. Several other mechanisms of αKlotho regulation, besides the direct action on AT1 receptor, by Ang II have been proposed. It is known that oxidative stress can downregulate αKlotho expression [130]. Oral administration of a free radical scavenger in rats suppressed the downregulation of αKlotho, supporting the hypothesis that an increase in systemic oxidative stress modulates αKlotho expression [128]. There is accumulating evidence that Ang II stimulates formation of reactive oxygen species (ROS) through the NADPH oxidase system [131–133]. Indeed, Ang II receptor blockade reduced oxidative stress in vivo, suggesting that Ang II can modulate Klotho expression by influencing the formation of ROS [134]. Furthermore, Ang II can downregulate Klotho by upregulating TACE, a TNFα-converting enzyme, thereby identifying TACE inhibitors as a new potential therapeutic target in patients suffering from diseases characterized by low Klotho abundance in the kidney [135–137].

As mentioned above, FGF23 requires the presence of its coreceptor αKlotho in the kidney to exert its effects. In proximal renal tubules, FGF23 signaling inhibits $1,25(OH)_2D$ synthesis, and stimulates its catabolism by downregulating 1α-hydroxylase and upregulating 24-hydroxylase, respectively. Therefore, the effect of Ang II on αKlotho could be interpreted as an indirect modulation of vitamin D metabolism by influencing the FGF23-αKlotho axis.

Chronic kidney disease is characterized by low αKlotho and $1,25(OH)_2D$ levels and elevated circulating FGF23. High FGF23 levels are associated with left ventricular hypertrophy and heart failure [138]. Furthermore, increased circulating FGF23 additionally exacerbates $1,25(OH)_2D$ deficiency, possibly contributing to faster progression of CKD and increased mortality in these patients. Therefore, it seems plausible that targeting Klotho deficiency by blocking RAAS and thus normalizing $1,25(OH)_2D$ production may have beneficial effects in patients with CKD in regard to cardiovascular and renal outcomes. Indeed, RAAS inhibition and supplementation with vitamin D analogs are common treatment

strategies in patients with CKD [139]. However, they are usually considered separately, and their potential joint effect on the FGF23-αKlotho axis is not well investigated. Adapting the existing treatment to modulate renal αKlotho levels and the FGF23-αKlotho-vitamin D axis could prove to be a more efficient therapeutic option in patients with CKD.

6. Conclusions

The purpose of this review was to highlight the current knowledge about the molecular mechanisms underlying the interaction of vitamin D, FGF23, PTH and RAAS in health and disease. FGF23 is a key regulator of vitamin D metabolism. FGF23 lowers $1,25(OH)_2D$ biosynthesis by suppressing 1α-hydroxylase transcription, and increases its degradation by stimulating 24-hydroxylase transcription in proximal tubules of the kidney, thereby down-regulating renal $1,25(OH)_2D$ production. PTH on the other hand has the opposite effect on vitamin D metabolism: it stimulates $1,25(OH)_2D$ synthesis and inhibits its catabolism. Our understanding of the genomic mechanisms involved in the FGF23- and PTH-mediated regulation of vitamin D metabolism in the kidney has increased significantly during recent years. However, some major gaps in our knowledge still remain. For example, the signaling cascades mediating the FGF23-induced regulation of 1α-hydroxylase and 24-hydroxylase transcription in the kidney are still unknown. Furthermore, studies investigating the role of vitamin D in the cardiovascular system and the relationship between vitamin D and the RAAS have provided conflicting evidence. While there are several proposed mechanisms as to how vitamin D signaling can regulate RAAS, the regulation of vitamin D metabolism by RAAS is still an area largely unexplored.

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Month-of-Birth Effect on Muscle Mass and Strength in Community-Dwelling Older Women: The French EPIDOS Cohort

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Abstract: Background. Vitamin D is involved in muscle health and function. This relationship may start from the earliest stages of life during pregnancy when fetal vitamin D relies on maternal vitamin D stores and sun exposure. Our objective was to determine whether there was an effect of the month of birth (MoB) on muscle mass and strength in older adults. **Methods**. Data from 7598 community-dwelling women aged ≥ 70 years from the French multicentric EPIDOS cohort were used in this analysis. The quadricipital strength was defined as the mean value of 3 consecutive tests of the maximal isometric voluntary contraction strength of the dominant lower limb. The muscle mass was defined as the total appendicular skeletal muscle mass measured using dual energy X-ray absorptiometry scanner. The MoB was used as a periodic function in regressions models adjusted for potential confounders including age, year of birth, latitude of recruitment center, season of testing, body mass index, number of comorbidities, IADL score, regular physical activity, sun exposure at midday, dietary protein intake, dietary vitamin D intake, use vitamin D supplements, history and current use of corticosteroids. **Results**. A total of 7133 older women had a measure of muscle strength (mean age, 80.5 $\pm$ 3.8 years; mean strength, 162.3 $\pm$ 52.1 N). Data on total ASM were available from 1321 women recruited in Toulouse, France (mean, 14.86 $\pm$ 2.04 kg). Both the sine and cosine functions of MoB were associated with the mean quadricipital strength (respectively $\beta = -2.1$, $p = 0.045$ and $\beta = -0.5$, $p = 0.025$). The sine function of MoB was associated with total ASM ($\beta = -0.2$, $p = 0.013$), but not the cosine function ($\beta = 0.1$, $p = 0.092$). Both the highest value of average quadricipital strength (mean, 163.4 $\pm$ 20.2 N) and the highest value of total ASM (15.24 $\pm$ 1.27 kg) were found among participants born in August. **Conclusions**. Summer-early fall months of birth were associated with higher muscle mass and strength in community-dwelling older women.

Keywords: vitamin D; month of birth; muscle; pregnancy; older adults

1. Introduction

Besides its classical role in the regulation of bone metabolism, vitamin D has many non-skeletal biological targets mediated by the vitamin D receptor (VDR), which is a specific vitamin D hormone receptor. Vitamin D is involved in the health and function of skeletal muscles, and serum vitamin D concentrations are positively associated with muscle mass and strength in adults [1]. The clinical relevance is that vitamin D deficiency leads to poorer muscular performance and physical deterioration [2]. Importantly, it was proposed that this relationship could start from the earliest stages of life, during pregnancy [3].

During gestation, the fetus is completely reliant on maternal vitamin D stores [4]. The maternal serum 25-hydroxyvitamin D (25OHD) concentration is highly correlated to that of the umbilical cord of the fetus [4]. Moreover, since 90% of maternal vitamin D is synthesized in the skin under the action of solar ultraviolet-B (UV-B) rays, the mother's vitamin D status is mostly influenced by the season; with higher concentrations reported during summer and early fall [5]. Thus, in the absence of supplementation, fetal vitamin D concentration largely depends on the season of pregnancy [5]. This may explain why several conditions related to hypovitaminosis D have been previously linked to the month of birth (MoB); the children born in winter being more at risk of lower height and weight, and at risk of multiple sclerosis for instance [6].

Several studies have also brought evidence that the maternal serum 25OHD concentration and UV-B rays exposure during pregnancy influence the body composition in offspring, including bone mass, degree of adiposity, and muscle mass [7,8]. However, to the best of our knowledge, the relationship between the MoB and the muscle mass and function during adulthood has not been examined yet. We hypothesized that there could be an effect of the MoB on muscles in older adults, specifically that the summer-early fall MoB would be associated with better muscle mass and function. The objective of the present study was to determine whether there was an effect of the MoB on muscle mass and strength in community-dwelling older women.

2. Materials and Methods

2.1. Participants

We used for the present analysis data from the older women included in the 'EPIDOS' study (EPIDémiologie de l'OStéoporose), a French national prospective multicentric and observational cohort study originally designed to determine the risk factors for hip fracture among community-dwelling older women. Sampling and data collection procedures have been described in detail elsewhere [9]. In summary, from 1992 to 1994, 7598 women aged 70 years and older were recruited from electoral lists in five French cities (Amiens, Lyon, Montpellier, Paris and Toulouse). All included study participants had a full medical examination, which consisted of structured questionnaires, demographical measures including the month and year of birth, and a clinical examination.

2.2. Muscle Strength Measure

The maximal isometric voluntary contraction (MVC) strength of the dominant lower limb was measured with a strain gauge fixed to a chair, while the participant was seated, leg and ankle flexed at 90° angle. The leg tested was attached to the lever arm of the strain gauge and the seat height was adjusted to the leg length of the participant. Before carrying out the test, participants were offered to practice the isometric movement in order to warm up. Verbal instructions regarding the test procedure were given by a trained evaluator. Participants pushed as hard as possible against the dynamometer. Three MVC were recorded in Newton (N), and verbal encouragement was given each time to obtain the maximal score. The average MVC strength value was calculated from a set of three consecutive contractions and used for the present analysis.

2.3. Muscle Mass Measure

The total appendicular skeletal muscle mass (ASM) was measured using a dual energy X-ray absorptiometry (DXA) scanner (QDR 4500 W Hologic, Waltham, MA, USA) at enrollment only in the center of Toulouse, France. DXA measurements were performed by a trained technician, and the DXA machine was regularly calibrated. The total ASM was defined as the sum of the two upper and lower limb muscle masses, expressed in kilograms (kg). Data on the validity of body composition parameters of the EPIDOS-Toulouse cohort have previously been published [10].

2.4. Covariates

The following covariates were included as potential confounders in the statistical models: age, body mass index (BMI), number of comorbidities, regular physical activity, instrumental activities of daily living (IADL) score (from 0 to 8, best) [11], dietary protein and vitamin D intakes, use of vitamin D supplements, history of corticosteroids use, current use of corticosteroids, sun exposure at midday, season of evaluation, and study centers.

A physical examination and a health status questionnaire were conducted to assess comorbidities (i.e., hypertension, diabetes, dyslipidemia, coronary heart disease, chronic obstructive pulmonary disease, peripheral vascular disease, cancer, stroke, Parkinson's disease and depression). Weight was measured with a beam balance scale, and height with a height gauge. BMI was calculated according to the formula: weight (kg)/height^2 (m^2). The practice of a physical activity was considered regular if the participants practiced at least one recreational physical activity (i.e., walking, gymnastics, cycling, swimming or gardening) for at least one hour per week for at least the past month. Medications taken regularly had to be brought by the participants to the clinical center during the assessment [9]. The dietary intakes of vitamin D and protein were estimated from a self-administered food frequency questionnaire, as previously published [12]. The cutaneous synthesis of vitamin D was estimated using the following standardized question: "When weather is nice, do you stay more than 15 min exposed to the sun (face and hands uncovered) between 11 a.m. and 3 p.m.?" (yes/no), as previously published [13]. Finally, the season of evaluation was recorded as follows: spring from 21 March to 20 June, summer from 21 June to 20 September, fall from 21 September to 20 December, winter from 21 December to 20 March.

2.5. Statistical Analysis

We provide here a post-hoc analysis of the EPIDOS cohort study. Firstly, the participants' characteristics were summarized using means ± standard deviations or frequencies and percentages, as appropriate. As the number of observations was higher than 40, no transform was applied. Secondly, to determine the association of the MoB (independent variable) with muscle strength and muscle mass (dependent variables), we applied multiple linear regressions. The MoB was added as a periodic function: $\beta 1 \times \sin(2\pi \times \text{MoB}/12) + \beta 2 \times \cos(2\pi \times \text{MoB}/12)$, where $\pi = 3.1415\ldots$ and βi are regression coefficients. p-values < 0.05 were considered significant. All statistics were performed using Stata (version 14.1; College Station, TX, USA).

2.6. Ethics

Women participating in the study were included after having given their written informed consent for research. The study was conducted in accordance with the ethical standards set forth in the Helsinki Declaration (1983). The project was approved by the local ethics committee of each city.

3. Results

Among the 7598 women recruited in the EPIDOS cohort, 7133 had a measure of quadricipital strength (mean ± standard deviation age, 80.5 ± 3.8 years), and 1321 women (mean age, 80.3 ± 3.9 years; all from Toulouse, France) had a measure of total ASM.

The mean quadricipital strength on the cohort was 162.3 ± 52.1 N, and the mean ASM 14.86 ± 2.04 kg (Table 1).

Table 1. Summary of the participants' characteristics (*n* = 7133).

Characteristics	Cohort	
	Summary Value	**(95% CI)**
Demographical measures		
Age, years	80.5 ± 3.8 (70–98)	(80.4; 80.6)
Year of birth	1912 ± 4 (1893–1922)	-
Recruitment center, n (%)		
Amiens (49°54′ N)	1488 (20.9)	(20.0; 21.8)
Paris (48°51′ N)	1457 (20.4)	(19.5; 21.3)
Lyon (45°45′ N)	1365 (19.1)	(18.2; 20.0)
Montpellier (43°36′ N)	1492 (20.9)	(20.0; 21.8)
Toulouse (43°36′ N)	1331 (18.7)	(17.8; 19.6)
Season of testing, n (%)		
Spring	1922 (26.9)	(25.9; 27.9)
Summer	1876 (26.3)	(25.3; 27.3)
Fall	1843 (25.8)	(24.8; 26.8)
Winter	1492 (20.9)	(20.0; 21.8)
Clinical measures		
Body mass index, kg/m^2	25.3 ± 4.0 (14.5–42.1)	(25.2; 25.4)
Number of comorbidities, n (%)	3.4 ± 2.0 (0–24)	(3.36; 3.44)
IADL score, /8	6.3 ± 1.3 (0–8)	(6.27; 6.33)
Regular physical activity, n (%)	3490 (48.9)	(47.7; 50.1)
Sun exposure at midday, n (%)	3609 (50.6)	(49.4; 51.8)
Dietary protein intake, g/day	69.6 ± 16.2 (8.7–162.3)	(69.2; 70.0)
Dietary vitamin D intake, µg/week	63.1 ± 31.3 (0–278.0)	(62.3; 63.9)
Use of vitamin D supplements, n (%)	985 (13.8)	(13.0; 14.6)
History of corticosteroids use, n (%)	455 (6.4)	(5.8; 7.0)
Current use of corticosteroids, n (%)	146 (2.0)	(1.7; 2.3)
Muscles measures		
Mean quadricipital strength, N	162.3 ± 52.1 (17.3–507.3)	(161.1; 163.5)
Total ASM, kg *	14.86 ± 2.04 (9.33–22.78)	(14.75; 14.97)

Data presented as mean ± standard deviation [range] where applicable. ASM: appendicular skeletal muscle mass; CI: confidence interval; SD: standard deviation; IADL: Instrumental Activities of Daily Living; *: data available for 1321 participants.

As indicated in Table 2, every MoB were represented, with a maximum of participants born in March (9.7%) and a minimum born in September (7.4%), with no significant difference.

Table 2. Months of birth among the participants (*n* = 7133).

Month of Birth	Cohort			
	n	**Percentage**	**(95% CI)**	**Cumulative Percentage**
January	630	8.83	(8.17; 9.49)	8.83
February	602	8.43	(7.79; 9.07)	17.27
March	690	9.67	(7.79; 9.07)	26.95
April	568	7.96	(7.33; 8.59)	34.91
May	603	8.45	(7.80; 9.10)	43.36
June	573	8.03	(7.40; 8.66)	51.39
July	625	8.76	(8.10; 9.42)	60.16
August	598	8.38	(7.74; 9.02)	68.54
September	524	7.35	(6.74; 7.96)	75.89
October	527	7.39	(6.78; 8.00)	83.27
November	585	8.20	(7.56; 8.84)	91.48
December	608	8.52	(7.87; 9.17)	100.00

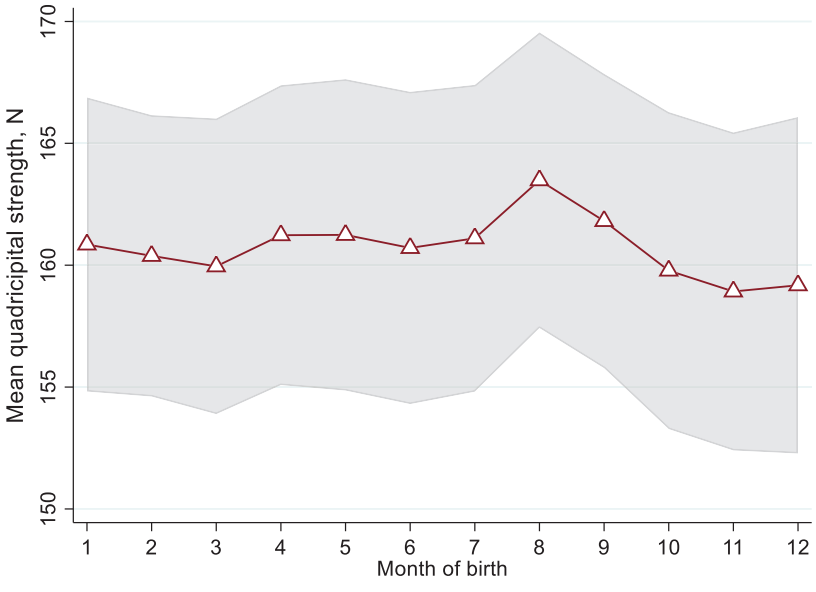
Multiple linear regression models showed that both the sine and cosine functions of MoB were associated with the mean quadricipital strength (respectively fully adjusted β = −2.1 with $p = 0.045$, and fully adjusted β = −0.5 with $p = 0.025$), which means that the association between the MoB and mean quadricipital strength could be modeled using a combination of an inverted sine function and an inverted cosine function (Table 3, Figure 1A). Moreover, only the sine function of MoB was associated with the total ASM (fully adjusted β = −0.2, $p = 0.013$), but not the cosine function (fully adjusted β = 0.1, $p = 0.092$), which means that the association between the MoB and the total ASM was modeled by an inverted sine function (Table 3, Figure 1B). Both the highest predicted value of average quadricipital strength (mean, 163.4 ± 20.2 N) and the highest value of total ASM (15.24 ± 1.27 kg) were found among participants born in August (Table 3).

Table 3. Average values of muscles' measures predicted with the trigonometric modelling by month of birth.

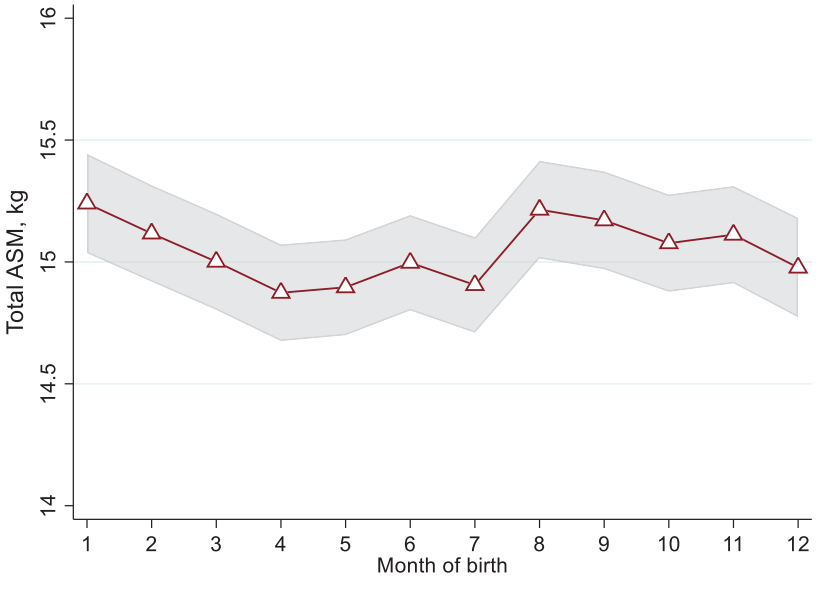
Month of Birth	Mean Quadricipital Strength, N	Total ASM, kg
January	161.0 ± 20.8	15.06 ± 1.20
February	160.0 ± 19.5	15.13 ± 1.19
March	160.0 ± 19.6	15.01 ± 1.17
April	161.0 ± 19.8	14.89 ± 1.22
May	161.2 ± 19.6	14.91 ± 1.15
June	160.9 ± 19.6	15.01 ± 1.18
July	160.6 ± 20.5	14.93 ± 1.24
August	163.4 ± 20.2	15.24 ± 1.27
September	161.6 ± 20.6	15.20 ± 1.29
October	159.8 ± 20.5	15.10 ± 1.19
November	158.8 ± 19.8	15.13 ± 1.21
December	159.0 ± 19.2	14.99 ± 1.21
Beta for sin (*p*-value)	β = −2.1, $p = 0.045$	β = −0.2, $p = 0.013$
Beta for cos (*p*-value)	β = −0.5, $p = 0.025$	β = 0.1, $p = 0.092$

Data presented as mean ± standard deviation.

We also found that the BMI (β = 2.6, $p = 0.001$), a regular physical activity (β = 10.5, $p = 0.002$), and the IADL score (β = 5.9, $p < 0.001$) were positively associated with the mean quadricipital strength, although the number of comorbidities (β = −3.4, $p = 0.019$), the indication for vitamin D supplements (β = −5.2, $p = 0.001$) and the history of corticosteroids use (β = −13.0, $p = 0.025$) were inversely associated with the mean quadricipital strength. Finally, a more recent year of birth (β = 0.4, $p = 0.001$), the BMI (β = 0.3, $p < 0.001$), and the dietary protein intake (β = 0.1, $p < 0.001$) were positively associated with the total ASM, although the current use of corticosteroids (β = −0.8, $p = 0.008$), the need for vitamin D supplements (β = −0.5, $p = 0.001$) and the winter season of evaluation (β = −0.4, $p = 0.016$) were inversely associated with the total ASM.



(**A**)



(**B**)

Figure 1. Trigonometric modelling by month of birth of (**A**) mean quadriceps strength (n = 7178), and (**B**) total appendicular skeletal muscle mass (ASM) (n = 1321). (**A**,**B**): Open triangles represent the average predicted values for respectively "Mean quadricipital strength" and "Total ASM" according to "Month of birth". The shaded area represents the average predicted values plus or minus the median of the standard error of the prediction. The two models are adjusted for age, year of birth, latitude of recruitment centre, season of testing, body mass index, number of comorbidities, IADL

score, regular physical activity, sun exposure at midday, dietary protein intake, dietary vitamin D intake, use of vitamin D supplements, history of corticosteroids use, and current use of corticosteroids.

4. Discussion

The main finding of this population-based study is that the month of birth was associated as a periodic function with the muscle mass and strength in a large cohort of community-dwelling older women, independently of all studied potential confounders. Summer-early fall MoBs, notably August, were associated with higher muscle mass and strength. These birthdates correspond to the participants born from pregnant women who were at the end of the second trimester and at the third trimester of pregnancy during the sunny period from May to August, i.e., the key moment for fetal muscle development.

Seasonality depends on individuals' responses to seasonal fluctuations of environmental constraints. A widely studied seasonality is the effect of MoB on human traits. For instance, previous studies reported that the MoB influences a number of organic diseases [14], mood [15], as well as some morphological traits [16]. Birthweight, a marker of prenatal supplies, depends on the MoB [17]. This effect may be sustainable as birthweight is associated with muscle mass and strength throughout the life course from childhood to older age [18]; consistent with a potential influence of early life on long-term muscle development. Precisely, an effect of MoB on height and weight was also reported during childhood, and even in later life [16]. Weber et al. [19], using a large sample of conscripts in Austria, showed that males born between February to July were taller than those born in the remaining months. Similar observations were made by Banegas et al. [20], who found that Spanish male adults born in June/July were taller than those born in December/January. However, to our knowledge, we provide here the first evidence of a MoB effect on the muscle mass and function in older adults.

Several hypotheses may explain our finding. A first explanation is that the apparent MoB effect on muscles is explained by the influence of MoB on comorbidities. On the one hand, winter MoBs are associated with greater risks of heart disease, cerebrovascular disease, malignant neoplasms, and chronic respiratory diseases, with potential adverse consequences on physical activity and muscle mass and strength in adulthood [21,22]. On the other hand, the MoB may play a role in mood disorders due to the changing length of the photoperiod, and may influence the preference of individuals to plan activities rather in the morning or in the evening. According to Caci et al., people born in March/April are eveningness with a depressive mood tendency, although those born in September/October would be morningness and with impulsivity-related personality [23]. This may have an impact on daily physical activity and thus on muscle quality. However, the number of comorbidities, the IADL score, the practice of a regular physical activity and the sun exposure habits were used as potential confounders in the present analysis, and did not alter the association of MoB with muscle mass and function. Moreover, this first set of explanation could not account for the MoB-related morphological differences reported in offspring. Thus the alternative possibility of a direct MoB effect on muscles should be considered, based on the seasonal changes of maternal exposure to UV-B radiation during pregnancy and the subsequent changes of maternal serum vitamin D concentration. In French latitudes, the sun exposure of pregnant women is deemed insufficient between October and May to allow normal vitamin D concentration [24]. As the fetus is completely reliant on maternal vitamin D stores [4], and as 90% of maternal vitamin D is synthesized under the action of solar UV-B rays, the fetus experiences seasonal changes of 25OHD concentrations throughout gestation, with potential consequences on musculature.

Emerging evidence has shown that intrauterine exposure to 25OHD during pregnancy exerts a range of effects on development of skeletal muscle [1,25], probably through the modulation of the expression of muscle transcription factors [26]. Vitamin D results in the induction of myogenesis, proliferation, differentiation and cellular apoptosis [1], and may also participate in the control of protein synthesis in muscle cells by increasing the anabolic effect of insulin and leucine on muscle cells [1]. Recently, two studies in pigs found that

improving maternal vitamin D status not only increased the number of fibers in longissimus dorsi of fetuses at day 90 of gestation [3], but also induced increased weight and muscle fiber cross-sectional area in psoas major and longissimus dorsi of weaning piglets [26]. In humans, maternal serum 25OHD concentration in pregnancy was positively associated with offspring height-adjusted handgrip strength and with offspring percent lean mass [27]. Evidence is also accruing that maternal serum 25OHD concentrations during pregnancy might influence offspring body composition in childhood [7,8]. Maternal antenatal serum 25OHD concentrations have been associated positively with bone mass [8] and negatively with fat mass [7]. Consistently, a population-based mother–offspring cohort study reported that maternal vitamin D status during late pregnancy could influence muscle strength of offspring at age 4 years [27]. Findings from the Mysore Parthenon Study, a prospective mother-offspring birth cohort in India, demonstrated greater arm muscle area at 5 and 9.5 years in children born to vitamin D–replete (serum 25OHD > 50 nmol/L) compared with vitamin D–depleted (25OHD < 50 nmol/L) mothers [7]. A positive association between maternal estimated UV-B exposure in the third trimester and offspring lean mass determined by DXA at 9.9 years of age was also observed in the Avon Longitudinal Study of Parents and Children (ALSPAC) [8]. All these observations support that vitamin D promotes both prenatal and postnatal skeletal muscle development, which may account for our findings, notably that people born in summer-early fall, when vitamin D status is optimal, are more prone to exhibit higher (i.e., better) muscle mass and strength in later life.

The implications for practice and research are manifold. First, our results support the idea that muscles changes related to early life hypovitaminosis D are persistent in adult age, suggesting a trait-like association between vitamin D status and muscles; consistent with the fact that hypertrophy of skeletal muscle fibers developed prenatally is the primary mechanism by which skeletal muscle growth occurs postnatally [28]. Second, they support the fact that the vitamin D status of pregnant women is crucial for fetal development and should be closely monitored in clinical routine. Offspring exposure to high levels of vitamin D appears essential for the muscles at the end of the second trimester and during the third trimester of pregnancy. Since 70% of pregnant women have hypovitaminosis D [4], these observations are relevant to public health and call for a precautionary approach based on maternal vitamin D monitoring and eventual repletion. Third, they provide a strong rationale for conducting clinical trials in pregnant women, which is expected to reveal long-term effects of vitamin D supplements on body composition, behavioral development and physical function. We propose that future clinical trials should focus on pregnancies that give birth in winter-early spring. In this perspective, our findings participate in further elucidating the profile of ideal target populations, which is an important step to provide effective guidelines on the proper use of vitamin D supplements during pregnancy.

Our results confirmed that the use of corticosteroids is associated with reduced muscle mass and strength [29], although more frequent physical activity is associated with increased strength [30], and higher dietary intakes of proteins are associated with increased muscle mass [31]. These consensual results strengthen the consistency of our study and confirm the relevance of the MoB effect we found on muscle mass and strength.

The strengths of this study include a large sample of older adults recruited in five centers with various latitudes. Additionally, the participants recruited were all born in a large period, from 1893 to 1922, in an era without vitamin supplementation D policy for pregnant women. This could have emphasized the role of seasonality and sun exposure. We also had the opportunity to measure the muscle mass with DXA, which is more accurate and relevant than anthropometric measures such as the calf circumference. Finally, regression models were applied to measure adjusted associations. Regardless, some potential limitations of our study should be considered. Firstly, the MoB is a proxy measure of maternal vitamin D status during pregnancy, and no information was available on the actual vitamin D status of the participants' mothers during pregnancy. Secondly, the study cohort was restricted to relatively vigorous older women who may be unrepresentative of older adults in general, especially regarding the musculature. The study participants

may have been also more motivated, with a greater interest in personal health issues, than the general population of older adults. Thirdly, the use of an observational design precludes inferring any causal inference. Fourthly, although we were able to control for important characteristics that could modify the association between MoB and muscles, residual potential confounders, such as the latitude of the birthplaces, might still be present. Then, the ASM was measured only in one center, Toulouse, and one latitude, 43°36′ N. Finally, no information on eventual premature birth was available, although premature delivery appears to be more frequent in mothers deficient in vitamin D [32].

5. Conclusions

In conclusion, our results show for the first time to our knowledge that the month of birth is associated as a periodic function with the muscle mass and strength in a large cohort of community-dwelling older women, with potential consequences on various health outcomes including diabetes mellitus, falls, fractures, and all-cause mortality [33]. The summer-early fall months of birth were associated with higher muscle mass and strength in late life. This suggests that enhancing maternal vitamin D status during pregnancy whether through sun exposure, diet or supplementation, might improve prenatal and postnatal muscle development. This new orientation may offer a powerful mechanism to better understand the muscular changes in older adults, and to act on their healthcare early in life by setting up vitamin D supplementation in pregnant women. However, formal testing of this hypothesis in an interventional setting should be undertaken before the development of any formal clinical recommendations.

Author Contributions: C.A. has full access to all of the data in the study, takes responsibility for the data, the analyses and interpretation, and the conduct of the research, and has the right to publish any and all data, separate and apart from the attitudes of the sponsor. Study concept and design: C.A. Acquisition of data: A.-M.S. Analysis and interpretation of data: G.T.D. and C.A. Drafting of the manuscript: G.T.D. and C.A. Critical revision of the manuscript for important intellectual content: A.-M.S., D.S.-R. and F.R.H. Statistical analysis: F.R.H. Obtaining funding: A.-M.S. Administrative, technical, or material support: A.-M.S. Supervision: C.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the ethical standards set forth in the Helsinki Declaration (1983). The project was approved by the local ethics committee of each city.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Patient level data are freely available from the corresponding author at cedric.annweiler@chu-angers.fr. There is no personal identification risk within this anonymized raw data, which is available after notification and authorization of the competent authorities.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

25OHD	25-hydroxyvitamin D
ASM	Appendicular skeletal muscle mass
BMI	body mass index
kg	kilograms
DXA	Dual Energy X-ray Absorptiometry
IADL	instrumental activities of daily living
MoB	month of birth
N	Newton
SD	standard deviations
UV-B	ultraviolet-B rays
VDR	vitamin D receptor

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Review

Novel CYP11A1-Derived Vitamin D and Lumisterol Biometabolites for the Management of COVID-19

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Abstract: Vitamin D deficiency is associated with a higher risk of SARS-CoV-2 infection and poor outcomes of the COVID-19 disease. However, a satisfactory mechanism explaining the vitamin D protective effects is missing. Based on the anti-inflammatory and anti-oxidative properties of classical and novel (CYP11A1-derived) vitamin D and lumisterol hydroxymetabolites, we have proposed that they would attenuate the self-amplifying damage in lungs and other organs through mechanisms initiated by interactions with corresponding nuclear receptors. These include the VDR mediated inhibition of NFκβ, inverse agonism on RORγ and the inhibition of ROS through activation of NRF2-dependent pathways. In addition, the non-receptor mediated actions of vitamin D and related lumisterol hydroxymetabolites would include interactions with the active sites of SARS-CoV-2 transcription machinery enzymes (M^{pro};main protease and RdRp;RNA dependent RNA polymerase). Furthermore, these metabolites could interfere with the binding of SARS-CoV-2 RBD with ACE2 by interacting with ACE2 and TMPRSS2. These interactions can cause the conformational and dynamical motion changes in TMPRSS2, which would affect TMPRSS2 to prime SARS-CoV-2 spike proteins. Therefore, novel, CYP11A1-derived, active forms of vitamin D and lumisterol can restrain COVID-19 through both nuclear receptor-dependent and independent mechanisms, which identify them as excellent candidates for antiviral drug research and for the educated use of their precursors as nutrients or supplements in the prevention and attenuation of the COVID-19 disease.

Keywords: vitamin D; lumisterol; SARS-CoV-2; anti-inflammatory; ACE2; M^{pro}; RdRp

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1. Introduction

COVID-19 is still the top health issue in the world. Vaccines approved for COVID-19 provide protection against some strains of SARS-CoV-2; however, new mutant strains develop in continuity and some of them escape immunity provided by current vaccines [1]. The infection from SARS-CoV-2 has severe adverse outcomes with a significantly higher mortality rate than influenza. The major cause of death in COVID-19 is acute respiratory distress syndrome (ARDS) caused by cytokine storm [2,3]. This enhanced hyperactivated innate immune response against the virus causes severe damage to the patient's body/organs which might be fatal (Figure 1). In this mini-review we will discuss how vitamin D and its derivatives can be helpful against the infection caused by SARS-CoV-2.

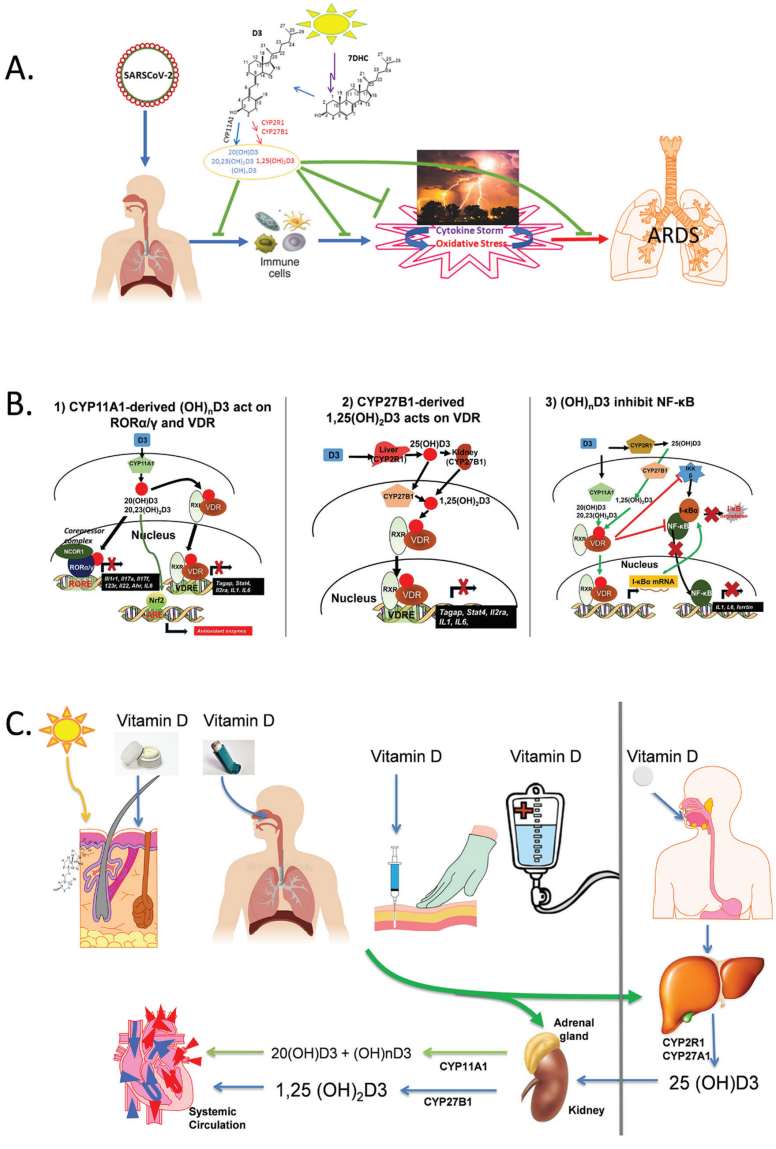

Figure 1. Possible mechanisms by which vitamin D can counteract the COVID-19 illness. In panel (**A**) it is proposed that the novel hydroxyderivatives of vitamin D3, in similar manner as $1,25(OH)_2D3$, inhibit cytokine storm and oxidative stress, with net attenuating effect on ARDS and multiorgan failure induced by COVID-19. Panel (**B**) proposes a mechanism of action of canonical and non-canonical vitamin D-hydroxyderivatives. Vitamin D signaling in mononuclear cells involves the activation of the VDR or inverse agonism on RORγ with downstream inhibition of inflammatory genes and the suppression of oxidative stress through the activation of NRF2. VDR, vitamin D receptor; RXR, retinoid X receptor; ROR, retinoic acid orphan receptor, RORE, ROR response element; ARE, antioxidant response element; VDRE, vitamin D response element; NRF2, transcription factor NF-E2-related factor 2. Panel (**C**) shows how different routes of vitamin D delivery impact vitamin D hydroxylation/activation patterns. Reprinted with permission from the publisher [86].

Vitamin D, a prohormone, is a fat soluble secosteroid, which is formed in the skin after the absorption of UVB energy by the B ring of 7-dehydrocholesterol (7DHC) [4–6]. The prolonged exposure of 7-DHC to UVB leads to the phototransformation of pre-vitamin D3 to tachysterol and lumisterol [4–6]. It can also be ingested from the diet and supplements. As a prohormone, it must be activated to exert the biological activity. In the classical activation pathway vitamin D3 is metabolized by several cytochromes P450 (CYPs) enzymes before being transformed to its known active form 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$) [7–10]. $1,25(OH)_2D3$ activates the nuclear vitamin D (VDR) receptor, which controls not only body calcium metabolism [9,11–13] but also many important physiological functions, including the regulation of the innate and adaptive immunity [6,9,14–17]. Vitamin D can be activated by two pathways known as a canonical, with sequential hydroxylation at C25 and C1α [18], and a non-canonical, activated by CYP11A1 [19,20]. The canonical pathway includes the metabolism of vitamin D3 to 25-hydroxyvitamin D3 (25(OH)D3) by CYP2R1 and CYP27A1 in the liver and final C1α hydroxylation in the kidney to the biologically active form $1,25(OH)_2D3$ by CYP27B1 [7–9]. This pathway also operates in the peripheral tissues, including skin [8,18,21–24].

The phenotypic effect of $1,25(OH)_2D3$ is predominantly mediated through an interaction with the VDR leading to the transcriptional activation of more than 3000 genes [4,6,8–10]. Non-genomic regulatory actions for $1,25(OH)_2D3$ were also described [6,8,10]. $1,25(OH)_2D3$, in addition of regulating body calcium metabolism, also regulates diverse functions on the systemic, tissue, and cellular levels [4,6,8–15,18–20]. Of general interest are the antioxidative and anti-inflammatory properties of $1,25(OH)_2D3$, which have been appreciated for almost two decades [4,6,8–15,18–20]. The latter includes the downregulation of proinflammatory cytokines production through the inhibition of NFkB [6,12–14].

Recently discovered non-canonical pathways of vitamin D activation are initiated by an obligatory enzyme of steroidogenesis, CYP11A1, in a complex process involving several CYPs and producing more than a dozen of hydroxyderivatives [8,20,25–27]. CYP11A1 is not only expressed in adrenals, placenta, and gonads [28] but also in immune cells [29] and other peripheral organs including skin [19,30]. The CYP11A1-derived hydroxyderivatives are non-calcemic or low calcemic [31–34] and can therefore be used at high concentrations for therapeutic purposes [35–39]. They are also detectable in natural products including honey [40] and human serum [25,41,42]. Similarly, to $1,25(OH)_2D3$, they can alter gene expression by binding on the genomic site of the VDR [33,43–46]. CYP11A1-derived vitamin D3 hydroxyderivatives also bind to other nuclear receptors, including aryl hydrocarbon receptor (AhR) [47–49], retinoic acid orphan receptors (ROR)α and γ [44,50], liver X receptors (LXR)α and β [51] and can change their expression and activities [27]. The CYP11A1-derived hydroxymetabolites of vitamin D3, including 20(OH)D3 and $20,23(OH)_2D3$, have demonstrated anti-inflammatory and anti-oxidative effects [27,36,42,46,52–56], which are similar to the effects described for the classical active form of vitamin D3, $1,25(OH)_2D3$ [6,12–14,18–20].

Novel pathways of 7DHC transformation by CYP11A1 [30,57,58], with the further phototransformation of the 5.7-dienal products to corresponding secosteroids [27,59–64] and the hydroxylations of lumisterol by CYP11A1 and CYP27A1, were also discovered [65–67], with their products being biologically active and detectable in human body and acting on RORα and γ as inverse agonists and as agonists on LXR α and β and on the non-genomic site of the VDR [51,66,68]. Most recently, an enzymatic activation of tachysterol was reported with the metabolic products acting on the VDR, AhR, LXRs, and RORs [63]. These metabolites exert similar biological effects as the classical active form of vitamin D [$1,25(OH)_2D3$] and they have their unique activity pattern towards various nuclear receptors, aside of the classical VDR/RXR complex [9].

Although SARS-CoV-2 infection in human cells involves multiple factors, in this review we are focusing on two main interactions listed below. The spike protein (S) of SARS-CoV-2 facilitates the entry of the virus into human cells by engaging angiotensin-converting enzyme 2 (ACE2) as their entry receptor [69] and further cellular serine protease TMPRSS2 is used for priming of S protein [70–72]. The association between ACE2 and Spike protein is critically im-

portant and current vaccines (mRNA) are developed to inhibit this interaction [73]. The actions of CYP11A-derived vitamin D3-hydroxymetabolites, canonical 1,25$(OH)_2$D3 and lumisterol hydroxymetabolites with SARS-CoV-2 replication machinery enzymes were previously explored [74], which included molecular modeling on classical vitamin D compounds [75]. The significance of these studies is further discussed in this review.

The SARS-CoV-2 virus replicates within host cells using its cellular and enzymatic components. M^{pro}, also termed 3CL protease, is a 33.8-kDa cysteine protease which helps in the maturation of functional polypeptides involved in the assembly of replication-transcription machinery [76–78]. M^{pro} digests the polyprotein at no less than 11 conserved sites, starting with the autolytic cleavage of this enzyme itself from pp1a and pp1ab, which are individual nonstructural proteins essential for viral genome replication [76]. Another enzyme important to the life cycle of SARS-CoV-2 is RdRp (RNA-dependent RNA polymerase), which catalyzes the replication [79] of RNA from an RNA template. SARS-CoV-2 use an RdRp complex for the replication of their genome and for the transcription of their genes [79]. M^{pro} and RdRp are enzymes required for viral replication and are not homologous to any gene in the human genome. Hence, they are very attractive targets for the development of anti-viral drugs against COVID-19. Therefore, we will further discuss how the hydroxymetabolites of vitamin D and of lumisterol can counter SARS-CoV-2 infection at different stages of this process.

2. CYP11A1-Derived Vitamin D and Lumisterol Hydroxymetabolites Exert Anti-Inflammatory and Antioxidant Effects

The cytokine storm is a response to viral infection, which causes immune cells to release several pro-inflammatory cytokines/chemokines (interferons, interleukins 1, 6 and 17, chemokines, colony-stimulating factors, and tumor necrosis factors (TNF)), leading to hyper inflammation and organ damage [80–82]. This process in the lung leads to acute lung injury and ARDS. Along with = ARDS, another factor which plays a role in damage to the tissue and cells is oxidative stress. It is secondary to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [83–85]. CYP11A1-derived vitamin D3 and lumisterol hydroxymetabolites exhibit potent anti-inflammatory activities through the inhibition of IL-1, IL-6, IL-17, TNFα and INFγ production and/or other pro-inflammatory pathways [25,36,37,43,46,50,52,54–56,68], which are similar to those mediated by classical 1,25$(OH)_2$D3 [4,6,8–15,18–20]. The anti-inflammatory effects of active forms of vitamin D can be mediated through the downregulation of NF-κB, involving action on VDR and inverse agonism on RORγ leading to the attenuation of Th17 responses (Figure 1B). These compounds also induce anti-oxidative and reparative responses with mechanism of action involving the activation of NRF2 and p53 signaling pathways [27,42,52,53,68,86]. Interestingly, the anti-viral role of NRF2 is also recognized [87]. The use of 1,25$(OH)_2$D3 has its limitations because of the toxicity that includes hypercalcemia [7,88]. However, CYP11A1-derived 20(OH)D3, 20(OH)D2, and 20,23$(OH)_2$D3 are not calcemic even at very high doses [31–34]. Hence, vitamin D and its metabolites can be used as economic nutritional supplements to counter the effects of the SARS-CoV-2 infection like cytokine storm [86,89].

3. Inhibition of the Interaction between ACE2 and SARS-CoV-2 Spike RBD

The ACE2 interacts with the receptor-binding domain (RBD) region of the spike protein [90,91]. SARS-CoV-2 and SARS-CoV-2 RBD are typically in standing up state and resting state, respectively. SARS-COV-2 RBD has higher binding affinity to ACE2, but its lying-down state makes it less accessible to ACE2 and other inhibitory or neutralizing agents [90,91]. The use of host protease (furin, TMPRSS2, cathepsins etc.) for its activation helps as a strategy to overcome the lying down state and maintaining its high binging affinity to ACE2 [90–92]. This interaction is critical and important for drug development against COVID-19 infection. Molecular modeling and simulation [93] evaluated the binding of vitamin D3 and its hydroxyderivatives to SARS-CoV-2 RBD, and their potential to inhibit

its interaction with ACE2. The study showed that vitamin D3 and its hydroxyderivatives can function as inhibitors of TMPRSS2 and inhibit the SARS-CoV-2 receptor binding domain (RBD) binding to the ACE2 [93]. Molecular dynamics (MD) simulations for the interactions of $1,25(OH)_2D3$ have shown the favorable binding free energy of ACE2, SARS-CoV-2 RBD, and TMPRSS2 with $1,25(OH)_2D3$ [93]. The binding free energy of ACE2, SARS-CoV-2, and TMPRSS2 with $1,25(OH)_2D3$ were -18.55 ± 4.16, -16.97 ± 1.69, and -21.04 ± 1.53 kcal/mol separately, further indicating that ACE2, SARS-CoV-2 RBD, and TMPRSS2 show favorable binding with $1,25(OH)_2D3$ [93]. The predicted interaction of $1,25(OH)_2D3$ with SARS-CoV-2 RBD and ACE2 could result in the conformation and dynamical motion changes of the binding surfaces between SARS-CoV-2 RBD and ACE2, leading to the interruption of the binding of SARS-CoV-2 RBD with ACE2 [93]. The interaction of $1,25(OH)_2D3$ with TMPRSS2 also caused the conformational and dynamical motion changes of TMPRSS2, which could affect TMPRSS2 to prime SARS-CoV-2 spike proteins [93]. These studies [93] have indicated that vitamin D3 and its biologically active hydroxymetabolites have the theoretical potential to prevent the cellular entry of SARS-CoV-2 by serving as the inhibitor of TMPRSS2 and blocking the binding of SARS-CoV-2 RBD with ACE2.

Molecular modeling was also used for the virtual screening of antiviral compounds to SARS-CoV-2 non-structural proteins [94]. The described interactions between spike protein and ACE2 can be also disrupted by other compounds, including vitamin D as described by other research groups [95–97]. These included interactions with vitamins, retinoids, steroids, vitamin D derivatives, and dihydrotachysterol as examples. The detailed mechanisms of action were discussed in [95–97]. In addition, vitamin D was identified as a potential inhibitor of COVID-19 Nsp15 endoribonuclease binding sites [98].

To confirm our predictions on novel vitamin D3 and lumisterol hydroxymetabolites, we have used an inhibitor screening kit (SARS-CoV-2 inhibitor screening kit, Acro Biosytems) (Table 1). The kit uses a colorimetric ELISA platform, which measures the binding of immobilized SARS-CoV-2 S protein and biotinylated human ACE2. Top compounds with best binding energy were theoretically predicted previously [93], selected for this assay, showing the inhibition of the interaction between ACE2 and RBD (Table 1). 20(OH)L3 showed the highest level of inhibition of the RBD-ACE2 interaction followed by 25*S*27(OH)L3 and 20(OH)D3 at concentrations of 2×10^{-7} M. The compounds were observed to be effective in μM concentrations, which is promising for future clinical and preclinical testing.

Table 1. Inhibition of ACE2 and RBD interaction by the hydroxymetabolites. Inhibition by the selected metabolites was observed concentration of 2×10^{-7} M using a SARS-CoV-2 inhibitor screening kit from Acro Biosytems. The assay followed the manufacture's protocol of the SARS-CoV-2 (B.1.617.2) Inhibitor Screening Kit (Spike RBD) (1 Innovation Way, Newark, DE 19711, USA). Data were analyzed by one way ANOVA using GraphPad Prism statistical software.

No.	Name of the Ligand	Inhibition in Enzyme Activity (%)	*p*-Value
1.	20(OH)D3	46.057	0.013
2.	$1,20(OH)_2D3$	29.222	NS *
3.	$1,25(OH)_2D3$	36.876	0.034
4.	$20,23(OH)_2D3$	36.152	0.018
5.	24(OH)L3	32.343	0.0265
6.	20(OH)L3	74.552	0.001
7.	25*S*27(OH)L	51.722	0.005
8.	$20,22(OH)_2L3$	13.074	NS *

NS * Not significant.

In addition, the treatment of HaCaT keratinocytes with these hydoxymetabolites changed the expression of ACE2 and TMPRSS2 in a metabolite-specific manner. 1,20$(OH)_2$D3, 1,25$(OH)_2$D3, and 24(OH)L3 suppressed the expression of ACE2, and TMPRSS2 expression was inhibited by 20(OH)D3, 1,20$(OH)_2$D3, 1,25$(OH)_2$D3, 20(OH)L3, and 24(OH)L3 (Supplementary Figure S1). This suggests that these compounds can inhibit the bonding of ACE2 and RBD not only by directly blocking the binding but also by altering the expression of these receptors' genes.

4. Inhibition of the Activity of the Replication Enzymes of SARS-CoV-2

SARS-CoV-2 replicates inside the host cell after its entry. The viral particles utilize host resources, but viral replication machinery plays crucial role in its replication. These viral specific factors do not share homologies with human proteins and are therefore targets for drug development against COVID-19 [74]. We selected two SARS-CoV-2 replication enzymes, RdRp or nsp12 and 3C-like protease (3CLpro or M^{pro}), based on their recognized importance for drug development [76]. Although there are reports that vitamin D and its metabolites have potential to inhibit other viral protein, we selected these proteins as we have had experimental potential to confirm our results [74]. 3CL-Chymotrypsin, such as Protease or Main protease (M^{pro}), is one of the two proteolytic enzymes that helps in cleaving the replicase polyprotein 1ab in SARS-CoV-2 at 11 specific sites, the recognition sites being Leu-Gln (Ser, Ala, Gly) in order to release 12 nsps (nsp4, nsp6-16) that are essential for viral replication as well as viral assembly [3,99]. This enzyme shares no common cleavage site with any human protease and its functional importance in the life cycle of the virus makes it an attractive target for drug development. Similarly, RNA-dependent RNA Polymerase (RdRp) is an enzyme that is responsible for the replication of RNA from an RNA template [100]. RdRp is another conserved protein of retroviruses and is also a proven target for the development of antiviral drugs [79]. We performed molecular docking on the active sites of these two enzymes and found that the hydroxyderivatives of vitamin D3 and lumisterol were binding efficiently on the active sites of these enzymes with similar affinities to known therapeutics danoprevir, lopinavir, and ritonavir serving as positive controls [74]. We further confirmed the inhibition of the enzyme activity in the presence of the compounds.

Danoprevir, lopinavir, and ritonavir were used as a standard for the comparison of predicted energies of the top 10 selected compounds [74,94]. A virtuous complementarity to the M^{pro} binding pocket was observed for top compounds, which indicates a possibility that these metabolites have ability to hinder the substrate accessibility, inhibiting the enzymatic activity in process. Significant interactions between the selected metabolites and the critically important residues of the M^{pro} substrate-binding pocket were observed (Figure 2) and predicting a block to the substrate-binding pocket of COVID-19 M^{pro} (Figure 2) [74]. The detailed analysis of the residues interaction with these metabolites was reported previously [74]. The inhibition of M^{pro} was confirmed using 3CL Protease, MBP-tagged (SARS-CoV-2) Assay (BPS Biosciences). The 25(OH)L3, 24(OH)L3, and 20S(OH)7DHC being most effective at inhibiting M^{pro} activity by 10–19% at a concentration of 2×10^{-7} M (Figure 3A). Similarly, selected metabolites showed interactions with critically essential residues of SARS-CoV-2 RdRp (Figure 4) [74]. These sterols and secosteroids presented a similar binding pattern to inhibitor remdesivir on RdRp active sites [74,101]. The binding prototype of the compounds showed a virtuous complementarity to the SARS-CoV-2 RdRp binding pocket predicting that they can inhibit the enzymatic activity (Figure 3B). These metabolites showed inhibitory activity ranging from 40–60% at a concentration of 10^{-7} M (Figure 3B) with 25(OH)L3 with an IC_{50} of 0.5 μM followed by 1,25$(OH)_2$D3 and 20S(OH)L3, which had an IC_{50} of 1 μM. Thus, our published work [74] has demonstrated that novel 7DHC, lumisterol, and vitamin D3 hydroxymetabolites have the potential to inhibit SARS-CoV-2 infection by restricting its replication cycle. Interestingly, unbiased retrospective analyses of microarray data obtained with epithelial cells indicated the antiviral effects of 20,23$(OH)_2$D3 with similar effects for 1,25$(OH)_2$D3 [86]. A potential role of

hydroxylumisterols appears to be strengthened by recent findings, showing their similarity in structure to 25(OH)L3, where 25-hydroxycholesterol can act as a potent SARS-CoV-2 inhibitor [102], and that cholesterol 25-hydroxylase inhibits SARS-CoV-2 [103] and oxysterols show anti-viral activity [104].

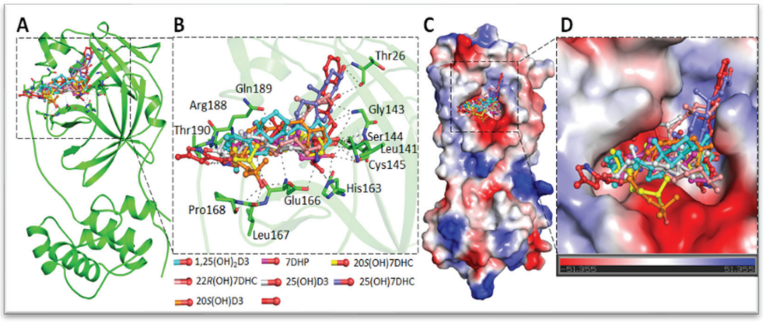

Figure 2. The binding pattern of identified compounds with SARS-CoV-2 M^{pro}. (**A**) structural representation of the protein in complex with selected sterols and secosteroids. (**B**) selected compounds blocking the binding pocket and making significant interactions with the functionally important residues of SARS-CoV-2 M^{pro}. (**C**) surface representation of conserved substrate-binding pocket of SARS-CoV-2 M^{pro} in complex with selected compounds. (**D**) zoomed view of the substrate-binding pocket of SARS-CoV-2 M^{pro} in complex with selected compounds. Reprinted with permission from the publisher [74].

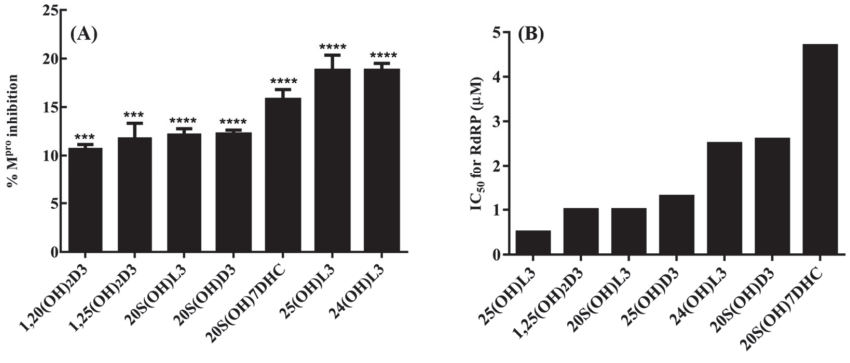

Figure 3. Enzyme inhibition by the selected sterols and secosteroids. (**A**) the M^{pro} enzyme inhibition by the selected metabolites at concentration of 2×10^{-7} M. The inhibition percentages were calculated using the formula: % inhibition = 100 × [1(X Minimum)/(Maximum–Minimum)]. Minimum = negative control without any enzyme (0% enzyme activity); Maximum = positive control with enzyme and substrate (100% enzyme activity). The test sets included enzymes, substrates, and the test compounds, and excitation at a wavelength of 360 nm and the detection of emission at a wavelength of 460 nm was observed for change in enzyme activity. The statistical significance of differences was evaluated by one-way ANOVA; *** $p < 0.001$ and **** $p < 0.0001$ for all conditions relative to ethanol blank, $n = 3$. (**B**) the RdRp enzyme activity inhibition by selected sterols and secosteroids. The inhibition percentages were calculated using the formula: % inhibition = 100 × [1 − (X-Minimum)/(Maximum–Minimum)]. Minimum = negative control without any enzyme (0% enzyme activity); Maximum = positive control with enzyme and substrate (100% enzyme activity). The statistical significance of differences was evaluated by one-way ANOVA; *** $p < 0.001$ and **** $p < 0.0001$ for all conditions relative to the ethanol blank, $n = 3$. RdRp, RNA-dependent RNA polymerase. Reprinted with permission from the publisher [74].

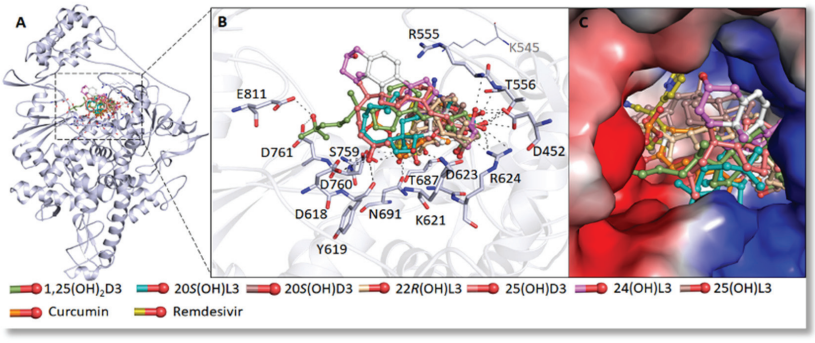

Figure 4. The binding pattern of identified sterols and secosteroids with SARS-CoV-2 RdRp. (**A**) structural representation of the protein in complex with selected compounds. (**B**) active site residues of the RdRp-binding pocket making significant interactions with each of the identified compounds. (**C**) surface view of the RdRp active site with the electrostatic potential from red (negative) to blue (positive) in complex with selected compounds. RdRp, RNAdependent RNA polymerase. Reprinted with permission from the publisher [74].

5. Hypothesis

Overall, the above considerations provide strong support for the ability of D3, L3, and 7DHC hydroxymetabolites to counter the different stages of SARS-CoV-2 infection and most of them are non-calcemic. These metabolites can attenuate cytokine storm and have an ability to inhibit the viral replication enzymes. A deficiency of these hydroxymetabolites may contribute to the transition of SARS-CoV-2 patients from asymptomatic to symptomatic. Vitamin D deficiency in the body will lead to reduced levels of the vitamin D hydroxymetabolites and consequently diminished capacity to attenuate cytokine storm. Anti-inflammatory effects by vitamin D and lumisterol hydroxymetabolites were observed at 0.1 μM concentration. Similarly, 20(OH)L3, 25*S*27(OH)L3, and 20(OH)D3 were able to inhibit RBD-ACE2 interaction, which is necessary for cellular entry of the virus. For M^{pro}, a significant inhibition has been observed at 0.1 μM of 25(OH)D3, which is close to its plasma concentration [8]. Of note, low pre-infection 25(OH)D3 levels are associated with a higher severity of the COVID-19 illness [105]. For RdRp, the IC_{50} for 25(OH)D3 was 1.3 μM, approximately one order of magnitude above its plasma concentration. For the hydroxylumisterols tested, plasma concentrations are unknown except for 20(OH)L3 where a value of 0.25 μM has been reported [66]. Also based on the enzymology, 25(OH)L3, which had the lowest IC_{50} (0.5 μM) for the inhibition of RdRp, is likely to have substantially higher concentrations. Interestingly, similar in structure 25(OH)cholesterol has been shown to have anti-SARS-CoV-2 activities [102,103], while cholesterol 25-hydroxylase generated anti-inflammatory environments [106] and oxysterols are recognized for anti-viral activities [104].

Therefore, defects in vitamin D or lumisterol delivery either orally as nutrients or supplements or their production in the skin after UVB exposure can lead to the deficiency of corresponding hydroxymetabolites; which have demonstrated anti-viral potential [74,86,93]. This family of compounds contains dozens of molecules [27], which can influence the different stages of SARS-CoV-2 infection. This will require further validation, as has been carried out for other molecules, including classical vitamin D3 derivatives [48,75,94–98]. Vaccines against SARS-CoV-2 are clearly a major advance in controlling COVID-19; however, new viral variants emphasize the need for alternative therapeutic or nutritional approaches. Therefore, consideration for novel vitamin D and L3 metabolites for anti-viral drugs that could attenuate COVID-19 is warranted.

6. Concluding Remarks

There are reports demonstrating a strong association for the pre-infection deficiency of vitamin D in hospitalized COVID-19 patients [100] and increased disease severity and

mortality [102,103]. The oral supplementation of vitamin D may affect SARS-CoV-2 infection outcomes. Several clinical trials are ongoing, assessing the ability of vitamin D to prevent COVID-19 infection and disease severity [104–110], showing its importance in managing COVID-19. However, the precise mechanism of vitamin D action against COVID-19 is still unresolved. Here, we have formulated a hypothesis for the mechanism of action of vitamin D and lumisterol hydroxymetabolites against COVID-19. It includes the enzymatic activation of vitamin D3 or sterol precursors with receptor-independent [74,93] or nuclear receptor-dependent activities downstream of VDR, LXR, and AhR activation or inverse agonism on RORs [27,51,86]. Similarly, tachysterol and its metabolites represent additional candidates for at least receptor-mediated activities [63]. We also acknowledge other mechanism-oriented work on classical vitamin D hydroxyderivatives in the prevention or therapy of COVID-19 that has been reported or reviewed recently [75,96,111–117]. Therefore, further clinical testing for their therapeutic use would represent an important step in understanding the beneficial actions of vitamin D3, lumisterol, and possibly tachysterol derivatives [63,97] in COVID-19 with significant implications for the nutritional approach. Vitamin D, lumisterol, or tachysterol ingested through the diet or as supplements would serve as prohormones for further activation in the body towards biologically active forms.

7. Patent

Patent application pending (WO2022006446A1), which includes the experimental portion of this work.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu1422477/s1, Figure S1: The change in the expression of *ACE2* and *TMPRSS2* in keratinocyte HaCaT cell line after treatment with listed vitamin D and lumisterol metabolites at 2×10^{-7} M [39].

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Vitamin D and the Ability to Produce 1,25(OH)$_2$D Are Critical for Protection from Viral Infection of the Lungs

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Abstract: Vitamin D supplementation is linked to improved outcomes from respiratory virus infection, and the COVID-19 pandemic renewed interest in understanding the potential role of vitamin D in protecting the lung from viral infections. Therefore, we evaluated the role of vitamin D using animal models of pandemic H1N1 influenza and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. In mice, dietary-induced vitamin D deficiency resulted in lung inflammation that was present prior to infection. Vitamin D sufficient (D+) and deficient (D−) wildtype (WT) and D+ and D− Cyp27B1 (Cyp) knockout (KO, cannot produce 1,25(OH)$_2$D) mice were infected with pandemic H1N1. D− WT, D+ Cyp KO, and D− Cyp KO mice all exhibited significantly reduced survival compared to D+ WT mice. Importantly, survival was not the result of reduced viral replication, as influenza M gene expression in the lungs was similar for all animals. Based on these findings, additional experiments were performed using the mouse and hamster models of SARS-CoV-2 infection. In these studies, high dose vitamin D supplementation reduced lung inflammation in mice but not hamsters. A trend to faster weight recovery was observed in 1,25(OH)$_2$D treated mice that survived SARS-CoV-2 infection. There was no effect of vitamin D on SARS-CoV-2 N gene expression in the lung of either mice or hamsters. Therefore, vitamin D deficiency enhanced disease severity, while vitamin D sufficiency/supplementation reduced inflammation following infections with H1N1 influenza and SARS-CoV-2.

Keywords: vitamin D; influenza; SARS-CoV-2; lung; inflammation

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1. Introduction

Low vitamin D status is associated with poorer outcomes following acute respiratory diseases including influenza [1]. Vitamin D supplements are touted as being useful in high doses for reducing the severity of seasonal influenza [2–4]. The recent emergence of severe acute respiratory syndrome (SARS)-coronavirus (CoV)-2 and the ongoing pandemic led to a renewed interest in high-dose vitamin D supplements to prevent and treat severe SARS-CoV-2 disease (i.e., COVID-19) [4]. Infection with SARS-CoV-2 results in local and systemic inflammation that when controlled may enhance survival and clinical outcomes of COVID-19 [5]. Severe respiratory illness can also be caused by influenza viruses or co-infection with influenza and coronaviruses. An association between low vitamin D status and severe COVID-19 was postulated, and accordingly, vitamin D supplementation was proposed to be beneficial to treat COVID-19 [6]. A recent systemic review concluded

that low circulating levels of vitamin D (serum 25(OH)D, 25D) were associated with more severe symptoms and higher mortality in patients with COVID-19 [7]. Interventions that control the lung inflammatory response to viruses have the potential to benefit the global population.

Vitamin D, and the active form of vitamin D ($1,25(OH)_2D$, 1,25D), has been implicated to play a role in the anti-viral response; however, this effect may be specific to different viruses. For example, 1,25D treatment of T cells from human immunodeficiency virus (HIV)-infected individuals in vitro resulted in a decrease in viral RNA transcription by a direct reduction in NF-κB, which reactivates proviral HIV [8]. Conversely, 1,25D treatment of respiratory syncytial virus (RSV)-infected human epithelial cells in vitro did not affect viral replication [9]. Production of antimicrobial peptides such as cathelicidin, β-defensin etc. and production of cytokines such as TNFα, IL-5, IL-1β, IL-6, IL-10, and type I interferons at the mucosal surface are important parts of the innate immune response against viruses [10,11]. Several studies show that 1,25D and other vitamin D analogs induce cathelicidin production in response to virus infection [12]. Cathelicidin LL-37 was shown to bind and kill viruses including influenza viruses in vitro [13–17]. Therefore, it is possible that vitamin D through the induction of cathelicidin could directly target SARS-CoV-2 and influenza. 1,25D also limits inflammatory responses by decreasing IFNγ, IL-6, and TNFα: however, the effects of 1,25D to reduce inflammation could be detrimental for the ability of the host to clear some viruses [18,19].

Lung epithelial cells are vitamin D targets since they express the vitamin D receptor (VDR) and are regulated by 1,25D treatments. In mice, 1,25D treatments reduced inflammation following lipopolysaccharide-induced lung injury and regulated angiotensin converting enzyme (ACE) expression in the rat lung epithelium [20]. In mice, infection with an influenza H9N2 virus induced mRNA for the VDR in the lung and 1,25D-treated animals had reduced lung inflammation [21]. Treating mice with the 1,25D precursor, 25hydroxyvitamin D (25D), also protected mice from subsequent H1N1 influenza infection [22]. 1,25D treatment of human bronchial epithelial cells suppressed IL-6 and protected the cells from oxidative damage [23]. VDR knockout (KO) mice had reduced expression of tight junction proteins such as zonula occludens-1, occludin, and claudins (2,4, and 12), suggesting an important role for the VDR in maintaining the integrity of the lung [24]. Vitamin D has direct effects on the lung epithelium, and 1,25D suppresses inflammation in the lung.

Extensive association studies in humans led to the proposal that high dose vitamin D supplements could be beneficial for protection from severe influenza and SARS-CoV-2 infections. Since cause and effect are extremely difficult to determine in human studies, we sought to evaluate the effects of vitamin D on the lung anti-viral response in animal models. The data from mice and hamsters suggest that vitamin D supplementation reduces inflammation in the lung following pandemic H1N1 and SARS-CoV-2 infection. D− mice had lung inflammation even without infection. We showed previously that feeding D− diets to mice that cannot produce 1,25D (Cyp KO) resulted in severe vitamin D deficiency [25]. Influenza disease was greatest in D− Cyp KO, and the least amount of disease was in D+ WT mice. The survival of D+ Cyp KO mice was significantly less than D+ WT mice following an influenza infection. Vitamin D supplementation reduced lung inflammation and *Ifnb* expression in the lung of mice following SARS-CoV-2 infection. Vitamin D treatments had no effect on the expression of viral RNA for either SARS-CoV-2 or H1N1 influenza in the lungs of hamsters or mice. Instead, the data support an important role for vitamin D and 1,25D in controlling the host inflammatory response to viruses in the lungs.

2. Materials and Methods

2.1. Animal Models

C57BL/6 WT, K18hACE2 (hACE, Jackson Laboratories, Bar Harbor, ME, USA), and Cyp KO (gift from Dr. Hector DeLuca, University of Wisconsin, Madison, WI, USA) mice were bred (mice) and housed (mice and hamsters) according to approved IACUC protocols at the Pennsylvania State University (University Park, PA, USA). For the experiments, age-

and sex-matched mice were fed: chow diets (D+) (Lab diets #5053, Arden Hills, MN, USA) or purified diets with (D+) and without (D−) vitamin D (Envigo, T.D. 89123, Madison, WI, USA). For some experiments, D+ mice were fed corn oil alone or corn oil with 1,25D. For some experiments, D− mice were fed corn oil alone or corn oil with one of two doses of vitamin D3 (Sigma-Aldrich, C9756, St. Louis, MO, USA). Age- and sex-matched Golden Syrian Hamsters were purchased from Envigo (Indianapolis, IN, USA) and maintained on the chow (D+) or D− diet (Envigo, T.D.120008) and orally fed corn oil or corn oil with vitamin D3. Serum was collected to monitor the vitamin D status of mice and hamsters.

2.2. Serum 25 Hydroxy Vitamin D (25D) Measurements

Serum 25D levels were measured using an ELISA kit and standards as per the manufacturer's instructions (25-OH D, Eagle Biosciences, Amherst, NH, USA). The limits of detection were 1.6 ng/mL 25D.

2.3. SARS-CoV-2 Infection

SARS-CoV-2 USA-WA-1/2020 (Centers for Disease Control and Prevention, BEI Resources, NIAID, NIH: NR-52281) was used for infecting both mice and hamsters. hACE2 mice (n = 7–8/group) were anesthetized using isofluorane and infected with 100−1000 $TCID_{50}$ units of SARS-CoV-2 in 50 μL phosphate buffered saline. Hamsters were sedated with ketamine (150 mg/kg), atropine (0.015 mg/kg), and xylazine (7.5 mg/kg) via intraperitoneal injection and intranasally inoculated with 10,000 $TCID_{50}$ units of SARS-CoV-2 in 100 μL Dulbecco's Modified Eagle Media. Hamsters were given atipamezole (1 mg/kg) subcutaneously to reverse the sedation. Equal numbers of males and females were used for all experiments, and body weights and symptoms were monitored daily until the endpoint criteria were reached or day 14 post-infection.

One series of experiments in hACE2 mice used standard D+ rodent chow (Lab diets #5053, Arden Hills, MN, USA) diets with oral dosing of corn oil or 10 ng/day of 1,25D diluted in 10 μL of corn oil beginning the day before infection and continuing until sacrifice. Additional experiments used mice or hamsters fed D− diets with or without oral vitamin D3 (n = 8–16/group). D− hACE2 mice were dosed orally with corn oil (D−), 0.125 μg vitamin D3/day (D+), or 2.5 μg vitamin D3/day (D++) beginning 8 weeks prior to infection and continuing throughout the experiment (n = 12–18/group). Hamsters were placed on D− diets with corn oil or 8 μg vitamin D3 (D+) in corn oil/day starting 11 days before infection and continuing throughout the experiment (n = 5–6/group).

2.4. H1N1 Infection

D+ and D− WT and D+ and D− Cyp KO littermates (n = 12–18/group) were fed identical diets with and without vitamin D. Mice were anesthetized with isofluorane to inoculate them intranasally with 10–30 $TCID_{50}$ mouse-adapted A/H1N1/California/04/2009 influenza in 50 μL Gibco's reduced-serum minimum essential media (ThermoFisher, 31985-070, Waltham, MA, USA) [26]. Body weight and clinical signs were monitored daily until they either reached end-point criteria or at day 14 post-infection.

2.5. Biocontainment and Animal Care and Use

All studies with SARS-CoV-2 were conducted in a biosafety level 3 enhanced (BSL3+) laboratory. This facility is approved for BSL3+ respiratory pathogen studies by the U.S. Department of Agriculture and Centers for Disease Control. Studies with pandemic H1N1 influenza were conducted under biosafety level 2 enhanced conditions. All animal studies were conducted in compliance with the Animal Care and Use Committee under protocol numbers: 202001693, 202001516, 202001440, and 202001638.

2.6. RNA Isolation and Quantitative PCR

Tissues were homogenized in TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA). Total RNA was extracted from the tissues using chloroform-isopropanol precipitation and

quantified using NanoDrop (ThermoFisher, Waltham, MA, USA). A total of 1–2 μg RNA was reverse transcribed using AMV Reverse Transcriptase (Promega, Madison, WI, USA). Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) and are listed in Supplementary Table S1. Gene expression was quantitated using the SYBR green mix (Azura Genomics, Raynham, MA, USA) and StepOne Plus system (Applied Biosystems, Carlsbad, CA, USA). Primers for the SARS-CoV-2 N gene were a commercial kit from IDT (Cat #10006713). Gene expression was calculated using the delta-delta C_T method using GAPDH and uninfected control tissues. Gene expression was normalized to day 0 uninfected controls.

2.7. Histology

Lung tissues were collected from hamsters and mice and fixed in 10% formalin. These tissues were embedded in paraffin, sectioned, and H & E stained by the PSU Animal Diagnostic Lab Histology lab. Scoring of lung pathology associated with SARS-CoV-2 infection was conducted by veterinary pathologists certified by the American College of Veterinary Pathologists that were blinded to vitamin D3 treatment status. The criteria for the histopathologic evaluation of the tissue sections were conducted as described for H1N1 infected mouse lungs (n = 2–5/group) [27–29], SARS-CoV-2 infected mouse lung sections (n = 4–6/group) [30,31], and hamster lung sections (n = 4/group) [32–34]. The individual parameters evaluated the extent of pneumonia, damage to alveoli, and lymphocytic infiltration by various specific parameters described in Tables S2–S4.

2.8. Statistical Analysis

Results are represented as the mean ± SEM. Statistical analysis was performed using Prism ver. 10 (GraphPad, San Diego, CA, USA) using multiple *t*-tests, one-way ANOVA, two-way ANOVA, mixed-effects analysis with Bonferroni multiple comparisons tests, the Kruskal–Wallis test with Dunn's multiple comparison, the unpaired *t*-test, and the log-rank survival test as applicable. Data were checked for normal distribution and outliers. $p < 0.05$ was the cutoff to determine significance.

3. Results

3.1. The Effect of Vitamin D on H1N1 Infection

To evaluate the role of vitamin D deficiency during respiratory virus infection, WT and Cyp KO mice were fed a D+ or D− diet. Regardless of the genotype, serum 25D levels were significantly different between mice fed the D+ or D− diet (Figure 1A). As expected, D+ Cyp KO mice accumulated 25D and had higher levels of 25D than D+ WT mice [25]. Following infection with pandemic H1N1 influenza, respiratory distress was evident in all mice by 7 days post-infection (Figure 1B). D+ WT mice showed the least amount of respiratory distress, and after day 10, D+ WT and D+ Cyp KO mice no longer exhibited symptoms of respiratory distress (Figure 1B). D− WT and D− Cyp KO mice had greater symptoms of respiratory distress that were not completely resolved by day 14 post-infection in the D− Cyp KO mice (Figure 1B). Only 1 of 18 D+ WT mice died following H1N1 infection (Figure 1C). D− WT mice had lower survival compared to D+ WT mice (Figure 1C). Conversely, both Cyp KO groups showed decreased survival; 62% from D+ Cyp KO and 57% from D− Cyp KO mice, respectively. The expression of the influenza M gene at day 4 post-infection was not different in the four groups of influenza-infected mice (data not shown). Lung sections from D− WT and D− Cyp KO mice (d0) but not D+ WT mice showed signs of inflammation even before infection (Figure 1D). The amount of alveolar hemorrhage was significantly more at day 4 post-infection in the D− Cyp KO compared to D+ WT or D− WT (Figure 1D). At day 14 post-infection, the lung sections of D+ Cyp KO, D− WT, and D− Cyp KO mice appeared more severe than D+ WT (Figure 1E). Therefore, vitamin D deficiency and the inability to produce 1,25D increased the susceptibility of mice to H1N1 influenza infection.

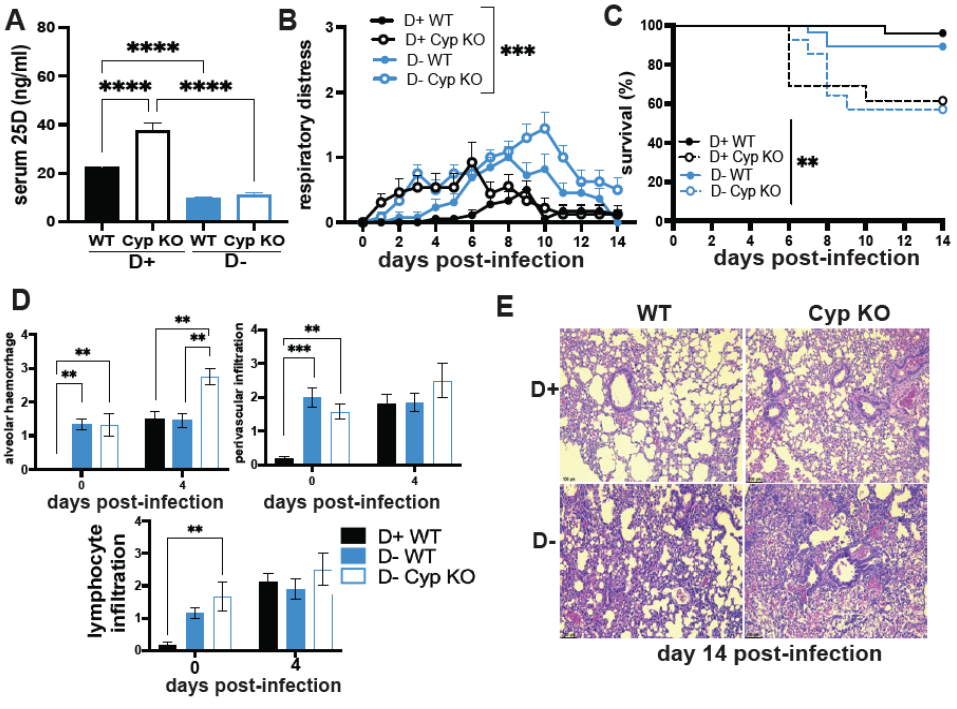

Figure 1. Vitamin D deficiency and Cyp27B1 KO increases susceptibility to H1N1 infection. D+ and D− WT and D+ and D− Cyp KO littermates were infected with H1N1 influenza (n = 12–18 per group). (**A**) Serum was collected at day 14 post-infection to measure 25D. Mice were monitored for (**B**) respiratory distress symptoms and (**C**) survival of D+ WT (n = 18), D+ Cyp KO (n = 13), D− WT (n = 13), and D− Cyp KO (n = 12) mice until day 14 post-infection. Lung tissues were collected for histology from uninfected and day 4 post-infected mice for histology. H & E-stained sections were scored for (**D**) lung alveolar hemorrhage, perivascular infiltration, and lymphocyte infiltration in D+ WT (n = 4) and D− WT (n = 4–5) and D− Cyp KO (n = 2–3) mice at day 0 and day 4 post-infection. (**E**) Representative histology of the lung of D+ WT (score = 3), D+ Cyp KO (score = 4), D− WT (score = 5.5), and D− Cyp KO (score = 6) at day 14 post-infection. Sections from D− WT, D+ Cyp KO, and D− Cyp KO show increased lymphocyte infiltration, alveolar hemorrhage and constricted bronchiolar spaces compared to D+ WT. Values are the mean ± SEM. Statistical significance was assessed using one-way ANOVA with Bonferroni multiple comparison test for (**A**) two-way ANOVA with Bonferroni multiple comparison test for (**B**,**D**) and log rank (Mantel–Cox) survival analysis for (**C**). ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3.2. Mouse and Hamster Models of SARS-CoV-2 Infection

SARS-CoV-2 does not effectively infect WT mice. The transgenic expression of human (h)ACE-2 was shown to allow SARS-CoV-2 infection in mice [31]. hACE-2 mice were infected with 100, 200, and 315 $TCID_{50}$ SARS-CoV-2, and the mice were evaluated for pre-determined euthanasia endpoints for up to 14 days post-infection. The dose of SARS-CoV-2 that resulted in the sacrifice of 50% of the mice was 200 $TCID_{50}$ (Figure 2A). The mice infected with 100 $TCID_{50}$ failed to reach the euthanasia endpoints, were sacrificed at day 14 post-infection and had only minimal weight loss (Figure 2A). The viral gene copy number for the nucleocapsid (N) protein was measured in the lungs of mice at 100 and 200 $TCID_{50}$. High copy numbers of the N gene were detected as early as day 2 post-infection and remained high until day 6 post infection (Figure 2B). The N gene copy number significantly decreased by day 14 post-infection (Figure 2B). Interestingly, the mice that

were infected with 100 $TCID_{50}$ had high amounts of the N gene in the lung even though they showed only mild symptoms of infection, and there were no differences between the N gene expression in the lung between the 100 and 200 $TCID_{50}$ inoculum (Figure 2A,B). Lungs from SARS-CoV-2-infected mice were used to measure mRNA for the 1alpha hydroxylase that produces 1,25D (*Cyp27B1*), the vitamin D receptor (*Vdr*), and the 24 hydroxlase that degrades vitamin D (*Cyp24A1*). There was no change in *Vdr* expression in the lung at day 2 or day 4 post-infection (Figure 2C). Conversely, *Cyp27B1* and *Cyp24A1* expression was higher at day 2 and day 4 post-infection than in the uninfected lung (Figure 2C). *Cyp27B1* expression was significantly lower in the day 4 than the day 2 post-infection lung (Figure 2C). Infection of the hACE2 mice with SARS-CoV-2 induced the expression of two genes that regulate vitamin D metabolism in the lungs.

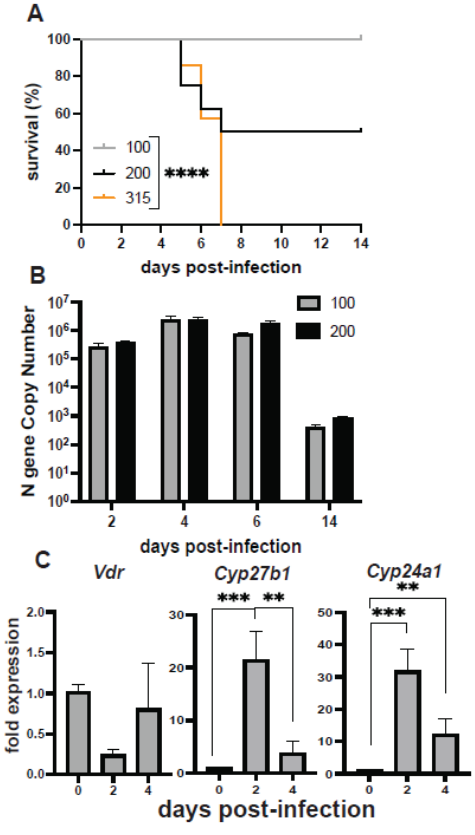

Figure 2. SARS-CoV-2 infection. K18-hACE2 mice were intranasally inoculated with 100 (n = 8), 200 (n = 8), and 315 (n = 7) $TCID_{50}$ of SARS-CoV-2 virus. Mice were sacrificed when they met predetermined endpoints (worsening conjunctivitis, lethargy, labored breathing, and/or dehydration) or at day 14 post-infection. (**A**) Mice were monitored for survival until day 14 post-infection. Mice were euthanized, and lung tissue was collected for (**B**) SARS-CoV-2 N gene expression at days 2–14 post-infection, (**C**) *Vdr, Cyp24A1*, and *Cyp27B1* mRNA gene expression (n = 4/group/timepoint) at day 0, day 2, and day 4 post-infection in the lung. Samples were normalized to uninfected control tissue. Values are the mean ± SEM. Statistical significance was assessed using log-rank test for trend (**A**), two-way ANOVA model for main effects only for (**B**), and one-way ANOVA with Bonferroni multiple comparison test on log-transformed expression values for (**C**). ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Hamsters infected with SARS-CoV-2 are reflective of human SARS-CoV-2 infection in that the virus infects the lower respiratory tract, causing similar respiratory sequelae [35]. We previously showed that hamsters infected with 10^5 $TCID_{50}$ SARS-CoV-2 lost weight shortly after infection, and none of the hamsters died following infection [33]. The weight loss peaked by day 6 post-infection, and the hamsters recovered completely by day 10 post-infection [33].

3.3. The Effect of Vitamin D on SARS-CoV-2 Infection

To establish differing vitamin D statuses, hACE2 mice were given a vitamin D deficient chow and were orally dosed with the vehicle (D−), 0.125 μg/day (D+), or 2.5 μg/day (D++) for 8 weeks prior to infection. Serum 25D levels were higher in D+ mice and significantly higher in D++ mice before and at 14 days post-infection (Figure 3A). The D+ dose was inadequate to raise serum 25D levels significantly over the D− values (Figure 3A). The survival of the SARS-CoV-2-infected mice was not affected by the D+ or the D++ treatments (Figure 3B). At day 6 post-infection the amount of N gene expression was the same in D− and D++ mice (Figure 3C). The expression of the *Vdr, Cyp27B1*, *Cyp24A1* was not different in D− and D++ mice at day 6 post-infection (Figure 4A). *Ifnb* and *Ifng* were induced by SARS-CoV-2 infection, while *Ifna* was not (uninfected control set at 1, Figure 4B). Expression of *Ifnb* was significantly lower in the D++ lung as compared to the D− lung at day 6 post-infection (Figure 4B). Mice surviving until day 14 post-infection showed no difference in lung histopathology scores between D− and D+ mice (Figure 3D,E). The D++ lung histopathology scores showed significantly reduced type II hyperplasia, significantly reduced alveolar remodeling, and lower (not significant) total histopathology scores (Figure 3D,E). The final series of experiments tested whether the active form of vitamin D (1,25D) could prevent the lethality of SARS-CoV-2 infection. There was no effect of 1,25D on survival from a lethal dose of SARS-CoV-2 (1,000 $TCID_{50}$) (Figure 3F). There was a trend for faster weight recovery in the surviving 1,25D-treated mice that did not reach significance (Figure 3F). There was an effect of D++ treatment to decrease lung histopathology and a trend towards faster recovery in 1,25D-treated mice infected with SARS-CoV-2.

Serum 25D levels in hamsters fed on the chow diet were not significantly different than serum 25D levels in hamsters fed D− diets for 4 weeks (Figure 5A). Therefore, to control for the diet, hamsters were fed D− diets and then fed orally with the vehicle (D−) or with 8 μg/day of vitamin D3 (D+) beginning 14 days before the SARS-CoV-2 infection and continuing throughout the experiment. Confirming the effectiveness of the dietary intervention, the serum 25D levels were significantly higher in D+ hamsters as compared to D− hamsters before infection on day 0, and this effect was maintained until day 14 post-infection (Figure 5A). The SARS-CoV-2 N gene was detected on day 3 but not at day 6 post-infection in the lungs (Figure 5B), and there was no difference in N gene expression between the D+ versus D− lungs (Figure 5B). Surprisingly, SARS-CoV-2 N gene expression was also detected in the colon tissues of hamsters at both day 3 and day 6 post-infection compared to uninfected control tissues (Figure 5B). Expression of the N gene in the colon was 1000-fold less than in the infected lung (Figure 5). N gene expression was significantly higher in the D− colon compared to baseline values from uninfected tissue controls but not different from uninfected controls in the D+ colon at day 3 post-infection (Figure 5B). The histopathology of the lungs showed significantly more damage at day 6 than day 3 post-infection (Figure 5C,D). There was no effect of vitamin D on the weight loss or histopathology scores following SARS-CoV-2 infection of the hamsters (Figure 5D,E).

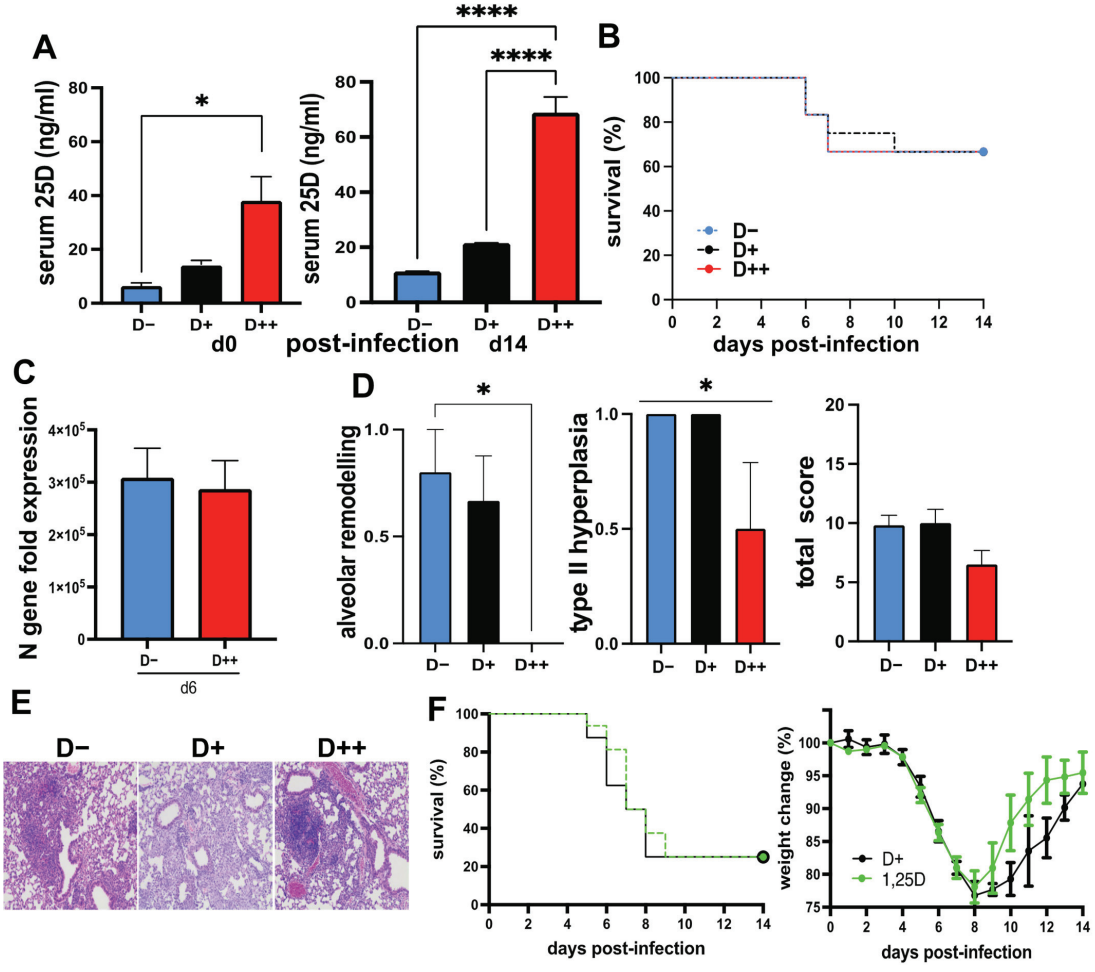

Figure 3. High dose vitamin D reduces lung inflammation following SARS-CoV-2 infection. hACE2 mice were fed vitamin D deficient (D−, $n = 18$), vitamin D sufficient (D+, $n = 12$), or vitamin D supplemented (D++, $n = 18$) diet and infected with 200 $TCID_{50}$ SARS-CoV-2. (**A**) Serum 25D was measured before and at day 14 post-infection, and (**B**) the survival of D−, D+, and D++ mice. (**C**) SARS-CoV-2 N gene expression in the lungs ($n = 6$ mice/group) at day 6 post-infection. Gene expression relative to uninfected D+ controls. (**D**) Alveolar remodeling, type II pneumocyte hyperplasia, and total histology score in D−, D+, and D++ mice ($n = 4$–6 mice/group) at day 14 post-infection. (**E**) Representative histology images for D− (score = 9), D+ (score = 11), and D++ (score = 5). D+ hACE2 ($n = 8$) mice at day 14 post-infection. $1,25(OH)_2D$ (1,25D)-treated D+ hACE 2 ($n = 16$) mice were infected with 1000 $TCID_{50}$ SARS-CoV-2. (**F**) Survival and body weight change over the course of infection. Values are the mean ± SEM. Statistical significance was assessed using one-way ANOVA with Bonferroni multiple comparison test for (**A**,**D**), log rank (Mantel–Cox) test for each of the groups for (**B**,**F**), unpaired *t*-test on log-transformed expression values for (**C**), and two-way ANOVA with Bonferroni multiple comparison test for (**F**). * $p < 0.05$ and **** $p < 0.0001$.

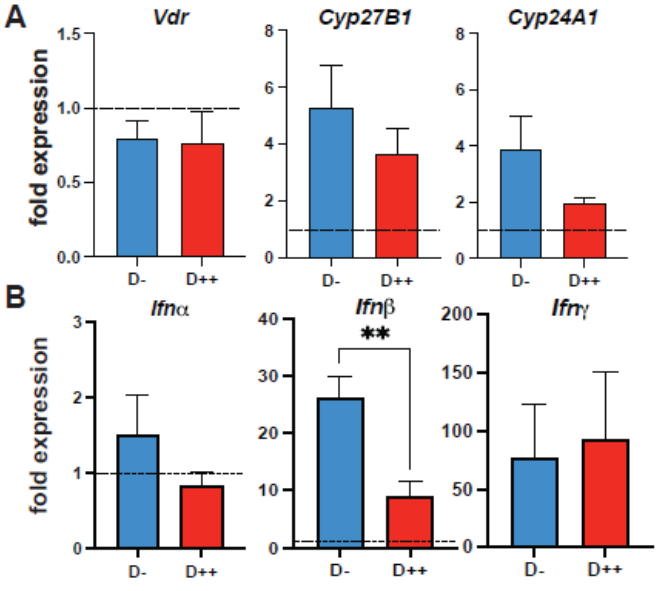

Figure 4. D++ mice have lower *Ifnb* at day 6 post-SARS-CoV-2 infection in the lung. Lung mRNA from day 6 SARS-CoV-2 infected D− ($n = 5$) and D++ ($n = 5$) mice. (**A**) *Vdr*, *Cyp27B1*, *Cyp24A1*, and (**B**) *Ifna*, *Ifnb*, and *Ifng* relative to *Gapdh* and uninfected controls set at 1 (dashed line). Values are the mean ± SEM. Statistical significance was assessed using unpaired *t*-test on log-transformed values. ** $p < 0.01$.

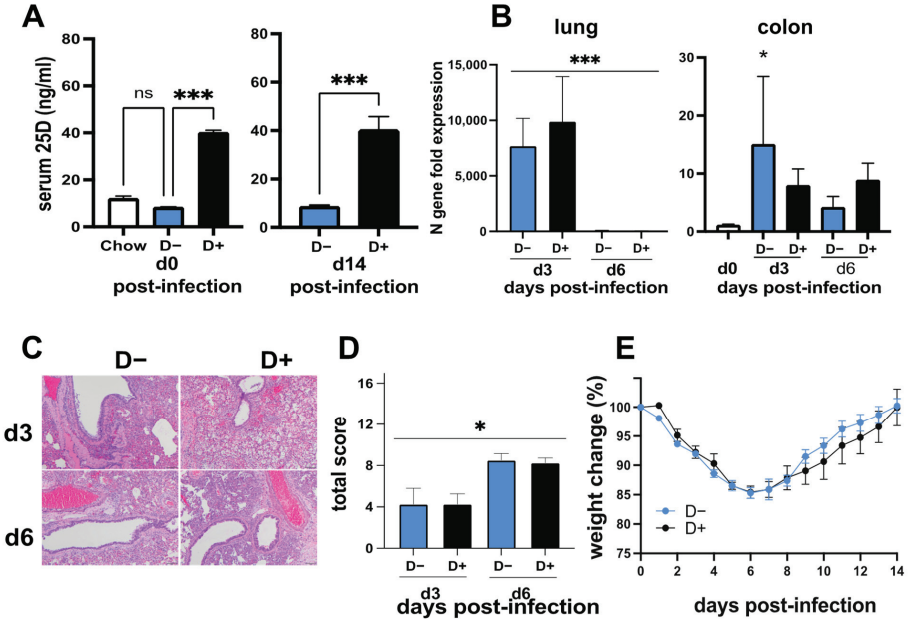

Figure 5. SARS-CoV-2 infection of hamsters. Hamsters were fed D− diets with corn oil (D−) or 8 µg D3/day dissolved in corn oil (D+) and then infected with SARS-CoV-2 virus. (**A**) Serum levels of 25D

in chow-fed, D+, and D− fed hamsters before (n = 2–10 hamsters/group) or from D− and D+ hamsters 14 days after (n = 5–6 hamsters/group) SARS-CoV-2 infection. Hamsters were euthanized at day 3 and day 6 post-infection, and tissues were collected for gene expression as well as histology. (**B**) SARS-CoV-2 N gene expression in the lung and colon relative to uninfected control (n = 4 hamsters per group and timepoint). (**C**) Representative histopathology sections of the lungs at day 3 (D− score = 6, D+ score = 4) and day 6 (D− score = 10, D+ score = 9) post-infection showed increased lymphocyte infiltration and lesions in D− and D+ hamsters at day 6 post-infection and (**D**) total histopathology scores (n = 4 hamsters/group) were determined based on the scoring criteria outlined in Table S4. (**E**) Change in body weight following infection with SARS-CoV-2 (n= 5–6 hamsters/group). Values are the mean ± SEM. Statistical significance was assessed using Kruskal–Wallis test with Dunn's multiple comparison (day 0) and unpaired t-test (day 14) for (**A**), one-way ANOVA with Bonferroni multiple comparison test on log-transformed expression values for (**B**), one-way ANOVA for (**D**), and two-way ANOVA with Bonferroni multiple comparison test for (**E**). * $p < 0.05$ and *** $p < 0.001$.

4. Discussion

Vitamin D deficiency resulted in lung inflammation in the absence of infection. Infected D− mice had more severe lung inflammation and respiratory symptoms than D+ or D++ mice when infected with either H1N1 influenza or SARS-CoV-2. The data point to shared effects of vitamin D to control inflammation in the lung following influenza or coronavirus infection. D− mice had significantly more inflammation than D+ mice following H1N1 influenza infection (Figure 1). High-dose vitamin D3 treatment (D++) resulted in some protection of mice from SARS-CoV-2 (Figure 3). In addition, 1,25D treatment showed a trend towards faster recovery of surviving mice from SARS-CoV-2 (Figure 3). Others have shown that 1,25D-treated mice had reduced lung inflammation [20,21], and treating D+ mice with 25D had a small protective effect on weight loss and lethality following H1N1 infection [22]. A recent clinical trial that used 25D in humans showed reduced mortality in hospitalized patients with COVID-19 [36,37]. However, it is unclear whether 25D treatment would be effective in mouse or hamster models of SARS-CoV-2. This is the first study that investigated the effects of vitamin D in animal models of SARS-CoV-2. The data suggest that vitamin D and 1,25D may be effective to protect the lung from SARS-CoV-2. SARS-CoV-2 infection induced *Cyp27B1* and *Cyp24A1* in the lung of mice suggesting a role for vitamin D metabolites in the lung response to SARS-CoV-2 infection (Figure 2). There are likely shared and unique mechanisms by which vitamin D regulates the host response to influenza versus SARS-CoV-2. A better understanding of the mechanisms by which vitamin D regulates the anti-viral response in the lung to both influenza and coronaviruses is needed to inform clinical studies.

Cyp27B1 KO mice cannot produce 1,25D, which induces Cyp24A1 and degrades 25D and 1,25D. Feeding Cyp27B1 KO mice D+ diets results in the accumulation of 25D (Figure 1A, [25,38]). 25D is a low-affinity ligand for the VDR, and it was shown that high amounts of 25D can replace the need for 1,25D for the regulation of calcium homeostasis and osteomalacia [39]. Previous experiments showed that D+ Cyp KO and D+ WT mice cleared a bacterial infection in the gut with similar kinetics [25]. Conversely, D+ Cyp KO mice had higher lethality and more severe inflammation than D+ WT mice when infected with H1N1 influenza (Figure 1). The effects of Cyp27B1 expression on host resistance to a bacteria could be different than the effects on host resistance to a virus. It would be interesting to determine the effect of the Cyp27B1 deletion on host resistance to other respiratory viruses including SARS-CoV-2. Conversely, the differential effect of Cyp27B1 could be due to the location of the infection in the gut versus the lung. Regardless, it seems that the ability of the host to produce Cyp27B1 is important for the mice to survive a H1N1 lung infection.

The dietary interventions to generate D−, D+, and D++ hACE2 mice resulted in D++, but not D+, mice having higher serum 25D than D− mice (Figure 4). Interestingly, the hamster studies suggest that the commercially available chow may not be adequate

to raise serum 25D levels (Figure 5A). The data suggest that the adequacy of vitamin D should be considered in evaluating studies that infect chow-fed hamsters with SARS-CoV-2. At day 6 post-SARS-CoV-2 infection, the D++ hACE2 mice had less inflammation and lower IFN-β in the lung than the D− mice (Figure 3). The results are consistent with the anti-inflammatory effects of vitamin D. Suppression of type-1 inflammatory cytokines by vitamin D underlies the effects of vitamin D and 1,25D to suppress immune-mediated diseases [40–42]. The benefits of 25D from influenza infection were associated with a reduction in IFN-γ in the lung [22]. Recently, Chauss et.al showed that 1,25D promotes anti-inflammatory responses by switching off IFN-γ production from Th1 cells and upregulating IL-10 [43]. IFN-γ and IFN-β production is essential for effective viral clearance; viruses have mechanisms to evade the IFN responses, and severe COVID-19 is associated with dysregulation of IFN responses [44–46]. Down-regulation of IFN-β by vitamin D is associated with protection from inflammation in the lung following a virus infection with either influenza or SARS-CoV-2.

Importantly, there was no effect of vitamin D on the SARS-CoV-2 N gene or H1N1 M gene expression in the lungs of mice or hamsters. This indicates that vitamin D did not reduce or inhibit viral replication in the lung. We found SARS-CoV-2 N gene expression in the hamster colons. Interestingly, D− colons had relatively more SARS-CoV-2 N gene expression than D+ colons. The implications of having SARS-CoV-2 in the colon but not the lung would need to be determined, and it would be important to quantitate live virus in the tissues. Unfortunately, we did not save colons from our SARS-CoV-2 mouse studies. Vitamin D is shown to be a strong inducer of cathelicidin LL-37 in human cells [47]. There is some evidence that LL-37 can directly kill some viruses including influenza viruses [13–17]. Treating mice with a high dose (500 μg/day) of human LL-37 peptide protected from lethal influenza infection and significantly reduced viral titers at day 3 post-H1N1 infection in the lung [48]. LL-37 inhibited binding of the SARS-CoV-2 spike protein containing pseudo viruses both in vivo and in vitro blocking entry via ACE2 [49]. There were no effects of vitamin D in vivo on the expression of viral genes for SARS-CoV-2 or H1N1 influenza in the lung. The cathelicidin peptides found in mice are not the same as the LL-37 in humans, and the mouse cathelicidin is not regulated by vitamin D [50]. The lack of a vitamin D effect on SARS-CoV-2 was shown in mice and hamster lungs. It is unclear whether the hamster cathelicidin gene has vitamin D response elements. Furthermore, no studies have been conducted to test the effect of vitamin D on SARS-CoV-2 in vitro. Therefore, an effect of vitamin D through the induction of anti-bacterial peptides, such as LL-37, that reduces viral titers cannot be ruled out.

The data from mouse H1N1 and mouse and hamster SARS-CoV-2 infection point towards a protective role of vitamin D against acute viral infection in the lung. The data are in line with several meta-analyses and observational studies suggesting a beneficial effect of vitamin D in the lung [51–54]. Vitamin D supplementation was reported to have a moderately protective effect during acute respiratory tract infections, such as influenza and COVID-19; however, this outcome was affected by a number of variables such as dose frequency, season of supplementation, and pre-existing conditions [51–54]. Patients with existing vitamin D deficiency, especially patients >80 years of age, are at a higher risk of being infected with SARS-CoV-2 and developing severe disease [51–54]. High-dose vitamin D supplementation with either calcifediol or cholecalciferol was shown to reduce overall mortality and severe outcomes such as intensive care admission [55–59]. Vitamin D deficiency is associated with acute respiratory distress syndrome (ARDS), and several clinical trials are underway to investigate whether vitamin D supplementation can reduce the development of ARDS [60]. However, a recent randomized controlled study (reported in medRxiv, [61]) that identified vitamin D deficiency and then treated it showed that vitamin D supplementation did not reduce the risk of acute respiratory infection or the risk of COVID-19 infection. With the limitations of performing a clinical study for a nutrient such as vitamin D, our study in animals suggests that vitamin D does control the host response to H1N1 and SARS-CoV-2 infection in the lung. The data point to an effect of

vitamin D to control the cytokine response and resolve inflammation in the lung following a viral infection. Understanding the mechanisms and timing of the vitamin D effects in animal models would inform future clinical trials.

There are several limitations of the current animal studies. There were technical limitations due to the cost, training, and restrictions needed to use the BSL3 facility for SARS-CoV-2 infections safely. Experiments in the BSL-3 facility had small sample sizes. In addition, viruses including H1N1 and SARS-CoV-2 do not naturally infect mice, and so the viruses used were passaged (H1N1), or a transgenic mouse was needed to allow infection (hACE) with SARS-CoV-2. In addition, vitamin D metabolism is not identical in animals and humans. To our surprise, hamsters fed on standard chow diets replete in vitamin D were found to have low serum 25D levels. It is possible that vitamin D metabolism in hamsters is different than in the mouse and humans, which might impact our results. Mechanistic studies in humans are difficult, so it is important to use animals to determine pathways and processes that are regulated by vitamin D. The data in humans and animals do support a role of vitamin D to control the inflammatory responses in the lung providing protection following a viral infection with H1N1 or SARS-CoV-2.

5. Conclusions

Together, the data support an important role for vitamin D and Cyp27B1 in the regulation of the host response to H1N1 and SARS-CoV-2 viruses. The role of vitamin D includes the restraining of the IFN response shortly after infection. Vitamin D-deficient hosts had pre-existing inflammation in the lungs that contributed to susceptibility to viral infection. Future experiments should continue to determine the mechanisms by which vitamin D regulates the anti-viral response in the lungs and whether there are differences in the effect of vitamin D on host resistance to H1N1 influenza and SARS-CoV-2.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14153061/s1. Table S1: Primer sequences for qPCR, Table S2: Histological evaluation of SARS-CoV-2-infected mouse lung, Table S3: Histological evaluation of SARS-CoV-2-infected hamster lung, and Table S4: Histological evaluation of H1N1-infected mouse lung.

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Institutional Review Board Statement: All studies with SARS-CoV-2 were conducted in a biosafety level 3 enhanced (BSL3+) laboratory. This facility is approved for BSL3+ respiratory pathogen studies by the US Department of Agriculture and Centers for Disease Control. Studies with pandemic H1N1 influenza were conducted under biosafety level 2 enhanced conditions. All animal studies were conducted in compliance with the Animal Care and Use Committee under protocol numbers: 202001693, 202001516, 202001440, and 202001638.

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A Single Vitamin D_3 Bolus Supplementation Improves Vitamin D Status and Reduces Proinflammatory Cytokines in Healthy Females

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Abstract: Vitamin D deficiency is a global health problem that not only leads to metabolic bone disease but also to many other illnesses, most of which are associated with chronic inflammation. Thus, our aim was to investigate the safety and effectiveness of a single high dose of vitamin D_3 (80,000 IU) on vitamin D status and proinflammatory cytokines such as interleukin (IL)6, IL8 and tumor necrosis factor (TNF) in healthy Saudi females. Fifty healthy females were recruited and orally supplemented with a single vitamin D_3 bolus (80,000 IU). All participants donated fasting blood samples at baseline, one day and thirty days after supplementation. Serum 25-hydroxyvitamin D_3 ($25(OH)D_3$), IL6, IL8, TNF, calcium, phosphate, parathyroid hormone (PTH) and blood lipid levels were determined. Serum $25(OH)D_3$ significantly increased one and thirty days after supplementation when compared with baseline without causing elevation in calcium or phosphate or a decrease in PTH to abnormal levels. In contrast, the concentrations of the three representative proinflammatory cytokines decreased gradually until the end of the study period. In conclusion, a single high dose (80,000 IU) is effective in improving serum vitamin D status and reducing the concentration of the proinflammatory cytokines in a rapid and safe way in healthy females.

Keywords: vitamin D deficiency; single high dose; vitamin D_3 supplementation; proinflammatory cytokines; IL6; IL8; TNF; $25(OH)D_3$

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1. Introduction

Vitamin D_3 is a micronutrient that can be synthesized in human skin from the cholesterol precursor 7-dehydrocholesterol through energy provided by the ultraviolet-B (UVB) component of sunlight [1]. Recent lifestyle and work–life changes towards indoor activities as well as the use of clothing and sunscreen for sunburn protection outdoors have reduced the chances of filling up vitamin D_3 stores. This results in far lower average vitamin D status in today's modern societies than in more traditionally living populations [2–5]. Even in sunny Saudi Arabia, a substantial proportion of the population is considered vitamin D-deficient [6]. This increases the risk not only of muscle weakness (sarcopenia) and early onset of osteoporosis but also leads to an increase in autoimmune diseases, such as type 1 diabetes, arthritis, multiple sclerosis, cancer, cardiovascular diseases and Alzheimer's disease [7,8]. Therefore, vitamin D deficiency is a global health problem that not only leads to musculoskeletal problems but also to many other illnesses, most of which are associated with chronic inflammation [9,10].

In the liver, vitamin D_3 is hydroxylated to 25(OH)D_3, which is the most stable vitamin D_3 metabolite circulating in the blood. Therefore, 25(OH)D_3 serum levels serve as a biomarker for the vitamin D status. In the kidneys, 25(OH)D_3 is further metabolized to the physiologically most active vitamin D metabolite, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) [11]. The lipophilic nature of 1,25$(OH)_2D_3$ allows the molecule to pass through cellular and nuclear membranes and to act in the nucleus as a high-affinity ligand to the transcription factor vitamin D receptor (VDR), i.e., 1,25$(OH)_2D_3$ has a direct effect on gene regulation [12,13]. Besides the kidneys, 1,25$(OH)_2D_3$ is also synthesized locally in a number of tissues and cell types expressing VDR. Taking all presently investigated tissues and cell types together, there are more than 20,000 VDR binding sites in the human genome, and significant changes in the transcriptome profile occur in over 1000 human genes [14].

Examples of vitamin D target tissues include immune cells such as T cells, B cells and monocytes, which are the major components of peripheral blood mononuclear cells (PBMCs) [15–17]. One hallmark of vitamin D's effects is the regulation of genes involved in the regulation of inflammatory processes. Accordingly, there is an interplay between vitamin D signaling and other signaling cascades involved in inflammation [18,19].

The impact of 1,25$(OH)_2D_3$ treatment on the expression of the proinflammatory cytokines IL6, IL8 and TNF was extensively studied in PBMCs from healthy donors, primary monocytes/macrophages as well as in monocytic cell lines, indicating that the VDR ligand causes their down-regulation on an mRNA and protein level [20–27]. Importantly, not only does the treatment of cell culture models with 1,25$(OH)_2D_3$ promote changes in gene expression, but also the supplementation of individuals with vitamin D_3 leads to the same results. Most of these studies were conducted on patients with diverse inflammatory diseases, such as COVID-19, colorectal cancer, irritable bowel syndrome, obesity and diabetes [28–34], and only a few studies were performed with healthy individuals [35–37].

Vitamin D intervention studies usually use different doses of vitamin D_3 supplementation either daily or weekly for several weeks or months, and only a few of them used a single high dose. The pharmacology of vitamin D shows that the proper half-life for dose periods is longer than daily supplementation, and many dosing regimens suggest that high vitamin D_3 doses at less frequent periods are more suitable and have become a broad practice [38]. Moreover, from an experimental point of view, the use of a single high vitamin D_3 dose is more suitable for observing the direct effects of vitamin D on the expression of its target genes, such as multiple cytokines, both on the mRNA and protein level. Accordingly, the aim of this study was to investigate the safety and effectiveness of a single high dose of vitamin D_3 (80,000 IU) on the vitamin D status and the serum levels of representative proinflammatory cytokines IL6, IL8 and TNF in healthy Saudi females.

2. Materials and Methods

2.1. Study Design and Participants

Fifty healthy Saudi females aged between 18 and 60 were recruited from King Abdul Aziz University and King Fahad Medical Research Center's staff and their families from January to December 2019. The total sample size was calculated based on a power analysis (using G*Power software, version 3.1.9.7, Düsseldorf, Germany) that indicated a 95% chance of a 0.5 effect size between the tested groups at the 5% level (two-tailed). Exclusion criteria included the presence of cancer, liver or kidney diseases, the intake of vitamin D supplements during the last three months, and non-Saudis. All participants received a single high dose of vitamin D_3 (80,000 IU) orally administered (Figure 1). This dose was chosen since previous experience in the vitamin D intervention studies VitDbol (https://clinicaltrials.gov/ct2/show/NCT02063334) (accessed on 19 September 2022) and VitDHiD (https://clinicaltrials.gov/ct2/show/NCT03537027) (accessed on 19 September 2022) indicated that 80,000 IU vitamin D_3 is a safe monthly dose in healthy individuals. This study was approved by the ethical committee of the Faculty of Medicine, King Abdulaziz University (reference number 30-18), and all participants provided written informed consent.

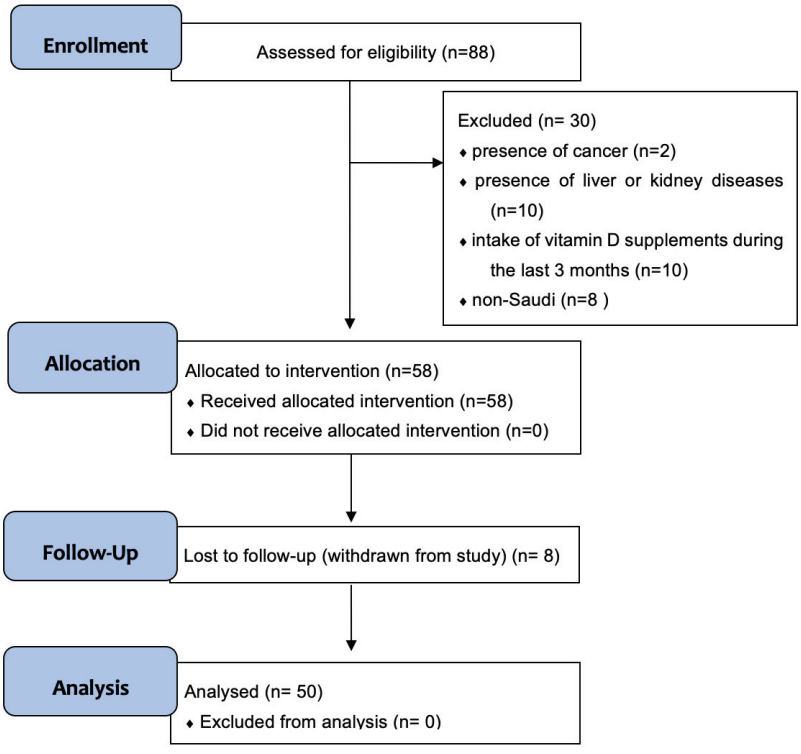

Figure 1. Flow chart showing the flow of the participants throughout the study. n = number of individuals.

2.2. Anthropometric Measurements

Height and weight were measured by using an electronic scale and a portable stadiometer from Seca (Hamburg, Germany), respectively, and the body mass index (BMI) was calculated for all participants. In addition, waist and hip circumference were measured using Seca tape, and the waist-to-hip ratio (WHR) was then calculated.

2.3. Biochemical Measurements

All participants donated fasting blood samples at three different time points; at baseline (day 0), after one day (day 1) and after thirty days (day 30) of oral administration of a single high dose of vitamin D_3 (80,000 IU). Serum was isolated and stored at −80 °C for later measurements of biochemical parameters including lipid profile, phosphorus (PHOS), calcium (CAL), parathyroid hormone (PTH), 25(OH)D_3 and proinflammatory cytokines.

Quantitative determination of serum cholesterol (CHOL), low-density lipoproteins (LDL) and triglycerides (TAG) was performed using a Siemens Dimension Vista instrument. Serum CAL and PHOS were measured using a kit from Siemens Healthcare Diagnostic Limited, Dimension Vista System UK (Cat. No K1023 and Cat. No K1061, respectively). Serum PTH was measured using a chemiluminescent microparticle immunoassay (CMIA) technique kit from Abbott (Cat. No 8K25). Serum vitamin D status was determined by measuring 25(OH)D_3 via the Abbott Architect 25-OH Vitamin D assay kit. Finally, the proinflammatory cytokines IL6, IL8 and TNF were measured using Human Interleukin 6 ELISA Kit by Bioassay Technology Laboratory (Cat. No E0089Hu), Human Interleukin 8 ELISA Kit by Bioassay Technology Laboratory (Cat. No E0089Hu) and Human Tumor Necrosis Factor Alpha ELISA Kit by Bioassay Technology Laboratory (Cat. No E0082Hu), respectively.

2.4. Statistical Analysis

All statistical analyses were performed using IBM SPSS software version 24 (SPSS Inc., Chicago, IL, USA) and graphs were represented using GraphPad prism 7. Data were presented as mean ± standard error of mean (SEM). Repeated measures one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to determine the significant differences in mean serum levels of 25(OH)D_3, IL6, IL8 and TNF, CHOL, TAG, LDL, PHOS, CAL and PTH between days 0, 1 and 30 of vitamin D_3 supplementation. The statistical significance threshold was taken as $p < 0.05$.

3. Results

Fifty females with a mean age of 29 years participated in this study. At baseline, their mean BMI was 23.6 kg/m^2 and their mean WHR was 0.77. All biochemical parameters including CHOL, LDL, TAG, PHOS, CAL, and PTH were in the normal range intervals indicating a good health status of all participants (Table 1). After supplementation with vitamin D_3, no changes were found in most biochemical parameters except in CHOL, PHOS and PTH levels. The changes in these parameters were minor and did not reach abnormal levels.

Table 1. Demographic and clinical characteristics of study participants at baseline, day 1 and day 30 following a single high dose of vitamin D_3 supplementation (n = 50).

	Baseline	Day 1	Day 30
Age (years)	28.9 ± 0.9		
Height (cm)	158.9 ± 0.7	NA	NA
Weight (kg)	59.9 ± 1.8	NA	NA
BMI (kg/m^2)	23.6 ± 0.7	NA	NA
Waist circumference (cm)	74.5 ± 2.1	NA	NA
Hip circumference (cm)	97.7 ± 2.5	NA	NA
WHR	0.77 ± 0.02	NA	NA
CHOL (mM)	4.34 ± 0.12	4.26 ± 0.12	4.13 ± 0.11 *
TAG (mM)	1.05 ± 0.07	2.39 ± 1.34	1.07 ± 0.08
LDL (mM)	2.78 ± 0.11	2.34 ± 0.18	2.53 ± 0.09
PHOS (mM)	1.24 ± 0.03	1.18 ± 0.03 *	1.24 ± 0.03
CAL (mM)	2.29 ± 0.02	2.24 ± 0.01	2.22 ± 0.02
PTH (pM)	5.25 ± 0.44	4.44 ± 0.30 *	4.17 ± 0.28 **

Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ when compared with baseline. NA: Data are not available.

The mean serum 25(OH)D_3 concentration at baseline was 41.9 ± 4.1 nM, and 72% of study participants had an insufficient vitamin D status of less than 50 nM (Table 2). The average vitamin D status significantly increased to 66.3 ± 3.5 nM at day 1 and 68.9 ± 2.5 nM at day 30 (Figure 2). This represents an average increase by 24.4 and 26.9 nM and a shift from deficiency and insufficiency to sufficiency for 76% and 94% of the study participants, respectively, at days 1 and 30 after vitamin D_3 bolus supplementation (Table 2).

Table 2. Prevalence of vitamin D deficiency among study participants at baseline, day 1 and day 30 following a single high dose of vitamin D_3 supplementation (n = 50).

Serum Vitamin D Status *	Baseline N (%)	Day 1 N (%)	Day 30 N (%)
Deficiency $25(OH)D_3$ < 30 nM	24 (48%)	0 (0%)	0 (0%)
Insufficiency $25(OH)D_3$ of 30–50 nM	12 (24%)	12 (24%)	3 (6%)
Sufficiency $25(OH)D_3$ > 50 nM	14 (28%)	38 (76%)	47 (94%)

* classification was based on US Institute of Medicine (IOM).

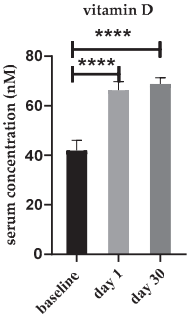

Figure 2. Mean serum $25(OH)D_3$ concentrations at baseline, day 1 and day 30 following a single high dose of vitamin D_3 supplementation (n = 50). Error bars show SEM. **** $p < 0.0001$.

Mean serum IL6 concentrations significantly decreased from 405 ± 30 ng/L at baseline to 350 ± 30 ng/L at day 1 and even 137 ± 20 ng/L at day 30 (Figure 3). This represents an average decrease by 55 and 269 ng/L, respectively. Similar trends were also found for serum IL8 concentrations, where baseline levels gradually decreased from 506 ± 40 ng/L to 455 ± 35 ng/L at day 1 and 192 ± 10 ng/L at day 30 (Figure 3) and for serum TNF levels, which significantly decreased from 165 ± 8 ng/L at baseline to 156 ± 7 ng/L at day 1 and 63 ± 3 ng/L at day 30 (Figure 3). Interestingly, neither Pearson nor Spearman correlation analysis provided any significant correlation between the vitamin D status and the expression level of the proinflammatory cytokines.

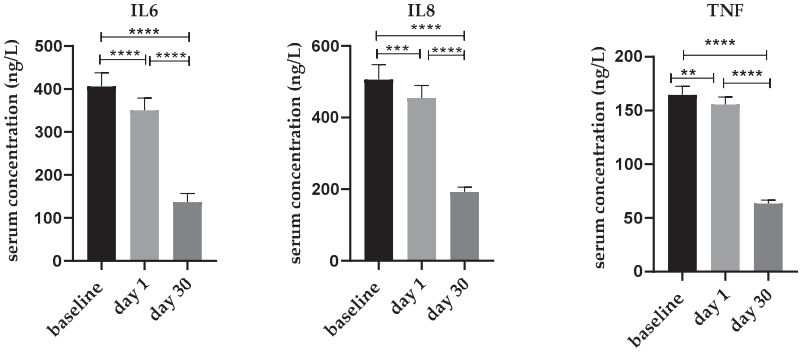

Figure 3. Mean serum levels of the proinflammatory cytokines IL6, IL8 and TNF at baseline, day 1 and day 30 following a single high dose of vitamin D_3 supplementation (n = 50). Error bars show SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

The purpose of this study was to investigate the effectiveness of a single high dose of vitamin D_3 (80,000 IU) on the vitamin D status and the representative proinflammatory cytokines IL6, IL8 and TNF in healthy Saudi females. The vitamin D_3 bolus increased the vitamin D status within a month by nearly 27 nM and achieved a shift in the study participants from vitamin D deficiency and insufficiency to sufficiency. In fact, the approximately 60% increase in vitamin D status was already visible within one day. This result is comparable to a previous study conducted in female adults supplemented with a single high dose of vitamin D_3 (100,000 IU) [39]. For comparison, when a lower dose was used (50,000 IU), the percent increase in serum 25(OH)D_3 concentrations was only 30% [40]. Other previous studies conducted on adults supplemented daily with different doses of vitamin D_3 ranging from 200 to 600 IU for 2 to 5 months showed a similar or lower percent increase in serum 25(OH)D_3 levels [41–44].

A potential chronic toxicity of vitamin D would result from the administration of doses far above the maximally recommended daily dose of 4000 IU vitamin D_3 for months or years that will increase serum 25(OH)D_3 concentrations to 250 nM or more. In addition to elevated serum 25(OH)D_3 concentrations, vitamin D toxicity can be diagnosed by severe hypercalcemia and by very low or undetectable PTH activity [45]. Accordingly, oral supplementation with a single high dose (80,000 IU) is sufficient to increase the level of serum 25(OH)D_3 in a rapid, suitable and safe way, as none of our study participants reached a vitamin D status of more than 125 nM. Moreover, no abnormal changes were found in either serum calcium or PTH levels after supplementation.

An association between high serum 25(OH)D_3 concentrations and low concentrations of the proinflammatory cytokines IL6, IL8 and TNF was reported previously [46–48]. In the present study, low serum 25(OH)D_3 concentrations at baseline were observed in concordance with the high concentration of the proinflammatory cytokines, but these correlations did not reach statistical significance. Importantly, a single vitamin D_3 bolus was sufficient to significantly increase the vitamin D status within one month and in parallel resulted in the reduction in protein levels of IL6, IL8 and TNF by 67, 62 and 61%, respectively, at the end of the study. The downregulation of the expression of the proinflammatory cytokines may be explained by the increased activation of VDR by an elevated vitamin D status. The latter may have caused a raise in 1,25$(OH)_2D_3$ levels in the nuclei of VDR-expressing PBMCs. Although the genes *IL6* and *TNF* are not known as primary vitamin D target genes, a network of secondary and indirect effects of VDR activation can lead to changes in their expression [49]. However, the *IL8* gene is known as a primary vitamin D target [50].

In contrast to our results, Smith et al. (2017) reported that a single high dose of vitamin D_3 (250,000 IU) did not change serum IL6 and IL8 levels in healthy adults, which could be due to the small sample size of their study [36]. Moreover, daily supplementation with low doses of vitamin D_3 (4000 IU) did not affect serum IL6 concentrations in healthy adults [35]. However, serum TNF concentrations were reported to decrease after supplementation of healthy male and female adults with 4000 IU vitamin D_3 for 20 days [37]. Studies conducted on patients with inflammation-related diseases showed that daily supplementation with different doses of vitamin D_3 ranging from 1000 to 50,000 IU for several weeks or months decreased not only serum TNF concentrations but also serum IL6 and IL8 levels [28–32,51–55]. Thus, a single high dose (80,000 IU) of vitamin D_3 is as effective in reducing proinflammatory cytokines as daily doses.

5. Conclusions

An important finding of this study was that oral supplementation with a single high dose (80,000 IU) is effective in improving the serum's vitamin D status and decreasing the concentration of the proinflammatory cytokines in a rapid, suitable and safe way in healthy females. This will help in preventing and reducing vitamin D deficiency, as well as related inflammatory diseases, in the general population. Further research needs to be performed

in order investigate the effectiveness of this single high dose on pro- and anti-inflammatory markers in various inflammatory diseases.

Author Contributions: H.M.A., S.A.A., S.O.K. and C.C. conceived the idea and designed the study. S.F.S. carried out the experiments. H.M.A., S.A.A. and S.F.S. analyzed the data. H.M.A., S.A.A. and H.F.A. performed the literature search and wrote the manuscript. S.O.K. and C.C. reviewed the original manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the ethical committee of the Faculty of Medicine, King Abdulaziz University (reference number 30-18).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available when requested.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

Vitamin D and Cancer: An Historical Overview of the Epidemiology and Mechanisms

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Abstract: This is a narrative review of the evidence supporting vitamin D's anticancer actions. The first section reviews the findings from ecological studies of cancer with respect to indices of solar radiation, which found a reduced risk of incidence and mortality for approximately 23 types of cancer. Meta-analyses of observational studies reported the inverse correlations of serum 25-hydroxyvitamin D [25(OH)D] with the incidence of 12 types of cancer. Case-control studies with a 25(OH)D concentration measured near the time of cancer diagnosis are stronger than nested case-control and cohort studies as long follow-up times reduce the correlations due to changes in 25(OH)D with time. There is no evidence that undiagnosed cancer reduces 25(OH)D concentrations unless the cancer is at a very advanced stage. Meta-analyses of cancer incidence with respect to dietary intake have had limited success due to the low amount of vitamin D in most diets. An analysis of 25(OH)D-cancer incidence rates suggests that achieving 80 ng/mL vs. 10 ng/mL would reduce cancer incidence rates by 70 ± 10%. Clinical trials have provided limited support for the UVB-vitamin D-cancer hypothesis due to poor design and execution. In recent decades, many experimental studies in cultured cells and animal models have described a wide range of anticancer effects of vitamin D compounds. This paper will review studies showing the inhibition of tumor cell proliferation, dedifferentiation, and invasion together with the sensitization to proapoptotic agents. Moreover, 1,25-$(OH)_2D_3$ and other vitamin D receptor agonists modulate the biology of several types of stromal cells such as fibroblasts, endothelial and immune cells in a way that interferes the apparition of metastases. In sum, the available mechanistic data support the global protective action of vitamin D against several important types of cancer.

Keywords: 25-hydroxyvitamin D; 1,25-$(OH)_2D_3$; antitumor action; breast cancer; case-control studies; colorectal cancer; cohort studies; ecological studies; epidemiological studies; randomized controlled trials; UVB; vitamin D

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1. Introduction

The role of vitamin D in reducing the risk of cancer incidence and death has been studied for years. A search of PubMed on 10 March 2022 searching for "cancer" and "vitamin D" or "vitamin D_3" in the title or abstract found 6732 publications starting in 1949. Of these, 523 were published prior to 2000; 1630 were published from 2000 through 2009; 1797 were published from 2010 through 2014; and 2782 were published in or after 2015. Publications with vitamin D and cancer in the title or abstract rose from 13 in 1990, 34 in 1995, 75 in 2000, 170 in 2005, 338 in 2010, 401 in 2012, and between 400 and 500 per year since then.

The earliest studies were ecological studies of cancer mortality rates with respect to indices of solar total or UVB radiation or laboratory studies of mechanisms of vitamin D metabolites on cancer cells. As time progressed, observational studies of cancer incidence with respect to serum 25-hydroxyvitamin D [25(OH)D] took place, and studies of the

mechanisms of vitamin on cancer incidence, progression, and metastasis were conducted. Later, randomized controlled trials (RCTs) of cancer risk with respect to vitamin D supplementation were conducted, and as more observational studies accrued, meta-analyses were conducted. Along the way, research approaches built on previous studies. However, since there are many sources of vitamin D, UVB exposure, diet, and supplements, and since 25(OH)D concentrations vary with time, both seasonally and over long periods, and since quantifying 25(OH)D concentrations can be uncertain and is not always conducted in studies, all such human studies of vitamin D and cancer are subject to error. There are also methodological issues, such as how to adjust for when 25(OH)D was measured. In addition, what was found in one group of people may not apply to other groups, such as those with different diets, geographical location, clothing, occupation, age, genetics, and BMI. Thus, all the epidemiological studies and RCTs have inherent limitations. However, by taking a comprehensive look at the findings from many types of studies and trying to identify those that are most reliable, a reasonable picture can emerge. What has emerged is that 25(OH)D concentrations play very important roles in the incidence, progression, and death for many types of cancer. While the roles of vitamin D in cancer are not fully understood, there is enough information for clinical and public health decisions to be made.

The epidemiology of vitamin D and cancer can be examined through the prisms of ecological studies, observational studies, and clinical trials. This review looks at findings from ecological studies of cancer risk with respect to indices of solar ultraviolet-B (UVB) doses, observational studies of cancer risk with respect to serum 25(OH)D concentration and oral vitamin D intake, and randomized controlled trials (RCTs) of cancer risk with respect to vitamin D supplementation.

Epidemiological data prompted the study of the putative anticancer action of vitamin D in the laboratory. Two important considerations in the study of the action of 1,25-$(OH)_2D_3$ and analogues in experimental cancer systems are the expression of vitamin D receptor (VDR), which is frequently low or absent, and the high doses of its ligands that are usually required to observe effects. A lack of VDR is linked to transcriptional (by silencing by DNA methylation or repression by SNAIL1/2), posttranscriptional (by several microRNAs) or posttranslational (phosphorylation, alteration of subcellular localization) inhibitory mechanisms, and low cell responsiveness to VDR ligands is often associated with upregulation of the 1,25-$(OH)_2D_3$ degrading enzyme CYP24A1 in tumor cells. These are two reasons for the absence of the 1,25-$(OH)_2D_3$ effects in some studies. An additional consideration is that, though fully convinced of the value of animal models, we will almost exclusively review studies performed in human systems in this paper.

2. Epidemiological Studies

2.1. Ecological Studies

Ecological studies treat defined populations as entities and compare health outcomes with respect to risk-modifying factors averaged for each population. The groups are usually defined by geographical location but also can be defined by other factors such as occupation. For vitamin D, various indices related to solar UVB dose can be used—for example, annual solar radiation, summertime solar UVB dose, and latitude. Other risk-modifying factors can be added to adjust for confounding factors. Ecological studies offer some advantages: the data required are generally readily available, often with large datasets, and the analyses are easy to do.

Thus, it is not surprising that the first epidemiological study linking vitamin D to a reduced risk of cancer, albeit indirectly, was an ecological study. In 1936, Peller reported that people who developed skin cancer from light exposure, such as from their occupation, had lower rates of internal cancers [1]. In 1937, he showed that sailors in the U.S. Navy, who had extremely high sun exposure, had eight times the expected rate of skin cancer but only 40% of the expected rate of internal cancers [2]. In 1941, Apperly showed that skin cancer mortality rates increased directly in a non-linear fashion with respect to a solar radiation index in the U.S., while total cancer mortality rates decreased in a linear

fashion [3]. Evidently, the fact that these three articles were related to vitamin D production went unnoticed until they were cited in a review published in 1993 by Ainsleigh [4].

In 1974, the brothers Cedric and Frank Garland were beginning graduate school at the Johns Hopkins School of Public Health. They attended a lecture by Robert N. Hoover, one author of the *Atlas of Cancer Mortality for U.S. Counties, 1950–1969* [5]. They were struck by the map for mortality, by county, for cancer of the large intestine except the rectum in white males. It showed low rates in three southwest states and high rates in approximately 15 northeast states. The Garlands reasoned that because vitamin D production is the most important health effect of sun exposure, vitamin D must reduce the risk of cancer in the large intestine (colon). They submitted manuscripts to several journals before one was finally accepted and published in the UK in 1980 [6]. They next found support for their hypothesis in terms of the reduced risk of colorectal cancer with respect to dietary vitamin D and calcium [7], prediagnostic serum 25(OH)D concentration, and risk of colon cancer [8]. They later published early ecological studies on solar radiation and the risk of breast cancer [9] and ovarian cancer [10]. Cedric Garland described their discovery and later work in an online posting at Grassrootshealth.net [11].

In 1999, the National Cancer Institute published the *Atlas of Cancer Mortality in the United States, 1950–1994* [12]. That revised edition used 10 colors (five shades each of blue and red) to show mortality rates for 38 cancers (see the breast cancer map in Garland's web post [11] as well as for other cancers at www.sunarc.org, both accessed on 24 February 2022) rather than only five in the earlier version [5]. Data were also displayed for 3053 counties and 506 state economic areas (totals of data for contiguous counties), and showed results for white people (including Hispanics) and black people separately. Through the previous work of one author (W.B.G.) at NASA in Virginia at the time, a map was available of surface-level solar UVB doses in the United States for July 1992 [www.sunarc.org (accessed 24 on February 2022)]. Solar UVB decreases with increasing latitude, albeit with higher doses at any latitude west of the Rocky Mountains than to the east. That effect is due to a combination of higher surface elevation in the west as well as a thinner stratospheric ozone layer owing to the prevailing westerly winds pushing the tropopause up as the air masses cross the Rocky Mountains. Inverse correlations were found for 11 cancers with respect to solar UVB doses for white Americans and several types of cancer for black Americans [13]. A new set of analyses, this time by state, included several risk-modifying factors: alcohol consumption, Hispanic heritage, lung cancer as an index of smoking, poverty status, and urban/rural residence [14]. However, the attribution to solar UVB did not change much between the two articles.

Later, a separate analysis regarding cancer mortality rates for black Americans was published [15]. Significant inverse correlations were found for lung cancer for males and breast cancer for females. The results for colon, esophageal, gastric, and rectal cancer suggested an inverse correlation with respect to solar UVB, but alcohol consumption rates and lung cancer mortality rates also had similar regression coefficients. As a result, UVB did not have a low enough *p*-value to satisfy the Bonferroni criteria. The results were weak because of the lower numbers of black participants in addition to having lower 25(OH)D concentrations [16].

Several ecological studies of UVB and cancer incidence or mortality rates have been published, particularly between 2002 and 2012 [17]. They helped encourage observational studies, mechanism studies, and clinical trials to explore the relationship between vitamin D and cancer. Single-country studies are preferred because people in individual countries tend to have many similarities, such as clothing preferences, diet, and religion, as well as differences, such as smoking, socioeconomic status, and urban/rural residences. Those comparisons can often be modeled. In addition, variations in solar UVB doses tend to be significant [18,19].

Table 1 outlines the more important solar single-country UVB–cancer ecological studies starting in 2002. Most are from mid-latitude countries, but one is from a subtropical country (Iran) and two encompass the Arctic Circle. Most studies used UVB data from NASA's

Total Ozone Mapping Spectrometer (TOMS) satellite instrument [20], but other indices were used as well, including latitude and global solar radiation.

Table 1. Characteristics of large single-country ecological studies of cancer incidence or mortality rates with respect to solar UVB doses.

Country(ies)	Solar UVB Index	Latitude (°N)	Incidence or Mortality; Years of Data	No. of Cases	Confounding Factors	Ref.
U.S.	Surface UVB, July 1992, TOMS	25–45	Mortality, 1950–1994	9.5 million, 1970–1994	None	[13]
Japan	Annual hours of solar radiation	30–45	Mortality, 2000	180,000	Fat intake for colon, rectum, and prostate; salt intake for stomach cancer	[21]
U.S. (white pop.)	Surface UVB, July 1992	25–45	Mortality, 1950–1994	9.5 million, 1970–1994	Alcohol consumption, Hispanic heritage, lung cancer (index for smoking), poverty, urban/rural residence	[14]
U.S.	300–320 nm, TOMS, north vs. south	25–45	Incidence, 1998–2002; mortality, 1993–2002	Incidence, 3.4 million; mortality, 3.5 million	Age, air quality, alcohol, exercise, income, outdoor occupation, poverty, smoking, urban/rural residence	[22]
Japan	Global solar radiation	30–45	Mortality, 1998–2002	~900,000	Dietary factors, smoking, socioeconomic conditions	[23]
China	TOMS, 305 nm	22–50	Incidence, 1998–2002; mortality, 1990–1992		Urban/rural residence	[18]
Russia	Latitude	43–69	Incidence, mortality, 2008	incidence, ~250,000; deaths, ~140,000	None	[24]
Nordic countries	Lip cancer less lung cancer incidence	55–70	Incidence, 1961–2005	2.8 million	Lung cancer	[25]

Pop., population; TOMS, NASA's Total Ozone Mapping Spectrometer satellite instrument.

One ecological study was based on data by occupation from a study involving 2.8 million cancer incidence cases from 15 million inhabitants of the five Nordic countries aged 30–64 years in the 10-year censuses from 1960 to 1990 [26]. The study included 53 occupational categories. A novel index, lip cancer less lung cancer, was used for long-term UVB exposure [25]. A suspected important risk factor for lip cancer was solar UVB exposure [27]. A study conducted in Denmark reported that outdoor workers employed for more than 10 years had twice the rate of lip cancer than nonmelanoma skin cancer [28]. Smoking also is a well-known risk factor for lip cancer. As expected, people in occupational categories associated with outdoor work, such as farmers, forestry workers, and gardeners, had the lowest cancer incidence rates.

Table 2 presents findings regarding the incidence of specific cancers for males and females with respect to the UVB indices used. Cancers are listed in descending order of incidence rates in the United States in 2009 to show that as the number of cases decreases, so does the likelihood of finding significant correlations with solar UVB. Note that the results from the United States [22], Russia [24], and the Nordic countries [25] are in good agreement.

Table 2. Ecological studies of cancer incidence rates with respect to indices of solar UVB doses.

Incidence [29] (×1000)	Cancer	USA [22]	China [18]	Russia [24]	Nordic [25]
219.4	Lung		–M, FNS, –R, –U		M, FNS
194.3	Breast	F	–F, –R, –U		M, F
192.3	Prostate	M		–M	MNS
147.0	Colorectal		M, F, R		
106.1	Colon	M, F			M, F
71.0	Bladder, urinary	M, F	–M, –F, –R, –U		M, F
68.7	Melanoma	–M, –F		M + F	M
66.0	Non-Hodgkin lymphoma	M, F			NS
57.8	Kidney	M, F		M + F	M, FNS
44.8	Leukemia	M, F	MNS, FNS, R, –U		
42.5	Pancreas	M, F		M + F	M, FNS
42.2	Uterus, corpus	F			FNS
40.9	Rectum	M, F			M, FNS
37.2	Thyroid	MNS, F			
35.7	Oral cavity and pharynx	–M, –F			
23.1	Oral				M
22.6	Myeloma	M, F		M + F	
22.6	Liver		–M, –F, –R, –U		M, FNS
22.1	Brain				M
21.6	Ovary	FNS			
21.1	Stomach (gastric)	M, F	M, F, R, –U	M + F	M?, FNS
16.5	Esophagus	M	M, F, R, –U	M + F	MNS
12.6	Pharynx		–M, –F, –R, –U	–(M + F)	
12.3	Larynx				M
11.3	Cervix	–F	F, R,–U		
9.8	Gallbladder	F			M
9.8	Biliary, other	M, F		M + F	
8.5	Hodgkin lymphoma	M, F			
8.4	Testis				NS
6.2	Small intestine	M, F			M
5.9	Skin, other	–M, –F		–(M + F)	–M
5.3	Anus, etc.	–M, –F			
3.6	Vulva	F			

F, female; FNS, female nonsignificant; M, male; MNS, male nonsignificant; R, rural residence; U, urban residence, –, direct correlation; ?, uncertain.

Table 3 is similar to Table 2 except for showing mortality rates, not incidence rates, and cancers are listed in descending order with respect to cancer mortality rates in the United States in 2009. Note the good general agreement between the findings for mortality rates in Table 3 with incidence rates in Table 2. The main exception is that solar UVB dose was inversely correlated with mortality rates for several cancers in China, for which it was directly correlated with incidence rates.

Table 3. Ecological studies of cancer mortality rates with respect to indices of solar UVB doses.

Mortality [29] (×1000)	Cancer	Japan [23]	USA [14]	USA [22]	China [18]	Russia [24]
159.4	Lung	M, F			M, F, R, U	
69.1	Colorectal	M			M, F, R	
49.9	Colon		M, F	M, F		M + F
40.6	Breast	FNS	M, F	F	F, R	–(M + F)
35.2	Pancreas	M, F	M, FNS	M, F		M + F
27.4	Prostate	MNS	MNS	M		M
21.9	Leukemia			M, F	MNS, FNS	
19.5	Non-Hodgkin lymphoma		M, F	M, F		

Table 3. *Cont.*

Mortality [29] (×1000)	Cancer	Japan [23]	USA [14]	USA [22]	China [18]	Russia [24]
19.2	Rectum		M, F	M, F		M + F
18.2	Liver	M		–M, –F	M, F, R	
14.6	Ovary		F	F		F
14.5	Esophagus	M	M, F	M	M, F, R	M + F
14.3	Bladder, urinary		M, F	M, F	M, F, R	M + F
13.9	Kidney		M, F	M, F		M + F
12.9	Brain			–M, –F		
10.6	Myeloma			M, F		M + F
10.6	Stomach (gastric)	M, FNS	M, F	M, F	M, F, U	M + F
8.7	Melanoma			–M, –F		M + F
7.8	Uterus, corpus		F	F		
7.6	Oral cavity and pharynx			–M, –F		
5.4	Oral		MNS, FNS			
4.1	Cervix		F	–F	–F, –R, –U	
3.7	Larynx		M, F?	MNS, FNS		M + F
3.4	Gallbladder	MNS, F	M, F	M, F		
3.4	Biliary, other			M. F		
2.9	Skin, other			–M, –F		–(M + F)
2.2	Pharynx				–M. –F, –R, –U	
1.6	Thyroid			MNS, F		
1.5	Bone and joint			–M, –F		
1.3	Hodgkin lymphoma		M, F	M, F		
1.1	Small intestine			MNS, F		
0.9	Vulva			F		F
0.7	Anus, etc.			–M, –F		M + F

F, female; FNS, female nonsignificant; M, male; MNS, male nonsignificant; R, rural residence; U, urban residence, –, direct correlation; ?, uncertain.

2.2. Observational Studies Based on Residential UVB Doses

Related to ecological studies of solar UVB and cancer risk are observational studies of ambient solar UVB doses and cancer risk. Cancer incidence data from the prospective National Institutes of Health—AARP Diet and Health Study were used with solar UVB dose data at residential locations to assess the relationship between UVB and cancer risk [30]. The study was limited to participants living in California, Florida, Georgia (Atlanta), Louisiana, Michigan (Detroit), Pennsylvania, and North Carolina. During the 9 years of follow-up, 75,917 participants developed cancer. Erythemal UV data for July from TOMS for 1978–1993 and 1996–2005 were used. Data were adjusted for age; sex; body mass index (BMI); caloric intake; intake of fruit, vegetables, and red and white meat; alcohol consumption; tobacco smoking; education; physical activity; and median household income. Over 9 years of follow-up, UV exposure was inversely associated with total cancer risk (highest vs. lowest quartile) and decreased risk of non-Hodgkin lymphoma and colon, squamous-cell lung, pleural, prostate, kidney, and bladder cancers (all $p_{trend} < 0.05$). UV exposure was associated with increased melanoma risk.

Another example is a nested case–control (NCC) study using 373 esophageal and 249 gastric cancer cases from the UK Biobank with respect to UVB doses at the residential location [31]. Annual solar UVB doses ranged from ~500 kJ/m^2 in the south to ~750 kJ/m^2 in the north. Five controls were matched to each case. Data were available for many cancer risk-modifying factors. Significant reductions were found for adjusted esophageal cancer, adjusted lower-third esophageal cancer, and adjusted gastric cancer, in agreement with ecological studies noted previously.

A further discussion of observational studies of cancer incidence and death with respect to solar UVB is in progress.

2.3. Observational Studies Based on Serum 25(OH)D Concentrations

Observational studies examine correlations between risk-modifying factors and health outcomes such as cancer incidence, survival, and mortality rates. Observational studies include cohort studies, both prospective and retrospective; case–control (CC) studies; and cross-sectional studies. Each type has advantages and disadvantages. For example, most observational studies regarding vitamin D use serum 25(OH)D concentrations as the index of vitamin D status, but assays used to measure 25(OH)D concentrations vary in quality [32]. Furthermore, serum 25(OH)D concentrations change with the seasons and over long periods [33]. Some studies use dietary vitamin D, i.e., oral vitamin D, including dietary sources and supplements. However, using dietary sources to assess vitamin D intake is problematic because diet generally accounts for less than 300 IU/d in the United States. Although meat is an important source of vitamin D as 25(OH)D [34], most food frequency tables do not include data on meat [35]. Some studies use personal or geographical solar UVB doses. This review emphasizes those that use serum 25(OH)D concentrations but will also include a few that used solar UVB doses.

Generally, CC studies of cancer risk report a stronger reduction with respect to serum 25(OH)D concentrations than do other observational studies. However, observational studies using serum 25(OH)D concentration from blood drawn before cancer diagnosis are generally considered more accurate than those in which blood is drawn near the time of cancer diagnosis.

Researchers have hypothesized that because RCTs have generally not been able to confirm findings from observational studies for many health outcomes, including cancer, having the disease may reduce 25(OH)D concentrations; that is, "reverse causation" [36,37]. However, that effect has been shown only for acute inflammatory diseases such as acute respiratory tract infections [38].

Although systemic inflammation may play a role in cancer risk, the inflammation does not rise as high as in, say, COVID-19. Reports on levels of C-reactive protein levels, an index of systemic inflammation, at the time of diagnosis show that for COVID-19, values can range from 1 to 120 mg/L as severity increases [39], whereas for cancer, they are between 1 and 4 mg/L [40]. Thus, systemic inflammation is not high at the time of cancer diagnosis. We are not aware of any other factor that could result in reverse causality regarding 25(OH)D concentrations for undiagnosed cancer. As will be discussed, the main reason for discrepancies between observational studies and RCTs of vitamin D and cancer is that the RCTs have not been properly designed and conducted.

Two articles reported that the longer the follow-up time in observational studies of 25(OH)D concentration and cancer risk, the lower the effect of 25(OH)D concentration [41,42]. The same effect has been found for all-cause mortality rates [43]. The reasons include that serum 25(OH)D concentrations change for several reasons and that 25(OH)D concentration near the time of diagnosis is more important than earlier concentrations, even though cancer may develop over a long period. Figure 1 in Grant's 2012 report [43] shows that the correlation coefficient between serum 25(OH)D concentrations repeated in the same group of participants drops to approximately 0.4 after 14 years.

Most observational studies of 25(OH)D concentration and cancer incidence are prospective cohort or NCC studies. An NCC study of 25(OH)D concentration and incidence of colorectal cancer (CRC) based on two Harvard cohorts [44] is reviewed here to show the complexity of such studies. The Health Professionals Follow-up Study (HPFS), with 18,225 male participants who supplied a blood sample, had 179 cases of CRC during follow-up periods up to 8 years. The analysis of results from the cohort was combined with results from the Nurses' Health Study (NHS) of women, of whom 32,826 gave blood samples, and 193 developed CRC during 11 years of follow-up [45]. In the HPFS, values for many factors were recorded at baseline in 1994, including season of blood donation, BMI, physical activity, aspirin use, smoking, alcohol intake, intake of vitamin D, calcium and retinol, and meat intake. Analyses were made for colon, rectal, and CRC with respect to quantiles of 25(OH)D, showing that though the trend in 25(OH)D concentrations was not significant

for HPFS alone, it was significant when combined with results from NHS. The pooled odds ratio (OR) for CRC for high versus low quintile of 25(OH)D was 0.66 (95% confidence interval [95% CI], 0.42–1.05; p_{trend} = 0.01). The risk of rectal cancer increased with respect to 25(OH)D in the HPFS but decreased in the NHS. Interesting findings also were shown for lifestyle characteristics, including BMI, physical activity, calcium intake, retinol intake, and effect of 25(OH)D measured in winter or summer. Thus, with 372 CRC cases, it was possible to find support for 25(OH)D concentrations reducing the risk of colon cancer and CRC.

A meta-analysis published in 2007 based on five NCC studies found a predicted 50 ± 20% reduction in CRC for 34 ng/mL vs. 6 ng/mL [46].

A pooled analysis of 12 NCC studies for CRC for men showed a relative risk (RR) of 0.93 (95% CI, 0.86–1.00), whereas the pooled analysis for 13 studies for women reported an RR of 0.81 (95% CI, 0.75–0.87) [47]. For men and women combined, the RR was 0.87 (95% CI, 0.75–0.87). A significant reduction in RR was shown for women between approximately 25 and 45 ng/mL, but no significant reduction was evident for men at any range. This analysis did not adjust for follow-up time between blood draw and cancer diagnosis. To examine the effect of follow-up time, plots were made of the ORs or RRs from the meta-analysis by McCullough and colleagues [47]. Table 4 shows the data used. Information regarding the relative weight for each study was not available, so plots were made of OR against follow-up time. Figure 1 shows the results. The RR for zero follow-up time should be approximately 0.75 for men and 0.77 for women. The regression fit to the data for men is OR = 0.74 + 0.031x years, r = 0.79, adjusted r^2 = 0.59, p = 0.002; the regression fit to the data for women is OR = 0.77 + 0.008x years, r = 0.25, adjusted r^2 = 0, p = 0.42. Thus, the lower effect of 25(OH)D on men versus that of women shown in Figure 1 in McCullough and colleagues [47] is due to not accounting for the degradation of the 25(OH)D effect with a longer follow-up time. Providing evidence that the results for men and women should be similar is supported by ecological studies in the United States [14].

Table 4. Data related to Figure 2 in McCullough and colleagues [47].

Study	Follow-Up (Years)	RR	Ref.
Men			
ATBC2	12.5	1.17	[48]
PHS	9.50	1.06	[49]
CLUE II	3.20	0.99	[50]
HPFS	6.30	0.99	[51]
JANUS	5.10	0.93	[52]
EPIC	3.60	0.86	[53]
MEC	1.50	0.86	[54]
CPS-II	3.20	0.83	[55]
JPHC	5.10	0.83	[56]
CARET	4.90	0.82	[57]
PLCO	5.40	0.81	[58]
ABCT1	3.50	0.77	[59]
Women			
ORDET	10.8	1.03	[60]
JPHC	5.10	0.94	[56]
JANUS	5.10	0.90	[52]
BGS	2.30	0.90	[61]
CLUE-II	9.00	0.87	[50]
WHI	3.20	0.87	[62]
NHS	9.60	0.84	[51]
CPS-II	3.20	0.77	[55]
WHS	8.00	0.77	[63]
EPIC	3.60	0.73	[53]

Table 4. *Cont.*

Study	Follow-Up (Years)	RR	Ref.
NYUWHS	12.3	0.72	[64]
PLCO	5.40	0.67	[58]
MEC	1.50	0.63	[54]

ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; BGS, Breakthrough Generations Study; CARET, Carotene and Retinol Efficacy Trial; CLUE II, Cancer Prevention Study II Nutrition Cohort; CPS-II, Cancer Prevention Study II; EPIC, European Prospective Investigation into Cancer and Nutrition; HPFS, Health Professionals Follow-up Study; JANUS, JANUS Serum Bank, Norway; JPHC, Japan Public Health Center-Based Prospective Study; MEC, multiethnic cohort study'; NYUWHS; New York University, Women's Health Study; ORDET, Hormones and Diet in the Etiology of Breast Cancer Risk; PHS, Physicians' Health Study; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; RR, relative risk; WHI, Women's Health Initiative.

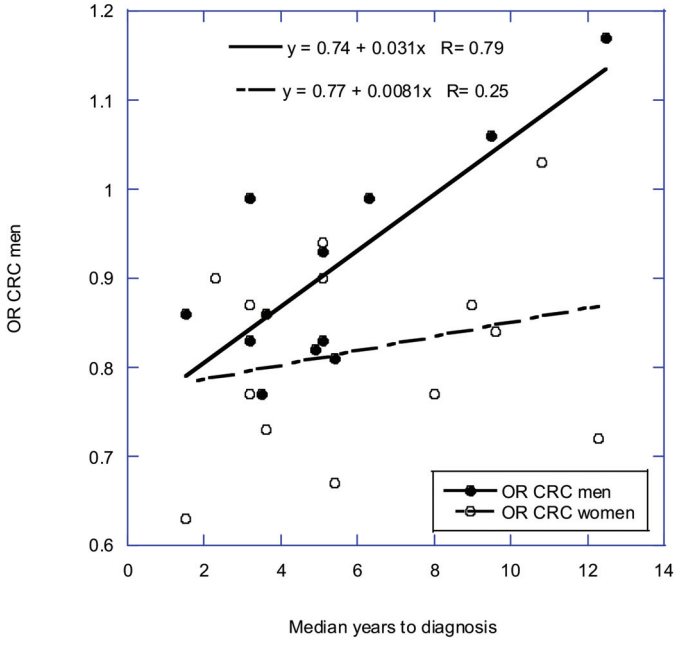

Figure 1. Plot of odds ratio (OR) for CRC against median years to diagnosis for data for men and women used in McCullough and colleagues [47].

In contrast to CRC, prospective and NCC studies with follow-up times greater than 4 years seldom show a significant inverse correlation between serum 25(OH)D concentration and incidence of breast cancer. Breast cancer can develop rapidly, with progression strongly affected by 25(OH)D concentration. Breast cancer is one of the few cancers that have a seasonality in diagnosis, with the highest diagnosis rates in spring and fall [65]. The authors of that study suggested that solar UVB, through producing vitamin D, lowers the risk of breast cancer in summer, whereas higher concentrations of melatonin reduce risk in winter. As a result, many more CC studies of breast cancer with 25(OH)D measured at the time of diagnosis exist than that for CRC.

CC studies of breast cancer incidence with respect to serum 25(OH)D concentrations in pre- and postmenopausal women are discussed first [66,67]. The premenopausal study included 289 cases and 595 matched controls; the postmenopausal study included 1394 cases and 1365 controls. In the premenopausal study, the adjusted OR (aOR) for 25(OH)D >24 ng/mL versus <12 ng/mL was 0.48 (95% CI, 0.29–0.70) and the p_{trend} value for the quantiles was 0.0006. In the postmenopausal study, the aOR for 25(OH)D >30 ng/mL versus <12 ng/mL was 0.31 (95% CI, 0.24–0.42) and the p_{trend} value of the

quintiles was <0.0001. In both studies, the risk increased more rapidly as 25(OH)D concentrations decreased below 12 ng/mL. Those two studies show that several individual factors affect cancer risk but, in general, have little impact on the role of 25(OH)D concentration.

The present study incorporated a search at Google Scholar and the National Library of Medicine's PubMed database for meta-analyses of cancer incidence or mortality rate with respect to serum 25(OH)D concentration. The most recent meta-analyses were favored. For several cancers, Table 5 includes more than one meta-analysis. Of the 44 studies listed as CC in the meta-analysis of breast cancer by Song and colleagues [68], 26 were true CC studies in which serum 25(OH)D concentration was measured near the time of cancer diagnosis for both cases and controls, with 14,851 cases and 30,979 controls. The remaining 18 studies were NCC studies or, in one case, a cross-sectional study. The number of breast cancer cases was 17,871, whereas the number of controls was 21,753. The analysis for cohort studies of breast cancer incidence in that study included the observational study of breast cancer incidence for participants in either two vitamin D plus calcium RCTs or the Grassrootshealth.net community-based cohort [69]. Because those participants generally had serum 25(OH)D measured every 6 months to 1–2 years, that study should have been combined with the CC studies. It reported an 82% lower risk of breast cancer for 25(OH)D concentration >60 ng/mL versus <20 ng/mL (rate ratio = 0.18 [95% CI, 0.04–0.62]).

Table 5. Meta-analyses of observational studies of incidence risk of individual cancer sites related to serum 25(OH)D concentration.

Cancer Site	*N* Studies, Cases, Controls	Type of Study	Follow-Up (Years)	RR (95% CI), High vs. Low	Ref.
All	8, —, —	Prospective, incidence	5–28	0.86 (0.73–1.02)	[70]
All	17, —, —	Prospective, mortality	5–28	0.81 (0.71–0.93)	[70]
Bladder	5, 1251, 1332	CC and NCC, incidence	0 (4), 12, 13	0.70 (0.56–0.88)	[71]
Bladder	2, 2264, 2258	Cohort, incidence	14, 28	0.80 (0.67–0.94)	[71]
Breast	44, 29,095, 53,060	CC and NCC, incidence		0.57 (0.48–0.66)	[68]
Breast	6, 2257, —	Cohort, incidence		1.17 (0.92–1.48)	[68]
Colorectal	11, —, —	1 CC, 9 NCC, 1 meta-analysis, incidence	0–20	0.60 (0.53–0.68)	[72]
Colorectal	6, 1252, —	Cohort, incidence	8–20	0.80 (0.66–0.97)	[72]
Colorectal	15, 6691, —	NCC, incidence		0.67 (0.59–0.76)	[73]
Head and neck	5, —, —	Cohort, incidence	7, 15	0.68 (0.59–0.78)	[74]
Liver	8, 992, —	Cohort, incidence	6–28	0.78 (0.63–0.95)	[75]
Liver	6, 776, —	Cohort, incidence	(0.75), 16–22	0.53 (0.41–0.68)	[76]
Lung	8, 1386, —	Cohort, incidence	7–26	0.72 (0.61–0.85)	[77]
Lung	9, —, —	7 Cohort, 2 CC, incidence		0.84 (0.74–0.95)	[78]
Lung	3, —, —	1 Cohort, 2 CC, mortality		0.76 (0.61–0.94)	[78]
Lung	12, —, —	7 Cohort, 5 CC		1.05 (0.95–1.16)	[79]
Ovarian	8, —, —	CC, cohort, NCC		0.86 (0.56–1.33)	[80]
Pancreatic	5, 1068, —	2 Cohort, 3 NCC, incidence	6.5–21	1.02 (0.66–1.57)	[81]
Pancreatic	5, 2003, —	Cohort, mortality	6.5–21	0.81 (0.68–0.96)	[81]
Prostate	19, 12,786	16 NCC, 3 cohort, incidence		1.15 (1.06–1.24)	[82]
Renal	5, —, —	4 Cohort (+1 CC, 3.5% weighting), incidence	(0), 7–22	0.76 (0.64–0.89)	[83]
Renal	1, —, —	CC, incidence	0	0.30 (0.13–0.72)	[83]
Thyroid	6, 387, 457	CC, incidence	0	Deficiency, 1.30 (1.00–1.69), $p = 0.05$	[84]

95% CI, 95% confidence interval; CC, case–control study; NCC, nested case–control study; parentheses for follow-up years indicate numbers for a very small percentage of the total; RR, relative risk; —, no data.

From the data in Table 5, it is apparent that CC and NCC studies report greater reductions in cancer risk for high versus low 25(OH)D concentration. The reason may be that cohort studies are conducted for longer than CC or NCC studies. That difference lowers the benefit due to 25(OH)D concentrations as a result of changes in 25(OH)D concentration, as discussed previously. Another finding is that studies of mortality rates show greater reductions than studies of incidence rates. That finding is similar to findings in RCTs of cancer as reported, for example, in the VITAL study [85] as well as in a meta-analysis

of results from vitamin D–cancer RCTs [86]. The reason for that finding is probably the presence of many risk-modifying factors that affect cancer incidence but few factors other than vitamin D that affect angiogenesis around tumors, cancer progression, and metastasis into stromal tissue.

Table 6 presents findings from a few meta-analyses of observational studies of vitamin D intake, both from diet and from supplements, and cancer risk. The reductions in cancer risk from oral intake are generally much lower than what is found with respect to serum 25(OH)D concentration studies, largely because differences in oral intakes did not have an observable effect on serum 25(OH)D concentrations. In addition, results with respect to serum 25(OH)D concentrations were not given.

Table 6. Meta-analyses of observational studies of the risk of incidence of individual cancer sites related to vitamin D intake.

Cancer Site	*N* Studies	Type of Study	RR (95% CI), High vs. Low Vitamin D Intake	Ref.
Breast	17	8 CC, 9 cohorts	0.97 (0.92–1.07), per 400 IU/d	[68]
Colorectal	12	CC	0.75 (0.67–0.81)	[72]
Colorectal	6	Cohort	0.89 (0.80–1.02)	[72]
Head and neck	3		0.75 (0.58–0.97)	[74]
Lung	6	Cohort	0.89 (0.83–0.97)	[77]
Lung	5	Cohort	0.85 (0.74–0.98)	[79]
Renal	4	CC	0.80 (0.67–0.95)	[83]
Renal	4	Cohort	0.97 (0.77–1.22)	[83]
Overall cancer death			0.84 (0.74–0.95)	[87]

CC, case–control study; NCC, nested case–control study.

Table 7 presents estimates of the OR for maximum 25(OH)D concentration compared with minimum concentration for several cancers. The reviews obtained from these values did not give numerical values, so they were estimated by inspecting the graphs.

Table 7. Estimates of odds ratio for maximum 25(OH)D concentration compared with minimum concentration for several cancers.

Cancer	Min 25(OH)D (ng/mL)	Max 25(OH)D (ng/mL)	OR (95% CI)	Ref.
All, inc	2	25	~0.6	[70]
Bladder, inc	3	30	~0.55 (0.35–0.70)	[71]
Breast, inc (Song et al.)	5	85	~0.2 (0.1–0.3)	[68]
Breast, inc	15	70	0.18 (0.04–0.62)	[69]
Colorectal, inc	4	55	~0.4 (0.3–0.5)	[73]
Colorectal, inc	10	50	~0.7 (0.4–1.0)	[88]
Liver, inc	4	30	0.35 (0.21–0.48)	[76]
Liver, inc	5	30	~0.6 (0.5–0.7)	[75]
Lung, inc	6	21	0.87 (0.76–0.97)	[89]
Lung, inc	10	24	0.80 (0.61–0.98)	[78]
Lung, mort	10	42	0.37 (0.25–0.53)	[78]
Prostate, inc	0	60	~1.3 (1.1–1.8)	[82]
Prostate, mort	4	43	~0.55 (0.2–1.1)	[90]

Inc, incidence; mort, mortality; OR, odds ratio.

Figure 2 shows the plot of OR for cancer incidence against the difference between minimum and maximum 25(OH)D concentration. The plot indicates a nearly linear relationship between serum 25(OH)D concentration and OR. The linearity between OR and 25(OH)D concentration is supported by results in the breast cancer study by McDonnell and colleagues [69]. Many studies have few participants with 25(OH)D concentrations above 40 ng/mL, thereby limiting the ability to investigate the effects of higher 25(OH)D concentrations.

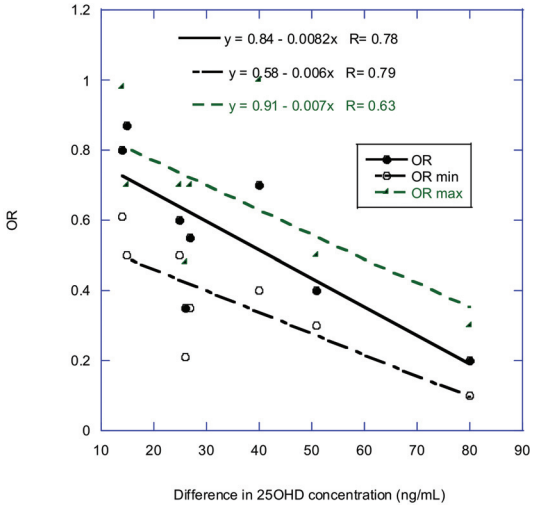

Figure 2. Plot of OR for cancer incidence versus the difference between minimum and maximum 25(OH)D concentration, using data from Table 7, omitting data for all cancer, breast cancer in McDonnell and colleagues [69], and data for prostate cancer.

2.4. RCTs of Vitamin D and Cancer Risk

According to a review published in 2019 [86], nine RCTs have studied how vitamin D supplementation affects cancer incidence, of which five also studied the effect on cancer mortality rate. The relative risk of vitamin D supplementation in the treatment versus placebo groups for cancer incidence was 0.98 (95% CI, 0.93–1.03), whereas for cancer, the mortality rate was 0.87 (95% CI 0.79–0.96). Results did not change significantly if they were analyzed by daily intake versus nondaily intake in a large bolus or attained 25(OH)D concentration >40 ng/mL. However, as pointed out in a recent review by Pilz and colleagues, RCTs rarely found a significant benefit from vitamin D supplementation [91].

The information on most of the trials discussed in [86] plus another published thereafter are presented in Tables 8 and 9. As can be seen in Table 8, none of the trials were well designed based on what is now known. Not all trials measured baseline 25(OH)D concentration and when they did, the concentrations were almost always above mean population values. Only five reported achieving 25(OH)D concentrations, and both baseline and achieved concentrations were generally based on a fraction of all participants. Four trials used infrequent bolus doses, which were done to improve compliance but resulted in large variations in 25(OH)D concentration between doses since the half-life of 25(OH)D is approximately two weeks. Some of the trials also gave calcium to the treatment arm but not the control arm. In all cases, participants were permitted to take modest vitamin D supplement doses and solar UVB exposure was not controlled. The mean BMI was generally high in the trials, which is a problem since those with higher BMI do not have the same response for a similar change in 25(OH)D concentration as those with lower BMI. For example, the VITAL study [86] reported that participants with BMI <25 kg/m^2 of body surface area had a significantly reduced risk of cancer from vitamin D supplementation (hazard ratio = 0.76 [95% CI, 0.63–0.90]) but not for higher BMI categories, even though the change in 25(OH)D was near 12 ng/mL for all three BMI categories. The apparent reason is that obesity is an important risk factor for cancer and vitamin D has a limited ability to overcome the mechanisms whereby obesity increases risk of cancer [92]. Finally, only a few of the trials were explicitly designed with cancer incidence a primary outcome.

Table 8. Characteristics of ten RCTs that investigated the effect of vitamin D supplementation on risk of cancer incidence and/or mortality rate.

Location	Mean Baseline and Achieved 25(OH)D (ng/mL), Treatment Arm	Vitamin D Dose (IU) Frequency in Treatment Arm	Duration (Years)	Mean BMI (kg/m^2)	Original Purpose	Reference
UK		100,000/ (4 months)	5.5	24 ± 3	fracture incidence, cause of death	[93]
USA		400/day + 1 g/day Ca	7	28?	colorectal cancer incidence, mortality	[94]
Nebraska, USA	29, 38	1100/day + 1.5 g/day Ca; 1.5 g/day Ca	4	29 ± 6	fracture incidence	[95]
Australia	21, 24–48	500,000/year			falls and fractures	[96]
England, Scotland		800/day; 1 g/d Ca; 800/day + 1 g/day Ca	3			[97]
Nebraska, USA	33, 44	2000/day + 1500 mg/day Ca	4	30 ± 7	cancer	[98]
New Zealand	26, –	100,000/mo	3.3 ± 0.8	28 ± 5	disease incidence with respect to bolus dose of vitamin D	[99]
USA	30, 41	2000/day	5.3	31	cancer and cardiovascular disease risk	[85]
Australia	31 ± 10, 46 ± 12	60,000/ month	5	27?	mortality by disease	[100]

Table 9. Outcomes of ten RCTs that investigated the effect of vitamin D supplementation on risk of cancer incidence and/or mortality rate with respect to intention to treat.

Location	Number of Participants, Cancer Cases, Deaths, Treatment Arm	Number of Participants, Cancer Cases, Deaths, Non-Vitamin D Arm	RR, Incidence (95% CI)	RR, Mortality (95% CI)	Reference
UK	1345, 163, 63	1341, 147, 72	1.11 (0.86–1.42)	0.86 (0.61–1.20)	[93]
USA	18,176, 1634, 344	18,106, 1655, 382	0.98 (0.91–1.05)	0.89 (0.77–1.03)	[94]
Nebraska, USA	446, 13, –	733, 37, –	0.76 (0.38–1.55)		[95]
Australia	1131, 7	1125, 10	0.70 (0.27–1.82)		[96]
England, Scotland	1306, 182, 78; 1311, 189, 95	1343, 187, 73; 1332, 165, 83	1.24 (0.80–2.28)	1.26 (0.73–3.26)	[97]
Nebraska, USA	1156, 45, –	1147, 64, –	0.70 (0.47–1.02)		[98]
New Zealand	2558, 302, –	2550, 293, –	1.01 (0.81–1.25)		[99]
USA	12,927, 793, 154	12,946, 824, 187	0.96 (0.88–1.06)	0.83 (0.67–1.02)	[85]
Meta-analysis for ten incidence trials and five mortality rate trials			0.98 (0.93–1.03)	0.87 (0.79–0.96)	[86]
Australia	21,315, –, 221	10,662, –, 189		1.15 (0.96–1.39)	[100]

Only one outcome based on intention to treat was significantly reduced, that of cancer mortality rate in the VITAL trial [85]. Nonetheless, a meta-analysis of five trials found a significant reduction in the cancer mortality rate [86].

The main problem with vitamin D RCTs seems to be that they are generally designed and conducted by following guidelines for pharmaceutical drugs rather than nutrients. For drugs, the only source of the agent is assumed to be what is given to participants in the treatment arm, and a linear dose–response relationship is presumed. Neither assumption is valid for vitamin D. As a result, participants generally have mean 25(OH)D concentrations

above the population's mean values, participants are given small doses of vitamin D, and participants in both the treatment and control arms are permitted to take additional vitamin D supplements as well as produce vitamin D through solar UVB exposure.

Robert Heaney outlined the guidelines for nutrient RCTs in 2014 [101], which were updated in 2018 [102]. The principal guidelines adapted for vitamin D are that:

- Baseline 25(OH)D concentrations should be measured and used as a criterion for inclusion in the study;
- The vitamin D dose should be large enough to increase 25(OH)D concentration to the point at which it would have an observable effect on health outcomes;
- Achieved 25(OH)D concentrations should be measured;
- Conutrient status must be optimized to ensure that vitamin D is the only nutrient-related limiting factor in the response.

No RCT investigating the role of vitamin D in reducing risk of cancer appears to have followed those guidelines.

Some secondary results of the vitamin D–cancer RCTs have yielded useful information. The VITAL study also reported that African American participants had a trend for reduced risk of cancer incidence (hazard ratio = 0.77 [95% CI, 0.59–1.01]). According to the report's supplementary material for African Americans who supplied 25(OH)D concentration values, the baseline 25(OH)D was 25.0 ng/mL, and the achieved 25(OH)D concentration was 39.7 ng/mL. Those values are in contrast to 31.4 and 42.4 ng/mL, respectively, for non-Hispanic white participants.

In addition, two RCTs showed some effect of vitamin D plus calcium supplementation on risk of cancer [95,98]. When those data were pooled with data from the Grassroots Health volunteer cohort and analyzed by achieved 25(OH)D concentration, the incidence rate of breast cancer for women with 25(OH)D concentrations $\geq$60 versus <20 ng/mL had a rate ratio of 0.18 (95% CI, 0.04–0.62; $p = 0.006$).

3. Perspectives on Epidemiological Studies

3.1. Ecological Studies

As would be generally expected, incidence and mortality rates are generally inversely correlated with solar UVB indices unless UVB exposure is linked to increased risk, such as that for melanoma and other skin cancer. The direct correlation with oral cavities and the pharynx in the United States is consistent with UVB exposure's being a risk factor for lip cancer. Solar UVB exposure increases human papillomavirus (HPV) concentrations, as evidenced by peak rates of positive Pap smears for cervical cancer in Denmark in August [103]. HPV is a risk factor for head and neck cancer [104]. HPV is also hypothesized to be an important risk factor for melanoma [105].

The finding that the incidence rates for several cancers are directly correlated with solar UV in China, whereas most of the cancer mortality rates are inversely correlated, is probably owing to the fact that air pollution levels are much higher in northern than in southern China [106]. In addition, vitamin D generally reduces the risk of cancer mortality rates rather than incidence rates. The reasons may include that although many factors affect cancer incidence, few factors affect cancer progression and metastasis.

Because the countries included are different in many respects, including diet, ethnicity, latitude, and pollution level, ecological studies offer strong evidence that UVB irradiance affects cancers similarly regardless of many other factors.

An important reason why ecological studies have shown robust relationships between indices of solar UVB doses is that they included many cases of cancer. Researchers conducting earlier ecological studies were more likely than researchers of more recent studies to find significant correlations with UVB doses because people back then spent more time in the sun without concern for skin cancer or photoaging, and obesity rates were lower.

3.2. Observational Studies

Several findings are important from the analyses presented regarding observational studies.

First, the inverse relationships between serum 25(OH)D concentration and cancer incidence or mortality rates are similar to those between solar UVB and cancer reported in ecological studies. The primary exception is for head and neck cancer; serum risk was inversely correlated with both serum 25(OH)D concentration and vitamin D intake. However, ecological studies showed direct correlations between solar UVB and both incidence and mortality rates for oral cavity/pharynx and pharynx cancers, although one study reported an inverse relationship for laryngeal cancer [25].

Secondly, a long follow-up time was again found to significantly decrease the observed beneficial effect of 25(OH)D concentration. For example, the meta-analysis of CRC risk with respect to 25(OH)D concentration by Hernandez-Alonso and colleagues [72] had 11 studies (one CC, nine NCC, and one meta-analysis) and six prospective cohort studies. The OR for the CC study was 0.45 (95% CI, 0.36–0.57). For the nine NCC studies, the mean follow-up time was near 8 years, and the OR was 0.63, whereas for the prospective cohort studies, the mean follow-up time was 13 years, and the OR was 0.80 (95% CI, 0.66–0.97).

Some parties have argued that CC studies with 25(OH)D concentration measured near the time of diagnosis would be the best type of observational study due to possible reverse causality [53]. There is no evidence to indicate that having undiagnosed cancer reduces 25(OH)D concentration other than perhaps decreasing with the progression cancer stage. Thus, CC studies, which are easier to conduct than prospective studies, are preferred.

The epidemiological and mechanical evidence regarding solar UVB exposure and vitamin D presented here generally satisfy Hill's criteria for causality in a biological system (based on Kosh's postulates) [107–109]. The only weakness is that RCTs have not yielded strong support, largely because they were poorly designed and conducted. However, as argued by Dr. Thomas R. Frieden, former head of the U.S. Centers for Disease Control and Prevention, in a review in *The New England Journal of Medicine*, RCTs have substantial limitations [110]. The review tabulates the strength and limitations of 11 study designs, including RCTs, prospective cohort, retrospective cohort, case-control, and ecological studies. It concludes by stating that there is no single, best approach to the study of health interventions, and clinical and public health decisions are almost always made with imperfect data.

3.3. Historical Overview

Many of the articles reviewed regarding epidemiological studies of solar UVB dose or exposure and vitamin D played important roles in developing the understanding of the role of vitamin D in reducing risk of cancer incidence and mortality rates. Table 10 lists a few of them in chronological order. Note that the importance of some of the articles, notably those reported prior to 1980, was not recognized until many years later.

Table 10. List of epidemiological studies that had important findings in the history of solar UVB exposure and/or vitamin D and cancer.

Year	Finding	Reference
1936	Sun exposure can cause skin cancer but reduce risk of internal cancer.	[1]
1937	US Navy personnel highly exposed to sun had high skin cancer rates but low internal cancer rates.	[2]
1941	Cancer mortality rates for whites in the U.S. found inversely related to a solar radiation index while skin cancer (melanoma) mortality rates were directly related.	[3]
1980	Annual solar radiation dose inversely correlated with colon cancer mortality rate, USA, vitamin D production suggested.	[6]
1985	Dietary vitamin D and calcium inversely correlated with colorectal cancer incidence.	[7]
1989	Serum 25(OH)D concentration inversely correlated with colon cancer incidence.	[8]

Table 10. *Cont.*

Year	Finding	Reference
1990	Annual solar radiation dose inversely correlated with breast cancer mortality rate in the U.S.	[9]
2002	Mortality rates for thirteen types of cancer are inversely correlated with solar UVB doses in the U.S., 1970–1994.	[13]
2006	A Harvard cohort study finding that incidence of several types of cancer were inversely correlated with predicted 25(OH)D concentration.	[111]
2006	An ecological study in the U.S. finding that incidence and mortality rates for many types of cancer were inversely correlated with solar UVB doses.	[22]
2007	A meta-analysis presenting a 25(OH)D concentration-colorectal cancer incidence relationship.	[46]
2007	An RCT conducted in the U.S. finding that vitamin D supplementation significantly reduced risk of all-cancer incidence rate.	[95]

4. Mechanisms Introduction

The first experimental studies supporting this effect of 1,25-$(OH)_2D_3$ were reported in 1981. They addressed the inhibition of human melanoma cell proliferation and the induction of the differentiation of mouse myeloid leukemia cells and were by D. Feldman's and T. Suda's groups, respectively [112,113]. Since then, many laboratories have described a high number of antitumoral effects of 1,25-$(OH)_2D_3$ on a variety of molecular mechanisms and cellular processes during carcinogenesis. Previous reviews have discussed some of these mechanisms in particular cancer types [114–119]. In this review, we update the current knowledge on 1,25-$(OH)_2D_3$ antitumor mechanisms.

4.1. Inhibition of Tumor Cell Proliferation

1,25-$(OH)_2D_3$ exerts an antiproliferative action on tumor cells by direct and indirect mechanisms that are partially redundant and sometimes function simultaneously in target cells. Of note, this action is mostly independent of *TP53* tumor suppressor gene status.

Direct mechanisms. In many cancer cell types, 1,25-$(OH)_2D_3$ directly arrests the cell cycle in the G_0/G_1 phase by downregulating cyclin-dependent kinases (CDKs: CDK4, CDK6) and repressing the genes that encode cyclins D1 and C (*CCND1, CCNC*) and CDK inhibitors $p21^{CIP1/WAF1}$ (*CDKN1A*), $p27^{KIP1}$ (*CDKN1B*) and p19 (*CDKN2D*) [116,119]. The induction of $p27^{KIP1}$ expression takes place at the promoter/transcriptional level and posttranslationally by the inhibition of its degradation [120–122]. These effects hamper retinoblastoma (Rb) protein phosphorylation and thus the activation of the E2F family of transcription factors, which trigger a series of target genes that are critical to entering the cell cycle from the quiescent state. In addition, an Rb-independent G_1 arrest has been described that is probably a consequence of the repression of the *MYC* oncogene [123]. Thus, 1,25-$(OH)_2D_3$ represses *MYC* expression via direct [124] or indirect transcriptional inhibition by antagonism of the Wnt/β-catenin pathway [125,126], the induction of cystatin D [127] or the MYC antagonist MAD/MXD1 [128], by repressing long non-coding *(lnc)RNA CCAT2* [129] or by promoting MYC protein degradation [130] in several carcinoma cell types.

In some systems (colon and gastric cancer cells), 1,25-$(OH)_2D_3$ downregulates other proliferative genes such as *FOS*, *JUN*, *JUNB*, and *JUND* proto-oncogenes, *G0S2* (G_0/G_1 switch 2), and *CD44*, while it upregulates *GADD45A* (growth arrest and DNA damage 45a), *MEG3* (Maternally expressed gene 3, a lncRNA) and *NAT2* (N-acetyltransferase 2) [131–134]. Additionally, 1,25-$(OH)_2D_3$ induces antiproliferative genes such as *CEBPA* (CCAAT-enhancer-binding protein-α) and *IGFBP3* (insulin-like growth factor binding protein-3) in breast, prostate, or colon carcinoma cells, respectively [131,135,136]. IGFBP3 mediates the induction of $p21^{CIP1/WAF1}$ by 1,25-$(OH)_2D_3$ in prostate carcinoma cells [136], and microRNA *miR-145* the repression of *CDK2*, *CDK6*, *CCNA2*, and *E2F3* genes and the antiproliferative effect

of 1,25-$(OH)_2D_3$ in gastric cancer cells [137]. In breast carcinoma and anaplastic thyroid cancer cells, 1,25-$(OH)_2D_3$ causes G_2/M phase arrest probably as a consequence of the downregulation of CDK2 activity due to the E2F blockade by non-phosphorylated Rb protein [138]. vitamin D analogues also inhibit proliferation through induction of G_1 phase arrest of some hematological cancer cells (lymphoma, myeloma, B-cell acute lymphoblastic leukemia and acute myeloid leukemia) [139].

Indirect mechanisms. 1,25-$(OH)_2D_3$ interferes with several mitogen signaling pathways in a context-dependent fashion. Thus, 1,25-$(OH)_2D_3$ decreases the expression of epidermal growth factor receptor (EGFR) and promotes its ligand-induced internalization in colon carcinoma cells [140,141]. Additionally, it diminishes EGFR signaling through the induction of E-cadherin and the repression of SPROUTY-2 and the renin-angiotensin system [125,142–144]. 1,25-$(OH)_2D_3$ and certain analogues interfere with the insulin-like growth factor (IGF)-I/II pathway by inhibiting IGF-II secretion and increasing IGFBP3 and IGFBP6 levels, and by inducing type II IGF receptor (IGFR-II), which accelerates IGF-II degradation and downregulates this pathway [145,146]. In oral squamous cell carcinoma cells, the 1,25-$(OH)_2D_3$ analogue Eldecalcitol antagonizes the mitogenic action of fibroblast growth factor (FGF)1/2 by repressing nuclear factor *kappa* B (NF-*k*B) and inducing *miR6887-5p*, which targets 3′UTR mRNA of heparin-binding protein 17/FGF-binding protein-1 (HBp17/FGFBP-1), a FGF2 chaperone [147,148]. In addition, 1,25-$(OH)_2D_3$ inhibits the mitogenic action of platelet-derived growth factor (PDGF)-BB in prostate cancer cells by downregulating PDGF receptor β [149]. The effect of 1,25-$(OH)_2D_3$ on hepatocyte growth factor (HGF) signaling is cell-type dependent. It is inhibitory in hepatocellular cells by reducing the expression of c-Met, the tyrosine kinase HGF receptor [150] and in promyelocytic leukemia cells by downregulating HGF RNA [151], but activating in some non-tumoral cell types [152].

1,25-$(OH)_2D_3$ also diminishes the proliferation of breast cancer cells by inhibiting estrogen synthesis and signaling through estrogen receptor (ER)α [153] and by downregulating RAS expression and the phosphorylation of its downstream effectors MEK and ERK1/2 [154]. The inhibition of pituitary transcription factor (Pit)-1 is another antiproliferative effect of 1,25-$(OH)_2D_3$ in breast cancer cells. Pit-1 expression is higher in tumors than in normal breast. It regulates growth hormone (GH) and prolactin (PRL) secretion and leads to increased cell proliferation, invasiveness, and metastasis [155]. 1,25-$(OH)_2D_3$ reduces Pit-1 expression and the increase in cell proliferation either directly or indirectly through GH and/or PRL [156].

Another indirect mechanism of the antiproliferative effect of 1,25-$(OH)_2D_3$ is the regulation of miRs. Thus, *miR-22* is induced by 1,25-$(OH)_2D_3$ and contributes to its antiproliferative effect on colon carcinoma cells 1,25-$(OH)_2D_3$ [157] and has antitumor effects in other carcinomas. Additionally, a recent study indicates that *miR-1278* sensitizes cells to 1,25-$(OH)_2D_3$ by suppressing the expression of CYP24A1 [158].

Transforming growth factor (TGF)-β is a strong inhibitor of epithelial cell proliferation in normal cells and at early steps in the tumorigenic process. 1,25-$(OH)_2D_3$ activates latent TGF-β and induces the expression of type I TGF-β receptor, which sensitizes breast and colon carcinoma cells to the growth inhibitory action of TGF-β [159,160]. Of note, TGF-β signaling is blocked in around 30% of colon cancers due to mutation of the genes encoding TGF-β receptor type II, SMAD2, or SMAD4. In contrast, TGF-β promotes at late stages epithelial-to-mesenchymal transition (EMT), migration, invasion, immunosuppression, and metastasis. As discussed in the following sections, these protumorigenic effects of TGF-β on tumor and stromal cells later in carcinogenesis are counteracted by 1,25$(OH)_2D_3$.

Concordantly with the association between low vitamin D status and poorer overall survival and progression-free survival in myeloid and lymphoid malignancies [161], in several types of leukemic cells, 1,25-$(OH)_2D_3$ regulates essential pathways for survival and proliferation such as TLR, STAT1/3 or PI3K/AKT that are induced by immune cell–cell or cytokine activation [162,163].

4.2. Sensitization to Apoptosis, Combined Action with Chemotherapy and Radiotherapy

Obviously, 1,25-$(OH)_2D_3$ per se does not induce apoptosis or any other type of cell death. However, it controls the expression of genes involved in apoptosis in cell systems in a way that is compatible with sensitization to the induction of apoptosis by other agents. Thus, in colon, prostate, and breast carcinoma cells, 1,25-$(OH)_2D_3$ upregulates several pro-apoptotic proteins (BAX, BAK, BAG, BAD, G0S2) and suppresses survival and anti-apoptotic proteins (thymidylate synthase, survivin, BCL-2, BCL-XL). In this way, it favors the release of cytochrome C from mitochondria and the activation of caspases 3 and 9 that lead to apoptosis promoted by a variety of signals [116,117]. Moreover, 1,25-$(OH)_2D_3$ induces apoptosis in ovarian carcinoma cells by caspase 9 activation [164] and by downregulation of telomerase reverse transcriptase (hTERT) via the induction of *miR-498* [165,166]. Intriguingly, while the aforementioned effects seem to be independent of the *TP53* gene, a study has proposed that mutant p53 protein interacts physically with VDR in breast cancer cells, converting the ligand into an anti-apoptotic agent by mechanisms that remain unclear [167].

In addition, 1,25-$(OH)_2D_3$ and metformin have additive/synergistic antiproliferative and proapoptotic effects in colon carcinoma and other types of cells, which are modulated but not hampered by *TP53* status [168]. Moreover, in an in vitro model developed to evaluate the crosstalk between tumor-associated macrophages and colon carcinoma cells, 1,25-$(OH)_2D_3$ restored the sensitivity of these cells to TRAIL-induced apoptosis by interfering with the release of interleukin (IL)-1β by macrophages [169]. Interestingly, the *TP53* mutation and suppression of *miR-17~92* polycistron are highly toxic in non-small lung cancer cell lines due to the upregulation of VDR signaling [170].

Based on these data, many completed and ongoing studies investigate the antitumor action of the combination of 1,25-$(OH)_2D_3$ and a variety of chemotherapeutic agents (5-fluorouracil, gemcitabine, paclitaxel, imatinib, and cisplatin, among others), inhibitors (of EGFR, HER2, HER4, JAK1/2 tyrosine kinases, estrogen or aromatase) and apoptosis inducers (dexamethasone, trichostatin A and 5-aza-2′-deoxycytidine, among others) in cells and animal models of several types of cancers see [116,119] and references therein. The definitive results of these studies are expected to constitute the foundation for clinical trials.

4.3. Regulation of Autophagy

Autophagy is a process of elimination of cytoplasmic waste materials and dysfunctional organelles that serves as a cytoprotective mechanism but that, when excessive, leads to cell death. vitamin D activates autophagy in many organs in healthy conditions to preserve homeostasis. It can also induce autophagy as protection against cell damage caused by intracellular microbial infection, oxidative stress, inflammation, aging, and cancer [171].

In cancer, VDR ligands trigger autophagic death by inducing crucial genes in several cancer cell types. Thus, 1,25-$(OH)_2D_3$ and its analogues de-repress the key autophagic MAP1LC3B (LC3B) gene and activate 5′-AMP-activated protein kinase (AMPK) via increased cytosolic Ca^{2+} and activation of Ca^{2+}/calmodulin-dependent protein kinase β in breast carcinoma cells [172]. In Kaposi's sarcoma cells [173] and myeloid leukemia cells [174], vitamin D compounds inhibit PI3K/AKT/mTOR signaling and activate Beclin-1-dependent autophagy. 1,25-$(OH)_2D_3$ also induces autophagy through the mTOR pathway in Pfeiffer diffuse large B lymphoma cells [175] and is mediated by activation of DNA damage-inducible transcript 4 (DDIT4), in cutaneous squamous cell carcinoma cells [176]. In addition, a recent study has shown that 1,25-$(OH)_2D_3$ promotes autophagy in acute myeloid leukemia cells by inhibiting miR-17-5p-induced Beclin-1 overexpression [177].

Moreover, 1,25-$(OH)_2D_3$ or EB1089 increase radiation efficiency via promotion of autophagic cell death in a VDR- and p53-dependent fashion in non-small cell lung cancer and breast cancer cells [178–181]. Additionally, synergy between 1,25-$(OH)_2D_3$ and temozolomide in tumor reduction and prolonged survival time has been reported in rat-cultured glioblastoma cells and in an orthotopic xenograft model [182].

4.4. Induction of Cell Differentiation, Inhibition of Epithelial-to-Mesenchymal Transition

Cell differentiation is usually, but not necessarily, linked to an arrest in proliferation, and both processes put a brake on tumorigenesis. Carcinoma is the most frequent type of solid cancer. Carcinomas originate from the transformation of epithelial cells in a process that involves the early loss of two key features of their differentiated phenotype: apical-basal polarity and adhesiveness (cell–cell and cell–extracellular matrix, ECM). Loss of epithelial differentiation results from the acquisition of a cellular program called epithelial-mesenchymal transition (EMT), which implies changes in gene expression, triggered by a group of transcription factors (EMT-TFs: mainly SNAIL1, SNAIL2, ZEB1, ZEB2 and TWIST1). EMT provides tumor cells with features of malignancy such as migratory capacity, stemness and diminished apoptosis that facilitate invasion and metastasis and possibly cause resistance to cytotoxic chemotherapy and radiotherapy, and to immunotherapy [183]. The EMT process is activated by a variety of agents and signals that induce or activate the EMT-TFs, such as TGF-β, Wnt, Notch, and ligands of several receptors with tyrosine kinase activity and cytokine receptors.

1,25-$(OH)_2D_3$ has a prodifferentiation effect on several types of carcinoma cells either by direct upregulation of epithelial genes and/or the repression of key EMT-TFs, as shown in [184,185]. In breast cancer cells, 1,25-$(OH)_2D_3$ promotes the formation of focal adhesion contacts, structures of binding to the ECM, by increasing the expression of several integrins, paxillin and focal adhesion kinase. Additionally, 1,25-$(OH)_2D_3$ reduces the expression of the mesenchymal marker N-cadherin and the myoepithelial proteins P-cadherin, integrins α_6 and β_4, and α-smooth muscle actin, which are associated with more aggressive and lethal forms of human breast cancer [186]. In colon carcinoma cells, 1,25-$(OH)_2D_3$ upregulates an array of intercellular adhesion molecules that are constituents of adherens junctions and tight junctions, including E-cadherin, occludin, claudin-2 and -12, and ZO-1 and -2 [125,131]. As mentioned by JoEllen Welsh in an excellent recent review [187], breast cancer heterogeneity is reflected in available model systems of this disease, including human breast cancer cell lines. These differ in the expression of VDR and other hormone receptors and in their global gene expression profile and phenotype. Consequently, results vary widely in laboratory studies of 1,25-$(OH)_2D_3$ and other VDR ligands, which show a heterogeneous, usually multilevel protective action that affects a variety of pathways (ERBB2/NEU-ERK-AKT, WNT/β-catenin, JAK-STAT, NF-κB, ERα). These studies have rendered only a few genes that are commonly regulated: *CYP24A1*, *CLMN*, *EFTD1* and *SERPINB1*.

Remarkably, the induction of E-cadherin by 1,25-$(OH)_2D_3$ in colon carcinoma cells has been reproduced in tumor cell lines derived from breast, prostate, non-small cell lung, and squamous cell carcinomas, usually associated with an increase in epithelial differentiation [184]. The mechanism of E-cadherin induction by 1,25$(OH)_2D_3$ in human colon cancer cells is transcriptional indirect. It requires transient activation of the RhoA-ROCK-p38MAPK-MSK1 signaling pathway [126]. Phosphatidylinositol 5-phosphate 4-kinase type II β is also needed for E-cadherin induction by 1,25-$(OH)_2D_3$ in these cells [188]. In agreement with the transcriptional regulation, 1,25-$(OH)_2D_3$ treatment causes partial demethylation of CpG sites of *CDH1* promoter in MDA-MB-231 triple-negative breast cancer cells [189]. In addition, 1,25-$(OH)_2D_3$ induces and/or redistributes several cytokeratins, F-actin, vinculin, plectin, filamin A and paxillin that modulate the actin cytoskeleton and the intermediate filament network, changing stress fibers and the ECM binding structures (focal adhesion contacts and hemidesmosomes) [125,126]. In summary, 1,25$(OH)_2D_3$ increases cell–cell and cell-ECM adhesion.

1,25-$(OH)_2D_3$ inhibits SNAIL1 and ZEB1 expression in non-small cell lung carcinoma cells, accompanied by an increase in E-cadherin expression, vimentin downregulation, and maintenance of epithelial morphology [190]. The 1,25-$(OH)_2D_3$ analogue MART-10 inhibits EMT in breast and pancreatic cancer cells through the downregulation of SNAIL1, SNAIL2 and TWIST1 in breast cancer cells [191,192]. 1,25-$(OH)_2D_3$ causes the downregulation of SNAIL1 and SNAIL2 in colon and ovarian carcinoma cells [193,194].

In addition, 1,25-$(OH)_2D_3$ induces several modulators of the epithelial phenotype that can influence the expression of these EMT-TF. Thus, it increases by a transcriptional indirect mechanism the expression of *KDM6B*, a histone H3 lysine 27 demethylase that mediates the induction of a highly adhesive epithelial phenotype in human colon cancer cells [195]. *KDM6B* depletion upregulates SNAIL1, ZEB1, and ZEB2 and increases the expression of mesenchymal markers fibronectin and LEF-1, and claudin-7. Accordingly, *KDM6B* and *SNAI1* RNA expression correlate inversely in samples from human colon cancer patients [195]. Furthermore, 1,25-$(OH)_2D_3$ directly upregulates the expression of cystatin D, which represses SNAIL1, SNAIL2, ZEB1, and ZEB2, and induces the expression of E-cadherin and other adhesion proteins such as occludin and p120-catenin. Accordingly, cystatin D and E-cadherin protein expression directly correlate in colon cancer, and loss of cystatin D is associated with poor tumor differentiation [127]. The *SPRY2* gene encodes SPROUTY-2, a modulator of tyrosine kinase receptor signaling that is strongly repressed by 1,25$(OH)_2D_3$ in colon carcinoma cells [143]. SPROUTY-2 promotes EMT through upregulation of ZEB1 and downregulation of the epithelial splicing regulator ESRP1. Consequently, SPROUTY-2 represses genes that encode E-cadherin, claudin-7, and occludin and the important regulators of the polarized epithelial phenotype LLGL2, PATJ, and ST14 [143,196].

The induction of differentiation seems to be a less important protective mechanism of 1,25-$(OH)_2D_3$ in hematological malignancies than in solid cancers. 1,25-$(OH)_2D_3$ induces differentiation almost exclusively of acute myeloid leukemia cells [197–199]. Thus, 1,25$(OH)_2D_3$ increases the expression of markers of the monocyte-macrophage phenotype such as CD14 and some proteins involved in phagocytosis and adherence to substratum, including CD11b [139,200]. A number of genes and proteins have been proposed as mediators of this prodifferentiation action of 1,25-$(OH)_2D_3$, such as PI3K, *CEBPB*, and *CDKN1A* [201–203]. Differentiation of acute myeloid leukemia cells was also described by the combination of 1,25-$(OH)_2D_3$ with l-asparaginase [204]. Interestingly, a recent study reports that liganded VDR has a strong prodifferentiation effect in acute myeloid leukemia cells harboring mutations in *IDH* gene encoding isocitrate dehydrogenase. This is the case because the oncometabolite 2-hydroxyglutarate that is produced by mutant IDH potentiates VDR signaling in a CEBPα-dependent manner [205]. In addition, prodifferentiation effects of VDR agonists have been reported in follicular non-Hodgkin's lymphoma cells, with increased expression of mature B-cell markers [206].

4.5. Antagonism of Wnt/β-Catenin Signaling Pathway

The Wnt/β-catenin signaling pathway is activated by several members of the Wnt family of secreted proteins (19 in humans) during ontogenesis and adult life, which play important roles in the development and homeostasis of many tissues and organs. The binding of these Wnt factors to plasma membrane co-receptor (Frizzled-LRP) complexes inhibits the degradation of β-catenin protein in the cytoplasm that is promoted by the products of tumor suppressor genes APC and AXIN, which leads to β-catenin accumulation and partial translocation into the cell nucleus. Nuclear β-catenin acts as a transcriptional co-activator of genes bound by the T-cell factor (TCF) family of transcriptional repressors [207]. The long list of β-catenin/TCF target genes includes some that are crucial for cell survival and proliferation (MYC, CCND1), EMT, migration/invasion, and other tumoral processes (Stanford University Wnt homepage: https://web.stanford.edu/group/nusselab/cgi-bin/wnt/) (accessed on 19 March 2022). These genes are active during ontogenesis but remain mostly silent in adult life except in some situations such as wound healing. Recent data suggest that Wnt factors only prime β-catenin signaling. This causes basal activation of the pathway that only becomes fully activated in the presence of R-spondin (RSPO)1–4. Upon binding to their membrane LGR4–6 receptors, the secreted RSPO family members inactivate two E3 ubiquitin ligases (RNF43, ZNRF3) that mediate Frizzled degradation. In this way, RSPOs extend Frizzled half-life at the cell surface and so potentiate Wnt signaling.

The Wnt/β-catenin pathway is an important player in cancer as it is aberrantly activated by mutation (APC, AXIN, CTNNB1/β-catenin, RSPO2/3, and RNF43 genes), overexpression of Wnt factors/receptors or silencing of Wnt signaling inhibitors (DICKKOPF/DKKs, SFRPs) leading to the activation or potentiation of carcinogenesis [208]. This is particularly important in colorectal cancer, as massive sequencing efforts have revealed that the mutation of at least one Wnt/β-catenin pathway gene is present in over 94% of primary tumors and metastases [209,210], while a variable proportion of other cancers (liver, breast, lung and leukemia, among others) also show abnormal pathway activation. Despite its clinical relevance, no inhibitors of the Wnt/β-catenin pathway have been approved up to now.

The first description of the antagonism of the Wnt/β-catenin pathway by 1,25-$(OH)_2D_3$ was reported in colon carcinoma cells by a double mechanism: (a) liganded VDR binds nuclear β-catenin, which hampers the formation of transcriptionally active β-catenin/TCF complexes, and (b) induction E-cadherin expression that attracts newly synthesized β-catenin protein to the plasma membrane adherens junctions. In that way, it decreases β-catenin nuclear accumulation [125]. Other mechanisms of interference of the Wnt/β-catenin signaling pathway by 1,25-$(OH)_2D_3$ have been subsequently described in colon, breast, ovarian, hepatocellular, renal, head, and neck carcinomas, and in Kaposi's sarcoma, see [211]. These mechanisms include the increase in AXIN, TCF4 or DKK1 level, modulation of TLR7, reduction of total or nuclear β-catenin, and enhancement of LRP6 degradation [212–217]. In addition, a paracrine mechanism of Wnt/β-catenin signaling has been proposed based on interruption by 1,25-$(OH)_2D_3$ of the secretion of the Wnt stimulator IL-β by environmental macrophages [218].

4.6. Inhibition of Angiogenesis

1,25-$(OH)_2D_3$ inhibits cancer angiogenesis by acting at two levels: tumor cells and endothelial cells. In diverse types of carcinoma cells (colon, prostate, and breast), the antiangiogenic action of 1,25-$(OH)_2D_3$ relies to a great extent on its ability to inhibit two major angiogenesis promoters: it suppresses the expression and activity of hypoxia-inducible factor (HIF)-1α, a key transcription factor in hypoxia-induced angiogenesis, and of vascular endothelial growth factor (VEGF)-A. Additionally, 1,25-$(OH)_2D_3$ induces the angiogenesis inhibitor thrombospondin-1 [219,220]. In colon tumor cells, modulation of the angiogenic phenotype is also mediated by the control of genes encoding inhibitors of differentiation (ID)-1/2 and by the repression of DKK4, a weak Wnt antagonist that promotes angiogenesis and invasion and is upregulated in colon tumors [219,221]. 1,25-$(OH)_2D_3$ alone and more strongly in combination with cisplatin suppresses VEGF activity in ovarian cancer cells [222]. By modulating VEGF receptor (VEGFR) 2, 1,25-$(OH)_2D_3$ or calcipotriol, it enhances the efficacy of the VEGFR inhibitor Cediranib in malignant melanoma cells [223]. Another antiangiogenic mechanism of 1,25-$(OH)_2D_3$ is the reduction of IL-8 secretion by prostate cancer cells through the inhibition of NF-κB [224]. Intriguingly, variable and sometimes opposite effects of 1,25-$(OH)_2D_3$ on angiogenesis have been reported, as in a xenograft breast cancer model, where it inhibits TSP-1 and increases VEGF expression [225]. Likewise, 1,25-$(OH)_2D_3$ induces VEGF synthesis and action in some non-tumoral cell systems, see [152].

1,25-$(OH)_2D_3$ also has inhibitory effects on tumor-derived endothelial cells. It reduces their proliferation and sprouting in vitro and diminishes the blood vessel density in xenograft tumors in breast, squamous cell carcinoma, bladder and prostate cancer models [226–230].

4.7. Inhibition of Cancer Cell Migration, Invasion and Metastasis

1,25-$(OH)_2D_3$ inhibits the migratory and invasive phenotype of cancer cells as a result of its effects on the cytoskeleton and adhesive properties and on the expression of proteases, protease inhibitors and ECM proteins. To a variable extent, these effects are linked to inhibition of EMT and the TGF-β and Wnt/β-catenin signaling pathways.

As mentioned above, in carcinoma cells, 1,25-$(OH)_2D_3$ induces E-cadherin and other proteins of adhesion structures and modulates actin and intermediate filament networks, which results in increased cell–cell and cell–ECM adhesion [125,186,194,217,231–233]. By promoting intercellular adhesion via upregulation of E-cadherin, 1,25-$(OH)_2D_3$ suppresses prostate cancer cell rolling and adhesion to microvascular endothelial cells, which is a step in extravasation that precedes metastasis [234]. In addition, vitamin D deficiency increases breast cancer metastasis to the lung by enhancing EMT and the CXCL12/CXCR4 chemokine axis [235].

1,25-$(OH)_2D_3$ reduces breast, renal, and prostate carcinoma cell migration and invasion by downregulating the expression and/or activity of N-cadherin, the ECM components tenascin C and periostin, several integrins and metalloproteases (MMP-1, -2, and -9) and serine proteases (plasminogen activator), while it upregulates protease inhibitors and the pro-adhesive actin cytoskeleton adaptor protein PDLIM2 [236–240]. In triple-negative breast cancer cells, 1,25-$(OH)_2D_3$ decreases hyaluronic acid synthesis [241], and inhibits bladder cancer cell migration partially via the induction of miR-101-3p [242]. In pancreatic adenocarcinoma cells, 1,25-$(OH)_2D_3$ ameliorates the pro-invasive action of tumor necrosis factor (TNF)-α by decreasing the expression of miR-221 and increasing that of the tissue inhibitor of metalloproteinase (TIMP)-3 [243].

4.8. Stromal Effects: Cancer-Associated Fibroblasts

Today, the critical role of stroma in the carcinogenic process is clear. Fibroblasts are the main cellular component of tumor stroma (Cancer-Associated Fibroblasts, CAF). This is a heterogeneous cell population of multiple origins (tissue-resident fibroblasts, myeloid precursors, pericytes and adipocytes, among others) and features that is acquired via the change to an "activation phenotype". It is thought to promote cancer invasion, angiogenesis and metastasis; inhibit the immune response; and reduce intratumoral delivery and the activity of chemotherapeutic agents [244,245]. However, the protective effects of CAF have also been described in some systems, and reprogramming their phenotype is accepted as a more advisable strategy than their elimination [246,247]. Early studies showed that VDR agonists have antifibrotic and antitumoral effects by antagonizing TGF-β in the intestine, liver, and pancreas [248–252].

1,25-$(OH)_2D_3$ regulated over one hundred genes in human CAF isolated from tumor biopsies of five breast cancer patients [253]. The induced gene signature reflects an antiproliferative and anti-inflammatory effect of 1,25$(OH)_2D_3$. Importantly, 1,25-$(OH)_2D_3$ inhibits the protumoral action of human colon CAF by reprograming them to a less activated phenotype. Thus, 1,25$(OH)_2D_3$ reduces the capacity of CAF to alter the ECM and their ability to promote the migration of colon carcinoma cells [254]. 1,25-$(OH)_2D_3$ regulates over one thousand genes in colon CAF that are involved in cell adhesion, differentiation and migration, tissue remodeling, blood vessel development, and the inflammatory response. Remarkably, 1,25$(OH)_2D_3$ imposes a gene signature that correlates with a better prognosis for colon cancer patients [254]. Curiously, in contrast to the antagonism reported in colon carcinoma cells, 1,25-$(OH)_2D_3$ and Wnt3A have an additive, partially overlapping effect in colon fibroblasts [255,256]. In line with the results in colon CAF, 1,25$(OH)_2D_3$ decreases the amount of miR-10a-5p found in the exosomes secreted by human pancreatic CAF, which attenuates the promigratory and pro-invasive effects that these CAF exert on pancreatic carcinoma cells [257]. Of note, a recent study reported that calcipotriol promotes an antitumorigenic phenotype of pancreatic CAF by reducing the release of prostaglandin (PG) E_2, IL-6, periostin, and other factors. However, it reduces T-cell-mediated immunity [258]. Clearly, the action of VDR agonists on fibroblasts associated with distinct human cancers is a highly interesting, open line of research.

4.9. Effects on Cancer Stem Cells

Cancer stem cells (CSC) are supposedly a small population of cells present in tumors that are responsible for tumor initiation, growth, malignization, metastasis, and resistance

to therapies. They originate from the mutational and epigenetic alteration of normal stem cells that maintain the homeostasis of tissues in adult life and behave as a source of new functional differentiated cells following injuries or in aging. The characterization and study of CSC present two unresolved problems: (a) the lack of confirmed universal or even tissue-specific markers, and (b) the existence of cell plasticity in tumors that implies differentiation/dedifferentiation processes during tumorigenesis and thus the lack of a stable stem phenotype but, instead, interconversion of stem and non-stem cells.

At present, there are two systems to study CSC: organoid cultures generated by CSC present in patient-derived tumor biopsies and subcultures of established, immortal tumor cell lines enriched in populations of cells expressing putative CSC markers and/or selected by their capacity to grow in suspension. Clearly, fresh, primary organoids are a more valuable system. They are three-dimensional (3D), self-organized multicellular structures generated by normal stem cells or CSC (that allow matched normal and tumor organoids to be obtained from a patient) that grow embedded in an ECM covered by a complex, tissue-specific, usually serum-free medium [259,260]. Organoids recapitulate some of the features of a particular organ or tumor of origin and are quite stable genetically, and thus are considered a better system to study cancer processes than 2D cell lines grown for decades on plastic dishes [261]. $1,25(OH)_2D_3$ profoundly and differentially regulates the gene expression profile of colon cancer patient-derived normal and tumor organoid cultures. $1,25(OH)_2D_3$ induced stemness-related genes (*LGR5*, *SMOC2*, *LRIG1*, and others) in normal but not tumor organoids [262]. In both normal and tumor organoids, $1,25(OH)_2D_3$ reduced cell proliferation and the expression of proliferation and tumorigenesis genes that affected only a few Wnt/β-catenin target genes (*MYC, DKK4*). Importantly, $1,25(OH)_2D_3$ induced some features of epithelial differentiation in tumor organoids cultured in proliferation medium, such as microvilli, adhesion structures, partial chromatin condensation, and increased cytoplasmic organelles. These effects were also observed in rectal tumor organoids [263].

Concordantly, $1,25(OH)_2D_3$-regulated genes were involved in cell proliferation, differentiation, adhesion, and migration in another study using patient-derived colon organoids [264]. Moreover, MDL-811, an allosteric activator of the sirtuin (SIRT)6 deacetylase, reduced cell proliferation in colon carcinoma cell lines and patient-derived organoids and has a synergistic antitumoral effect in combination with vitamin D in $Apc^{min/+}$ mice [265]. However, conflicting data have been found in normal, nontumoral organoids: whereas $1,25\text{-}(OH)_2D_3$ increased the stemness genes and the undifferentiated associated cell phenotype in organoids from healthy colon and rectum tissues of a dozen individuals [262,263], it enhanced the differentiation of organoids established from a benign region of a radical prostatectomy from a single patient [266].

A series of studies have examined the action of VDR agonists on putative breast cancer stem or progenitor cells identified by some markers ($CD44^{hi}/CD24^{low}$ and/or $ADH1^{+}$) that can grow as floating, nonadherent spheres (mammospheres). In these systems, $1,25(OH)_2D_3$ or the BXL1024 analogue reduced the population of putative CSC and the formation of mammospheres and the expression of pluripotency markers (OCT4, KL-4), Notch ligands and target genes, and genes involved in proliferation, EMT, invasion, metastasis, and chemoresistance 32,467,291 [267–269].

Organoids formed by cells isolated from patient-derived xenografts (not obtained directly from human biopsies but on injection and growth in mice) that acquired resistance in vitro to Trastuzumab-emtansine (T-DM1; composed of the humanized monoclonal anti-HER2 antibody Trastuzumab covalently linked to the microtubule-inhibitory agent DMI) constitute an intermediate system to the two discussed above. In this system, two vitamin D analogues (UVB1 and EM1) reduce the formation and growth of organoids [270].

4.10. Effects on the Immune System

$1,25\text{-}(OH)_2D_3$ is an important modulator of the immune system, as reflected by the expression of VDR by almost all types of immune cells [271–273]. $1,25\text{-}(OH)_2D_3$ is an

enhancer of innate immune reactions against infections and tumor cells by activating the responsive cells (macrophages, natural killer (NK) cells, and neutrophils). Conversely, and in line with its accepted anti-inflammatory action (that may contribute to the inhibition of cancers associated with chronic inflammation), 1,25-$(OH)_2D_3$ is commonly presented as a repressor of the adaptive immune reactions by deactivating antigen-presenting cells (induction of tolerogenic dendritic cells) and $CD4^+$ type-1 helper T (Th1) response (production of interferon-γ, IL-1, IL-6, IL-12...), and by promoting the suppressive Th2 and Treg responses (production of IL-10, IL-4, IL-5, IL-13...) [273,274]. Moreover, in macrophages, 1,25-$(OH)_2D_3$ has been proposed to promote a switch from the pro-inflammatory M1 phenotype (producing IL-1β, IL-6, TNF-α, RANKL, COX) towards the anti-inflammatory protumoral M2 phenotype and to reduce the T-cell stimulatory capacity of macrophages [275,276]. This is somehow counterintuitive as it would represent a potential protumoral effect that cannot be easily attributed to a conserved evolutionary agent such as vitamin D. Some other studies discussed below have introduced putative explanations.

Since naïve T-cells express VDR at a very low level that increases only after activation of the T-cell receptor [277], the role of 1,25-$(OH)_2D_3$ may conceivably be related to the late downregulation of the activated adaptive response. This view agrees with the usual description of repressive 1,25-$(OH)_2D_3$ action in experimental settings following overstimulation of the cells, and it may constitute a safety mechanism to prevent undesirable long-lasting immune activation, potentially leading to inflammation or autoimmunity [278,279]. Concordant with this idea and the anticancer action of 1,25-$(OH)_2D_3$, a series of studies have revealed antitumor effects at the level of several types of immune cells.

Interestingly, a study in mice orthotopically implanted with breast tumors has revealed that vitamin D decreases tumor growth and increases the amount of tumor-infiltrating cytolytic CD8+ T-cells, a usual marker of antitumor response. This effect is lost in high-fat diet conditions [280]. Moreover, in pancreatic cancer, 1,25-$(OH)_2D_3$ inhibits the T-cell suppressive function of myeloid-derived suppressor cells [281].

An important mechanism of 1,25-$(OH)_2D_3$ is the inhibition of the NF-κB pathway. In turn, this causes the downregulation of multiple cytokines and their effects [282]. 1,25-$(OH)_2D_3$ inhibits NF–κB at different levels: by inactivating the p65 subunit of the NF-κB complex and upregulating the inhibitor subunit IκB. In addition, 1,25-$(OH)_2D_3$ inhibits the PG-endoperoxide synthase (PTGS-2, also known as COX-2) [283–285]. 1,25$(OH)_2D_3$ reduces the protumorigenic effect of PG E_2 in prostate cancer cells by inhibiting COX-2 and so decreasing the levels of PG E_2 and two PG receptors (EP2 and FP) [286]. Importantly, vitamin D and calcium favorably modulate the balance of expression of COX-2 and 15-hydroxyPG dehydrogenase, its physiological antagonist, in the normal-appearing colorectal mucosa of patients with colorectal adenoma [287]. vitamin D enhances the tumoricidal activity of NK cells and macrophages [288,289]. 1,25-$(OH)_2D_3$ probably has a dual effect of stimulating the differentiation from monocytes to macrophages and their cell killing activity, including antibody-dependent cell cytotoxicity (ADCC). It may later balance these effects by promoting the M1 to M2 phenotypic switch ([279] and references therein). In addition, 1,25-$(OH)_2D_3$ enhances the susceptibility of hematological and solid cancer cells to NK cell cytotoxicity through downregulation of *miR-302c* and *miR-520c* [289].

The potentiation of ADCC of macrophages and NK cells may be a relevant antitumor action of 1,25-$(OH)_2D_3$ in clinical cases, particularly in patients treated with antibodies, of which the major mechanism of action is ADCC. Thus, several studies have shown that vitamin D deficiency impairs the macrophage and/or NK cell-mediated cytotoxicity of Rituximab (anti-CD20) in diffuse large B-cell, follicular, and Burkitt lymphoma patients [288,290,291], and of Cetuximab (anti-EGFR) in colon cancer cell lines [292]. In addition, some evidence of benefit has been observed in breast cancer patients treated with Trastuzumab (anti-HER2) and in melanoma patients treated with Bevacizumab (anti-VEGF) [290,293].

Agents that target programmed death (PD)-1 or its ligand PD-L1 immune checkpoint inhibitors (ICI) have attracted great attention in cancer therapy. Interestingly, 1,25-$(OH)_2D_3$

upregulates PD-L1 in human (but not mouse)-cultured epithelial and immune cells [294], while vitamin D treatment increases PD-1 expression in $CD24^{+}CD25^{+int}$ T-cells in Crohn's disease patients [295] and PD-L1 in epithelial and immune cells in melanoma patients [296]. These data suggest the possibility of combined treatments with VDR agonists and these ICIs, and perhaps others in development.

In conclusion, it is conceivable that 1,25-$(OH)_2D_3$ works as a general homeostatic regulator of the immune system, ensuring an appropriate global defense against challenges like tumors and infections.

4.11. Animal Models

Many studies on animal diet, chemical, genetic, and xenograft models (mainly for colon and breast cancer) have shown the antitumor actions of vitamin D compounds. This in vivo action is difficult to dissect and probably results from a variable combination of mechanisms in the distinct systems that were assayed, including the inhibition of tumor cell growth, EMT, invasiveness, angiogenesis, and metastasis. Importantly, as occurs in cultured cancer cells, vitamin D antitumor action is mostly independent of *TP53* gene status [119,187].

4.12. Systemic Effects: Detoxification and Microbiome

4.12.1. Detoxification

The elimination of xenobiotics or the detoxification process involves chemical modification (phase I reactions: oxidation, hydrolysis, etc.) and subsequent conjugations to water-soluble molecules (phase II reactions) carried out by a large number of enzymes. 1,25-$(OH)_2D_3$ regulates some of these enzymes in the intestine and liver [297]. This may have a positive effect on the prevention of tumorigenesis and perhaps another more controversial impact on the inactivation of chemotherapeutic drugs [298].

1,25-$(OH)_2D_3$ induces CYP3A4, a major human drug-metabolizing enzyme, SULT2A, a phase II sulfotransferase, and members of the multidrug resistance-associated protein (MRP) family in colon carcinoma cells [299,300]. CYP3A4, SULT2A1, and MRP3 are involved in the elimination of lithocholic acid (LCA), a secondary bile acid LCA that induces DNA damage and inhibits DNA repair enzymes in colonic cells. Accordingly, LCA promotes colon cancer in experimental animals, and high levels of LCA have been found in colon cancer patients [301,302]. Interestingly, LCA binds weakly and activates VDR, and so it activates its own degradation [303]. Another example is enhancement by 1,25$(OH)_2D_3$ of the benzo[a]pyrene metabolism via CYP1A1 in macrophages [304].

4.12.2. Microbiome

Alteration of the intestinal microbiome (dysbiosis) is connected to colon cancer and possibly other neoplasias [305]. Many experimental studies in mice have shown that vitamin D deficiency promotes gut permeability, colon mucosa bacterial infiltration, and translocation of intestinal pathogens. These effects lead to changes in immune cell populations and gut inflammation, and cancer—an overall condition that is improved after vitamin D supplementation [306,307]. As bacteria lack VDR, the effect of vitamin D is mediated by the host. Importantly, genome-wide association analysis of the gut microbiome in two large cohorts of individuals identified VDR as a factor that influences the gut microbiota [308]. A conditioned medium from probiotic lactic acid bacteria showed increased expression of VDR and of its target *CAMP* gene encoding cathelicidin in cultured colon carcinoma cells and organoids. It protected against the inflammatory response induced by TNF-α [309]. The protective action against dysbiosis and the intestinal tumorigenesis of liganded VDR have been proposed to be at least partially mediated by the inhibition of the JAK/STAT pathway [310].

4.13. Discussion of Mechanistic Studies

The vast array of effects that 1,25-$(OH)_2D_3$ has in a wide variety of experimental systems of a high number of cancer types agrees with a selected evolutionary role in protection against tumoral processes. The underlying mechanisms include the control of tumor cell survival (autophagy, apoptosis) and phenotype (differentiation), and the inhibition of their proliferation, invasiveness, and metastasis; attenuation of the proliferation and phenotypic features of some CSC; modulation of the physiology of diverse non-tumoral stromal cells (fibroblasts, endothelial cells); and the regulation of several types of immune cells and responses. Table 11 summarizes the references corresponding to key studies focused on the most relevant topics of the anticancer action of vitamin D.

Table 11. vitamin D anticancer mechanisms in experimental model systems. List of key representative references.

Mechanism	Cancer Type Model	References
Inhibition of cell proliferation	Breast, prostate, colon, ovarian, gastric thyroid, hepatocellular, leukemias, lymphomas	[111,119–150,152–156,158,159,161,162]
Induction of differentiation	Leukemia, colon, breast	[112,124–126,138,176,185,187,196–205]
EMT inhibition	Colon, ovarian, breast, pancreas	[126,142,189–195]
Sensitization of autophagy	Colon, prostate, breast, ovarian, lung	[115,116,118,163–165,168,169]
Induction of autophagy	Breast, Kaposi's sarcoma, lymphoma, cutaneous squamous cell carcinoma, leukemia	[171–181]
Wnt/β-catenin antagonism	Colon, breast, ovarian, hepatocellular, renal, head and neck, Kaposi's sarcoma	[124,210–217]
Invasion, angiogenesis, metastasis	Colon, prostate, breast, ovarian, renal, pancreas	[193–216,218–223,230–242]
Cancer-associated fibroblasts	Breast, colon, pancreas, liver	[248,250,252–257]
Normal/cancer stem cells	Breast, colon, pancreas, liver	[261–269]
Detoxification and microbiome	Colon, perhaps other cancer types	[296–303,305–309]
Immune system regulation	Many	[272–288]
Combination with immunotherapy	Lymphoma, melanoma, colon, breast	[289–295]

Together, these effects reflect a multilevel anticancer action of vitamin D. Therefore, an appropriate vitamin D status of the organism should be maintained to minimize the risk and severe consequences of many neoplasias. Further supporting this, the toxicity of vitamin D supplementation is limited, acceptable, and clearly lower than that of current anticancer drugs and therapies. We are not aware of any other natural or synthetic compound that has such an array of antitumor activities combined with low toxicity. Doubtless, the available experimental results meet Koch's postulate for biological causality regarding the existence of a global mechanism of action behind the association between vitamin D deficiency and high incidence and, especially, the mortality of several major cancer types found in observational and epidemiological studies. Hopefully, the further development of current and possibly, novel studies on the wide range of mechanisms of VDR agonists in a variety of biological systems will allow us to elucidate the anticancer action of vitamin D (Figure 3).

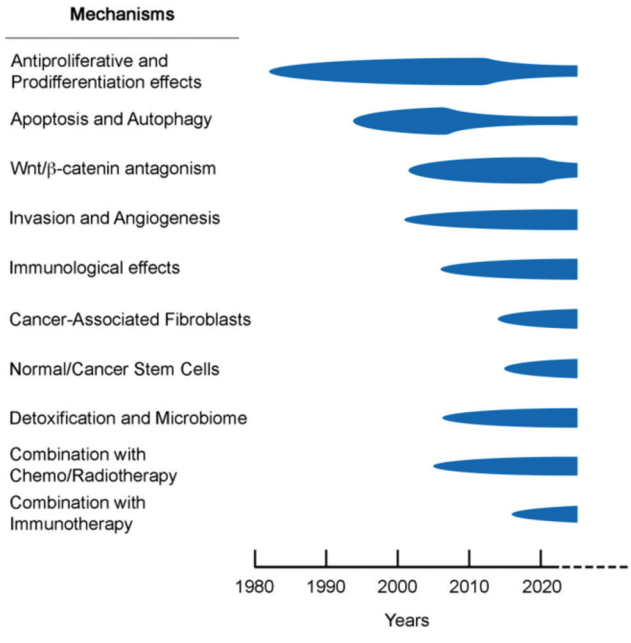

Figure 3. Time flow-chart of studies on the anticancer mechanisms of vitamin D compounds with some key references that are discussed in the text.

5. Outlook

On the basis of this review of ecological and observational studies, it seems that an efficient way to strengthen the links between vitamin D and cancer is to conduct more CC studies of cancer incidence. Such studies would measure 25(OH)D concentration, C-reactive protein, and other relevant factors, as well as obtain the history of UVB exposure, vitamin D supplementation, and dietary sources of vitamin D. The next step is to then find appropriate controls using, perhaps, the propensity score analysis, as done in a study of breast cancer survival with respect to de novo vitamin D supplementation [311]. In addition, care should be taken to investigate the effect of vitamin D supplementation and 25(OH)D concentration on cancer risk for various subgroups based on such factors as age, BMI, diet, ethnicity, geographical location, etc.

Future laboratory research on the anticancer action of vitamin D is desirable to develop a deeper understanding of the individual response to treatment with VDR agonists. To this end, *omics* studies using genomic, epigenomic, transcriptomic, proteomic, and metabolomic approaches must be integrated to understand and foresee personal susceptibility/sensitivity to each compound, which has been defined as "the personal vitamin D response index" [312]. Clearly, the characterization of biomarkers of compound activity and patient response in different cancer types will be important. Since 1,25-$(OH)_2D_3$ regulates the same pathways but distinct genes of them in mice and humans [313], studies should preferentially be carried out in human systems. Among them, it seems that primary cell cultures and organoids should be used instead of classical, long-term established cell lines.

Given the increasingly important role attributed to the stroma in tumorigenesis, the effects of vitamin D compounds on CAF, endothelial cells, and specific types of immune cells require attention. Likewise, the association of chronic inflammation with several types of cancer and the pro-inflammatory action of adipocytes suggest the interest in studying the effects of vitamin D in this context.

Another open field for research is combination therapies. Up until now, experimental studies have focused on the combination of VDR agonists and chemotherapeutic agents,

sometimes with radiotherapy. Obviously, this should be continued and extended to the exponentially growing field of cancer immunotherapies.

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Review

The Effect of Vitamin D and Its Analogs in Ovarian Cancer

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Abstract: Ovarian cancer is one of the deadliest cancers in women, due to its heterogeneity and usually late diagnosis. The current first-line therapies of debulking surgery and intensive chemotherapy cause debilitating side effects. Therefore, there is an unmet medical need to find new and effective therapies with fewer side effects, or adjuvant therapies, which could reduce the necessary doses of chemotherapeutics. Vitamin D is one of the main regulators of serum calcium and phosphorus homeostasis, but it has also anticancer effects. It induces differentiation and apoptosis, reduces proliferation and metastatic potential of cancer cells. However, doses that would be effective against cancer cause hypercalcemia. For this reason, synthetic and less calcemic analogs have been developed and tested in terms of their anticancer effect. The anticancer role of vitamin D is best understood in colorectal, breast, and prostate cancer and much less research has been done in ovarian cancer. In this review, we thus summarize the studies on the role of vitamin D and its analogs in vitro and in vivo in ovarian cancer models.

Keywords: ovarian cancer; vitamin D; vitamin D analogs

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1. Ovarian Cancer

Ovarian cancer (OC) is the seventh deadliest and the eighth most common cancer in women, affecting 313,000 women and causing 207,000 deaths in 2020 (International Agency for Research on Cancer). OC is also called the "silent killer" because it is usually diagnosed at a late stage when the chances of a cure are already very low. Most OCs are diagnosed at stage III (51%) or IV (29%), where the 5-year survival is only 42% or 26%, respectively [1]. See Figure 1 for the most important facts of ovarian cancer. Ovarian tumors arise not only, as previously thought, in tissues of the ovary, but recent data shows that in some cases they can also start in the distal fallopian tube [2]. OC is a highly heterogeneous disease. Heterogeneity is high not only among the different types of ovarian tumors, but also within a single tumor [3]. Based on its origin, OC has seven histological types: epithelial tumors, mesenchymal tumors, mixed epithelial and mesenchymal tumors, sex cord stromal tumors, germ cell tumors miscellaneous tumors, and tumor-like lesions [4]. Around 90% of all ovarian tumors are of epithelial origin [5]. Because of their high heterogeneity, epithelial ovarian carcinomas (EOC) are divided into subgroups by the World Health Organization according to cell type: serous tumors, mucinous tumors, endometroid tumors, clear cell tumors, seromucinous tumors, Brenner tumors, and other carcinomas [4]. According to the International Federation of Gynecology and Obstetrics (FIGO), OC has four stages based on macroscopic and microscopic examination before and after surgery as well as cytology [6]. At stage I and II, the tumor is present mainly in ovaries and fallopian tubes, whereas at stage III it has spread already to local lymph nodes and peritoneum outside the pelvis. At the highest stage IV, distant metastases are present [7].

Treatment of OC can be local or systemic. Selection of the therapy depends on the type and stage of the disease. First-line therapy is usually debulking surgery and chemotherapy with platinum-based compounds and taxanes. There are also other, less common treatments such as radiation therapy, hormone therapy, but also targeted therapy, which is

directed at genes and proteins specific for the cancers. The most common targeted therapy uses Poly(ADP-Ribose)-Polymerase (PARP) inhibitors. Unfortunately, current first-line chemotherapeutic treatments cause serious side effects such as dizziness, fatigue, nausea, vomiting, and diarrhea. That is why there is an urgent need to develop new adjuvant or curative therapeutic approaches that would decrease the required dose or duration of the classical chemotherapy, thus reducing side effect severity.

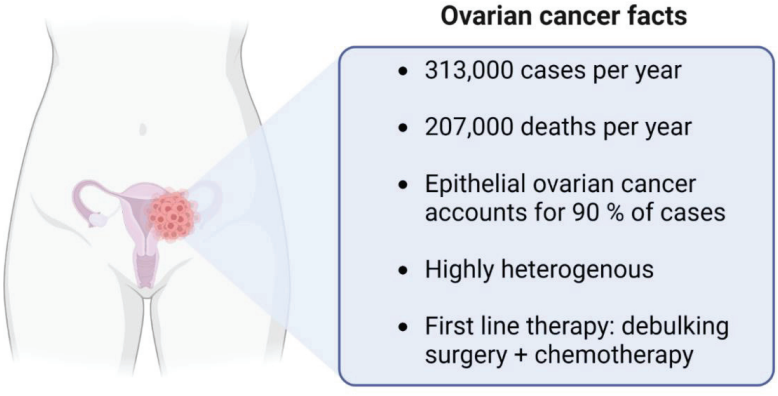

Figure 1. Ovarian cancer facts.

2. Vitamin D and Vitamin D Analogs

Ultraviolet rays transform 7-dehydrocholesterol in the skin to cholecalciferol, or vitamin D3 [8]. Vitamin D is then transported to the liver where it is hydroxylated on position 25 to the main circulating metabolite 25-hydroxyvitamin D3 (25D3) by 25-hydroxylase encoded by the gene *CYP2R1*. 25D3, bound to the vitamin D binding protein, is transported to the target tissues, where it is hydroxylated on the position 1α to the main active hormone 1,25-dihydroxyvitamin D3, or calcitriol (1,25D3), by the rate-limiting enzyme 1α-hydroxylase, coded by the *CYP27B1* gene [8]. The kidneys are the main site for this hydroxylation step, but many other tissues express *CYP27B1* and thus are able to synthetize the active hormone 1,25D3. Both 25D3 and 1,25D3 are degraded by the enzyme 24-hydroxylase, encoded by *CYP24A1* [8]. The main role of 1,25D3, bound to its receptor, the transcription factor vitamin D receptor (VDR), is the regulation of serum calcium and phosphorus homeostasis, but it also regulates the expression of many genes that might play a role in the development of cancer [9]. The main indicator of the organism's vitamin D status is serum 25D3, although the optimal status is still a subject of debate. Levels <12 ng/mL are considered as severe vitamin D deficiency, while sufficiency varies between 20-30 ng/mL, depending on the expert bodies or societies [10–12].

The main function of 1,25D3 is to maintain the proper levels of calcium and phosphorus in serum. Thus, higher doses of 1,25D3, which would be effective against cancer, can cause hypercalcemia as a side effect. For this reason, various synthetic vitamin D analogs have been developed (representative structures of parent compounds and analogs are shown in Figure 2). MT19c is a vitamin D2 (the plant-derived ergocalciferol) derivate, created by Diels–Alder cycloaddition of *N*-methyl,1,2,4-triazolinedione and esterification with bromoacetic acid [13]. This last modification is shared with the analog B3Cd, which is a 3-bromoacetoxy derivative of 25D3 [14]. EB1089 was one of the first developed vitamin D analogs. It is based on the 1,25D3 structure by elongating the side chain by one carbon and the introduction of terminal ethyl groups and introduction of double bonds at positions 22 and 24 [15]. The PRI-1906 and PRI-1907 analogs were designed based on the 1,25-hydroxylated form (1,25D2) of the plant-derived vitamin D2 (ergocalciferol), with an extended side chain and introduced double bonds. PRI-1906 carries terminal methyl and

PRI-1907 ethyl groups [16]. The analogs PRI-5201 and PRI-5202 are based on PRI-1906 and PRI-1907 by removing the methylidene group of the A-ring [17].

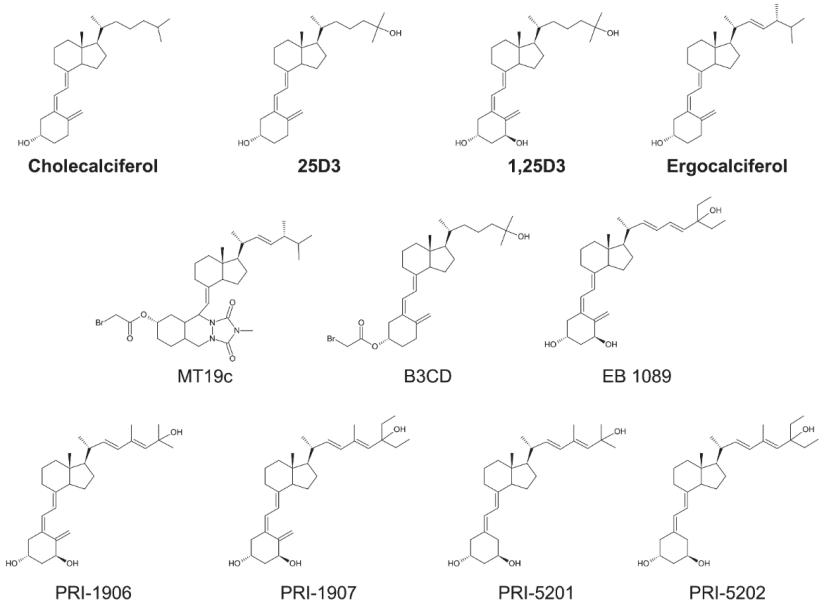

Figure 2. Chemical structures of the most important parent compounds (in bold) and the vitamin D analogs mentioned in the review.

3. Effect of Vitamin D on Ovarian Cancer Epidemiology

Several recent studies reviewed the anticancer effects of different vitamins on selected female malignancies [18–20]; in our review, we focus only on the effect of vitamin D in ovarian cancer. The role of vitamin D levels in epidemiology of OC is still unclear. One of the first studies on the correlation between vitamin D and cancer found that sunlight can be a protective factor against OC-associated mortality [21]. A report from Australia concluded that exposure to ambient ultraviolet radiation may reduce the risk of EOC [22]. In another cohort study from Australia, researchers have shown that higher 25D3 serum levels at the stage of diagnosis correlated significantly with longer survival of women with diagnosed invasive OC [23]. In a European population, a Mendelian randomized study found that genetically lower 25D3 levels correlated inversely with higher susceptibility to OC [24]. Additionally, predicted higher concentrations of 25D3 (based on GWAS studies) were associated with reduced risk of EOC [25]. However, current data on this topic are inconsistent. In another Mendelian randomized study, researchers concluded that vitamin D levels had no effect in seven cancers, including OC [26]. A further study found that genetically low plasma 25D3 concentrations were not associated with increased cancer risk and mortality rates [27]. Similarly, a study conducted in African women from Nigeria found no significant correlation between serum 25D3 levels and the risk of EOC [28]. A meta-analysis of 21 articles with almost one million participants concluded that vitamin D intake could not decrease the risk of OC [29].

4. Mechanism of Anticancer Activity in Ovarian Cancer Models In Vitro

The heterogeneity of OC is mirrored by the diversity of the existing cell lines. Even those cell lines that were obtained from the same type of tumors show high diversity in their sensitivity to vitamin D and its analogs [30]. This might be due to differences in their mutational landscape, but also in the expression of the components of the vitamin D system.

While VDR is present in most of the known cell lines, the expression is highly variable, as is also the expression of *CYP27B1* and *CYP24A1*. The results comparing the expression level of VDR in normal and malignant ovarian tissues are inconsistent. One study reported higher levels in ovarian tumors compared with healthy tissue [31], while others found that the VDR level was lower in tumors than in normal ovaries [32]. Treatment of OC cell lines with 1,25D3 or its analogs has no effect on VDR mRNA levels, while it might increase protein levels in a cell line-dependent manner [30,33]. Interestingly, the effect of 1,25D3 or its analogs on the expression of *CYP24A1* in different OC cell lines does not predict their anticancer effect [30].

4.1. Effect of 1,25D3 and Its Analogs on the Hallmarks of Cancer

Besides its major role to maintain calcium-phosphate homeostasis, 1,25D3 regulates most hallmarks of cancer. It inhibits proliferation, angiogenesis, and metastasis, induces differentiation and apoptosis, and regulates the immune system [34,35]. The anticancer effects of 1,25D3 were best documented for colorectal, breast, and prostate cancer [36–39]. Much less is known about the effect of 1,25D3 and its analogs in OC cells (Figure 3).

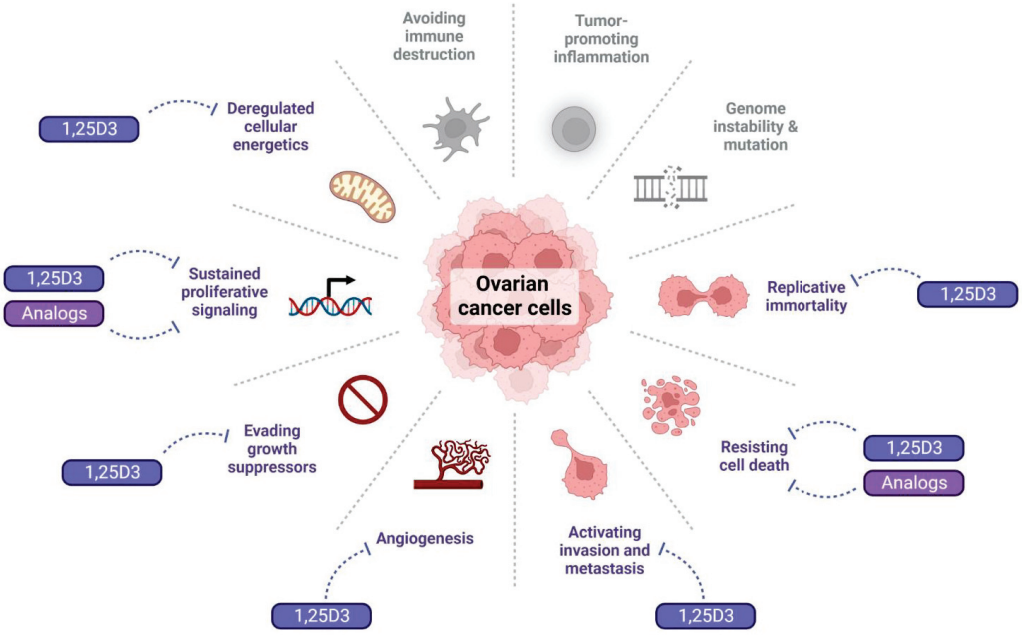

Figure 3. Impact of 1,25D3 and its analogs on the hallmarks of cancer in OC cells. In color, the hallmarks affected by 1,25D3 (see main text below), in gray, those where no relevant studies were found. The dotted blunt arrows indicate inhibition.

4.2. Effect of 1,25D3 and Its Analogs on Proliferative Signals

1,25D3 inhibited proliferation by reducing the cell number of a patient-derived high grade serous ovarian cancer (HGSOC) cell line, while no effect was seen in a further HGSOC line [30]. In OVCAR3 cells, 1,25D3 induced cell cycle arrest either at the G1/S or G2/M checkpoint [40,41]. The expression of several genes involved in the cell cycle was also inhibited in some (e.g., OVCAR3, CAOV3, OV2008) but not in other cell lines (e.g., OVCAR5, SKOV3). Interestingly, another study found that SKOV3 cells were sensitive to 1,25D3, which reduced both their proliferation and viability [42]. In OVCAR3 cells, 1,25D3 prevented cell cycle progression through the inhibition of the CKD2-Rb-E2F axis. In these

cells, 1,25D3 increased p27 and decreased cyclin E and A expression [40]. Proliferation of OVCAR3 and SKOV3 cells was inhibited also by the 25D3 analog B3CD [14].

Epidermal growth factor receptor (EGFR) is often upregulated in OC, conveying a proliferative advantage to these tumors [43,44]. In vitamin D-sensitive OC cells, 1,25D3 downregulated EGFR expression, reducing their proliferative potential [41]. The OVCAR3 cells were also sensitive to the 1,25D3 analog, EB1089, which was more active in reducing EGFR expression than 1,25D3 [41]. The relevance of the impact of vitamin D on EGFR is underlined also by the fact that EGFR seems to be one of the key genes associated with resistance to platinum therapy of OC [44].

We also observed that different HGSOC cell lines responded differently to various analogs of 1,25D2. While all tested analogs reduced cell number and viability of 13,781 cells, in the 14,433 and 8714 cells, none of the analogs affected cell viability significantly ([30] and unpublished data).

4.3. Effect of 1,25D3 and Its Analogs on Cell Death

Very often, p53 is mutated in ovarian tumors, reducing the apoptotic ability of these cells. Therefore, finding compounds that would induce apoptosis even in the presence of a mutated p53 is of utmost importance. Interestingly, 1,25D3 and the EB1089 analog were able to stimulate apoptosis through p53-independent ways, by upregulating Growth Arrest and DNA Damage-inducible 45 (GADD45), or the cyclin-dependent kinases p21 and p27 [45]. The pro-apoptotic role of GADD45 proteins is well documented and they regulate many cellular functions, e.g., DNA repair, cell cycle, and senescence [46]. In cell lines from clear cell ovarian carcinoma (ES-2, TOV-21G), papillary serous adenocarcinoma (OV-90) and endometrioid carcinoma (TOV-112D), 1,25D3 activated the intrinsic apoptotic pathway by reducing the membrane potential of the mitochondria, increasing cytochrome C release, and activating caspase 9 [47]. In the cell lines that expressed the progesterone receptor, the effect of 1,25D3 was significantly higher when given together with progesterone [48].

1,25D3 increased sensitivity of the ovarian epithelial adenocarcinoma cell line SKOV3 to radiation-induced apoptosis by supporting the formation of reactive oxygen species (ROS) [49]. Another study has shown that, in SKOV3 cells, 1,25D3 induced apoptosis and potentiated the cytotoxic effect of cisplatin, increasing the activity of caspase 3/7. It also increased expression of the pro-apoptotic protein Bax and the cleaved PARP in a dose-dependent manner [42]. B3CD induced apoptosis by activating the p38 MAPK pathway [14].

4.4. Effect of 1,25D3 and Its Analogs on Metastatic Potential

Epithelial to mesenchymal transition (EMT) is considered as the driver of invasion and metastasis. 1,25D3 inhibited EMT in SCOV3 cancer cells [50]. One of the first organs OC disseminates to is the peritoneum [51]. Vitamin D prevented the TGF-β-induced mesenchymal transition and thus the transformation of the peritoneal mesothelial cells into cancer-associated mesothelial cells (CAM) by maintaining high e-cadherin levels and blocking the upregulation of the EMT-associated markers α-smooth muscle actin, slug and the matrix metalloproteinases (MMP) 9 and 2, and that of thrombospondin-1, a gene involved in both the TGF-β and focal adhesion pathways [52]. Treatment of the germline-derived immortalized ovarian cancer cell line A2780 with 1,25D3 inhibited migration of the cells and their adhesion to fibronectin. Pre-treatment of the cells with 1,25D3 also reduced their metastatic potential when injected in immunodeficient mice [53].

Several long noncoding RNAs, e.g., lnc-BCAS1-4_1, play an important role in the regulation of EMT by 1,25D3 [54]. OC patients with high levels of the lncRNA *TOPORS Antisense RNA 1* (*TOPORS-AS1*) in their tumors had favorable overall survival compared with those expressing low levels. As *TOPORS-AS1* is a target for VDR, it has been suggested that the inhibitory effect of VDR in ovarian cancer cells could be mediated through *TOPORS-AS1* [55].

In a recent study, 1,25D3 inhibited the self-renewal capacity of ovarian cancer stem cells (CSC) by reduction of their sphere formation rate and inhibition of the expression of stem cell markers, such as CD44, SOX2, or OCT4 [56].

4.5. Effect of 1,25D3 and Its Analogs on Replicative Immortality and Angiogenesis

In the OVCAR3 cells, 1,25D3 inhibited a further hallmark of cancer, the replicative immortality, by downregulating activity and expression of the telomerase [57]. One mechanism by which 1,25D3 regulated telomerase expression in these cells was the upregulation of miR-498, which then degrades the telomerase mRNA, leading to their apoptotic cell death [58].

In SKOV3, 1,25D3 inhibited VEGF expression and activity and enhanced the anti-angiogenic effect of cisplatin [42].

There is not enough information on the role that vitamin D could play in regulating other hallmarks. Although it is known that vitamin D affects the immune system and plays an important role in inflammation, little is known about its effect on these hallmarks in OC. One study has shown that 1,25D3 was able to reduce the tumor promoting effect of M2 macrophages in ovarian cancer cells [59]. Another interesting finding was that the vitamin D target human cathelicidin, well known as an effector molecule of the innate immune system, promotes OC progression. It seems that OC cells induce the expression of cathelicidin in macrophages in a VDR-dependent manner [60]. This would suggest a detrimental effect of vitamin D on OC development.

5. Mechanism of Anticancer Activity in Ovarian Cancer Models In Vivo

The anticancer activity of 1,25D3 and its analogs in OC models has already been studied broadly in vitro but there are only a few studies about their effects in vivo. Figure 4 summarizes the observed effects found in in vivo studies.

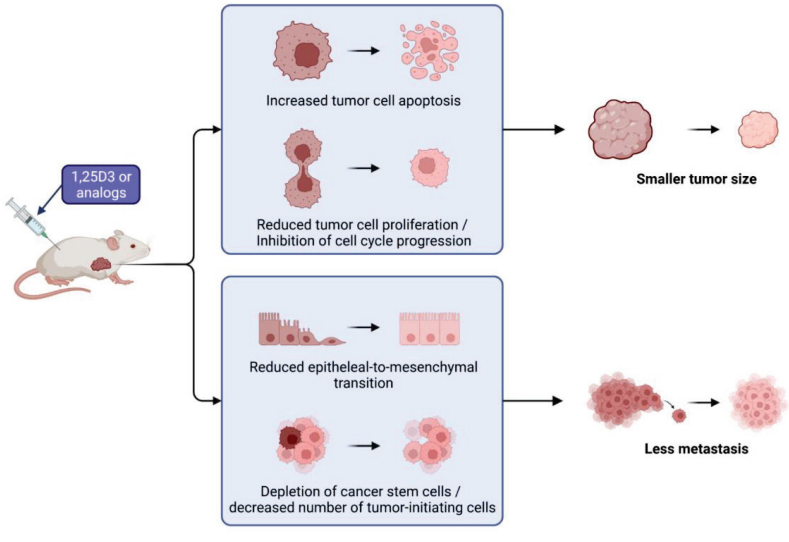

Figure 4. Effects of 1,25D3 and its analogs in in vivo models of ovarian cancer.

In a mouse model of peritoneal metastasis, vitamin D3 protected the microvilli on the peritoneum, thus preventing the interaction of CAMs with cancer cells by inhibiting Smad-dependent TGF-β signaling, thus inhibiting peritoneal dissemination of the ES-2 OC cells [52].

Vitamin D affects at different stages of the carcinogenesis process. In a mouse model of OC, induced by 7,12-dimethylbenz[a]anthracene (DMBA), 1,25D3 administration reduced

tumor size significantly at the stage of initiation, promotion and entire period of the experiment. The general condition of the mice in the treated groups was significantly better than in the untreated controls [61]. Mice treated with vitamin 1,25D3 also had lower levels of CA125, which is considered a potential ovarian tumor marker [61,62].

1,25D3 as an anticancer agent is known to also regulate CSCs. Srivastava et al. studied the effect of 1,25D3 on OC stem cells in vivo in a mouse xenograft tumor model using the 2008 cell line. 1,25D3 delayed tumor growth and depleted the ovarian CSCs. The effect on the CSCs was studied in isolated xenograft tumor cells by measuring CD44 and CD117 positive cells, which are markers for stem cells. 1,25D3 significantly reduced the CSC population in ovarian xenografts in vivo [63].

Only a few analogs of vitamin D3 or 25D3 were tested in vivo in ovarian cancer models. MT19c reduced tumor growth in the SKOV3 xenograft model in nude mice and in a syngeneic rat ovarian cancer models and decreased the expression of genes involved in energy metabolism. The treatment reduced the expression of EGFR, and inhibited PI-3 kinase [13]. The 25D3 derivative B3CD was tested on the SKOV-3 xenograft model. The compound delayed tumor growth in the majority of the mice, and in some cases even led to full regression. However, in some of the mice, B3CD accelerated tumor growth [14]. The study with the 1,25D3 analog EB 1089 found that EB 1089 suppressed the growth of OVCAR3 tumor xenografts in mice. Histological analysis of tumor sections showed that EB 1089 induced apoptosis and decreased proliferation in the tumor [45].

More in vivo studies are needed to understand if vitamin D analogs should be carried further into clinical trials.

6. Future Perspectives

The impact of vitamin D and its analogs on OC is still unclear, although the in vitro studies are promising. Further to the research we summarized, a few studies reported that 1,25D3 is able to potentiate the effect of some of the chemotherapeutics used in the treatment of OC. 1,25D3 increased the cytotoxic effect of carboplatin and paclitaxel in serous-, mucinous-, and endometrioid-type OC cell lines [33]. Although it has been shown that 1,25D3 is a PARP inhibitor [64], the studies to test if it would potentiate the effect of the PARP inhibitors used in OC treatment are still missing. PARP inhibitors are used for the therapy of recurrent OC, however, the majority of the patients develop resistance, mediated by cancer stem cells [65]. As 1,25D3 was shown to reduce the number of CSCs in OC [63], this suggests a high clinical potential in preventing resistance to this class of drugs. Therefore, it would be of utmost importance to understand what makes OC cells responsive or resistant to the anticancer effects of vitamin D and its analogs. More needs to be done especially in vivo to gain a clear picture about the impact and potential application of vitamin D and its less calcemic analogs in OC.

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Review

Vitamin D Derivatives in Acute Myeloid Leukemia: The Matter of Selecting the Right Targets

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Abstract: Acute myeloid leukemia (AML) is an aggressive and often fatal hematopoietic malignancy. A very attractive way to treat myeloid leukemia, called "differentiation therapy", was proposed when in vitro studies showed that some compounds are capable of inducing differentiation of AML cell lines. One of the differentiation-inducing agents, all-*trans*-retinoic acid (ATRA), which can induce granulocytic differentiation in AML cell lines, has been introduced into clinics to treat patients with acute promyelocytic leukemia (APL) in which a PML-RARA fusion protein is generated by a chromosomal translocation. ATRA has greatly improved the treatment of APL. Since 1,25-dihydroxyvitamin D (1,25D) is capable of inducing monocytic differentiation of leukemic cells, the idea of treating other AMLs with vitamin D analogs was widely accepted. However, early clinical trials in which cancer patients were treated either with 1,25D or with analogs did not lead to conclusive results. Recent results have shown that AML types with certain mutations, such as isocitrate dehydrogenase (IDH) mutations, may be the right targets for differentiation therapy using 1,25D, due to upregulation of vitamin D receptor (VDR) pathway.

Keywords: acute myeloid leukemia; blast; 1,25-dihydroxyvitamin D; analogs; all-*trans*-retinoic acid; differentiation; immunomodulation

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1. Introduction

Acute myeloid leukemia (AML) is a malignancy of the myeloid blood lineage, characterized by the rapid growth of abnormal cells (blasts) in the bone marrow. The blast cells overgrow bone marrow, preventing normal blood cell production, and expanding to circulation, where they are unable to function properly. Since leukocytes produced in bone marrow belong to the immune system, every AML is accompanied by an immune deficiency resulting in vulnerability to infections. In addition, inability to produce appropriate amounts of red blood cells and platelets results in anemia and bleeding [1].

The primary goal in the treatment of AML is an elimination of leukemic blasts. However, chemotherapy blocks not only the proliferation of blasts, but also the proliferation of immune cells, an essential step in immune cells' activation. Therefore, chemotherapy-induced immunodeficiency adds to leukemia-induced immunodeficiency [2].

AML is a relatively rare disease which constitutes about 1% of all malignancies. It is a disease common in elderly people and very rare in children, with about 25% of cases diagnosed among adults aged 65–74 years and 34% among these aged 75 and older [3]. AML is the most heterogeneous hematologic malignancy with about 200 known underlying mutations [4]. For more than 40 years, all AML patients have been treated using standard intensive chemotherapy, combining anthracycline and cytarabine. For patients who responded with complete remission after intensive chemotherapy, stem cell transplantation was their treatment of choice [3]. However, it should be remembered that most AML patients are elderly and not fit for either intensive chemotherapy or stem cell transplantation. Understanding disease heterogeneity has allowed for the development of lower-intensity and more targeted treatments for elderly patients who are unfit for intensive treatments [3].

Leukemic blasts are inhibited in their differentiation by either genetic abnormalities or by gene-expression anomalies. These cells do not express the proteins important for the function of their normal counterparts. Therefore, finding a method of forced differentiation of leukemic blasts seemed to be a particularly attractive solution for AML patients. Differentiation therapy is based on forced transcription of the genes that are crucial for the function of normal counterparts to leukemic blasts. This concept has been based on the findings concerning normal hematopoiesis, where the eventual cell fate is governed by spatiotemporal fluctuations in transcription factor concentrations, which either cooperate or compete in driving target-gene expression [5]. Some of these transcription factors have critical roles in lineage selection [6], while others govern cell cycle exit and expression of lineage-specific genes [7]. There are several reasons why transcription factors in leukemic blasts do not operate properly: one of them may be epigenetic silencing of the gene, while the others are mutations [8,9]. The general idea of this type of therapy is presented in Figure 1.

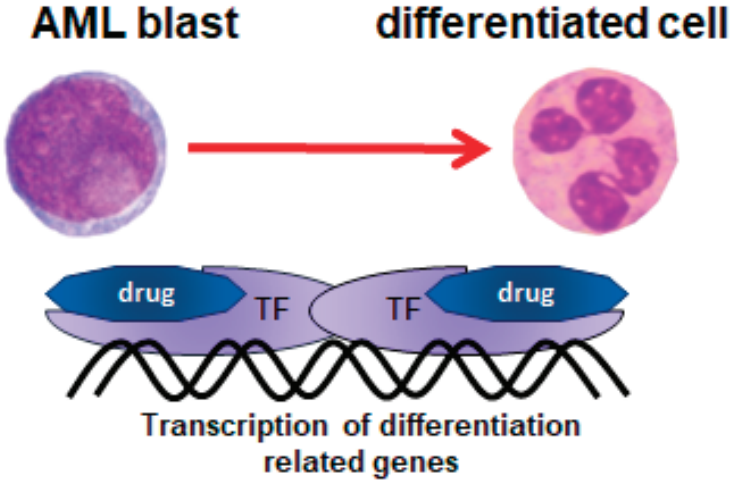

Figure 1. The general idea of differentiation therapy. AML—acute myeloid leukemia; TF—transcription factor.

2. All-*trans*-Retinoic Acid (ATRA)

Acute promyelocytic leukemia (APL) is a subtype of AML characterized by uncontrolled expansion of blasts, which are blocked at the promyelocytic stage of hematopoiesis. Cytogenetically, APL is characterized by a translocation between the long arms of chromosomes 15 and 17 [t(15;17)]. This aberration leads to the fusion between the promyelocytic leukemia gene (*PML*) located on chromosome 15q21, and the retinoic acid receptor α gene (*RARA*) from chromosome 17q21, forming the chimeric oncogene *PML-RARA* [10]. In its first description in 1957, APL was considered to be the most malignant form of AML, accompanied by severe bleeding and very short survival time [11]. Retinoic acid receptor α (RARα) is a nuclear receptor activated by two metabolites of retinoic acid (RA): all-*trans*-RA (ATRA) or 9-*cis*-RA. When dimerized with a retinoid X receptor α (RXRα), it binds to response elements located in the promoters of target genes, activating their transcription. In the absence of the ligand, RARα/RXRα induces chromatin condensation and repression of transcription [12]. Activated RARα/RXRα regulates many genes crucial for myeloid differentiation, for example these encoding transcription factors PU.1 and CCAAT/enhancer-binding proteins α and ε (C/EBPα and C/EBPε) [13–15].

Fusion protein in APL contains the *N*-terminal part of PML protein and the C-terminal part of RARα, and in terms of function it influences transcription. ATRA at physiological concentrations is unable to release complexes of co-repressors from PML-RARα, leading to

transcription blockade [16]. It has been noticed, however, that supra-physiological concentrations of ATRA are able to cause the exchange of co-repressors to co-activators, activating the transcription of genes responsible for granulocytic differentiation [17]. Importantly, the blasts lose their immortality following differentiation processes, and start to die by apoptosis [18]. In fact, surprisingly, the very first demonstration that ATRA is capable of inducing granulocytic differentiation was in using HL60 cell line, which is not an APL subtype [19]. However, in clinical situations only patients who have the t(15;17) mutation respond to ATRA treatment, which was reported for the first time in 1988 [20]. Despite experiencing rapid remission when treated with ATRA alone, the patients suffered from relapse within 6 months. Arsenic trioxide (ATO) used in the patients who relapsed after initial treatment with ATRA had significantly improved results [21,22]. The mechanisms of beneficial action of ATO in APL are SUMOylation, ubiquitination, and eventual degradation of the PML part of the fusion protein [23]. Most of the current protocols combine ATRA, ATO, and cytostatics, such as cytarabine or idarubicin. Using these protocols, complete remission (CR) can be achieved in 90–100% of patients, while overall survival (OS) rates can be achieved in 86–97% of patients [24]. This highlights the great success of differentiation therapy, indicating that the proper combinations of drugs with complementing mechanisms of action are needed.

There were many attempts to widen the success of ATRA therapy beyond APL subtypes of AML. There were some clinical trials in which ATRA was added to chemotherapy [25]. Analysis of one trial suggested that the beneficial effects of ATRA were restricted to the subgroup of patients with a mutated nucleophosmin 1 (NPM1) gene, and without fms-like tyrosine kinase 3 (FLT3)-internal tandem duplication (ITD) [26]. Unfortunately, in other trials this beneficial effect was not observed [27,28]. In fact, in some cases ATRA may even worsen the patient's situation, as it was in the case of the patient with t(4;15)(q31;q22) translocation, resulting in the expression of the TMEM154-RASGRF1 fusion protein. This patient was treated with ATRA and died from rapid disease progression, which was related to ATRA-induced activation of RARγ, a RAR isoform responsible for hematopoietic stem cell renewal and proliferation [29].

3. 1,25-Dihydroxyvitamin D_3 (1,25D)

The possibility to use 1,25-dihydroxyvitamin D_3 (1,25D) in differentiation therapy originated from a study published in 1981, where mouse myeloid leukemia cells exposed in culture to 1,25D were induced to differentiate into functional macrophages [30]. This discovery was extended to human HL60 cells soon after [31,32]. The beneficial actions of 1,25D against AML were also presented in mouse models of this disease [33,34].

The idea to use 1,25D against cancers originated from epidemiological studies. These studies indicated an association between an increased risk of developing colorectal cancer and a low level of 25D in the blood [35,36], as well as an increased risk of developing breast cancer and a low blood level of 25D [37,38]. The role of 1,25D in solid cancers has been discussed in a detailed manner in another paper from this Special Issue [39].

1,25D is an active metabolite of vitamin D, which, despite being named a "vitamin", is a steroid hormone [40]. It is produced by the human body from cholesterol and, similarly to other steroid hormones, its effective concentration is strictly regulated by feedback mechanisms. Vitamin D is produced from 7-dehydrocholesterol in human skin when exposed to UV light. Activation of vitamin D is controlled by cytochrome P450 mixed-function oxidases (CYPs) and occurs in two steps: 25-hydroxylation followed by 1α-hydroxylation [41]. The first stage of activation occurs in the liver, where vitamin D undergoes enzymatic hydroxylation by 25-hydroxylase (CYP2R1/CYP27A1), converting it to 25-hydroxyvitamin D (25D). Then, 25D is transported to the kidneys, where it undergoes further hydroxylation at C-1 by 1α-hydroxylase (CY27B1) and results in the formation of the active metabolite, 1,25D. Hydroxylation of 1,25D at carbon atom C-24, catalyzed by 24-hydroxylase of 1,25D (CYP24A1), is the first step of its inactivation. Since the gene encoding CYP24A1 is the most strongly upregulated 1,25D target, it provides negative

feedback to the activity of 1,25D and controls the effective concentration of this highly active compound [42]. The metabolism of vitamin D is presented in Figure 2.

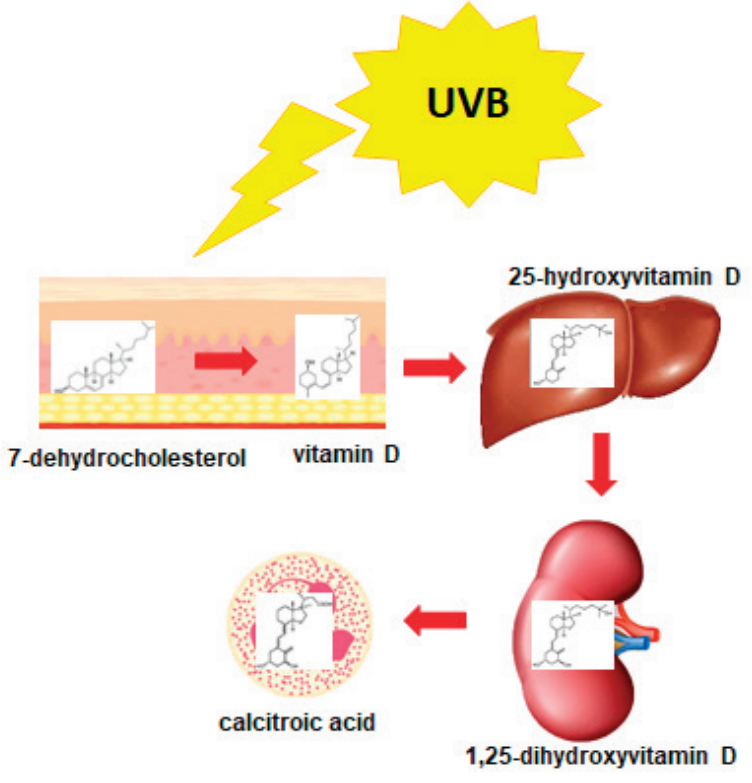

Figure 2. Vitamin D metabolism. Vitamin D is produced in human skin from 7-dehydrocholesterol following exposure to UVB. Then, vitamin D undergoes two hydroxylations: at C-25 in the liver by 25-hydroxylase, and at C-1 in the kidneys by 1α-hydroxylase. Degradation of 1,25-dihydroxyvitamin D (1,25D) into inactive metabolite (calcitroic acid) occurs by hydroxylation at C-24 by 24-hydroxylase in all cells which express vitamin D receptor (VDR).

The major and most well known role of 1,25D is to maintain the calcium phosphate homeostasis of the organism [43], but it is well-documented that 1,25D regulates other vital processes, such as differentiation and proliferation of the cells [40]. The vitamin D receptor (VDR), similarly to RARα, is the nuclear receptor which after binding its ligand translocates to the cell nucleus, where it acts as a ligand-activated transcription factor. VDR, after binding 1,25D, heterodimerizes with RXRα in order to regulate transcription of target genes [44]. There are hundreds of VDR-regulated genes [45], many of them responsible for maintaining calcium phosphate homeostasis [43]; however, there are also many genes involved in immune functions, exemplified by CD14, encoding a macrophage co-receptor for bacterial LPS [46]. The overview of 1,25D/VDR intracellular pathway is presented in Figure 3.

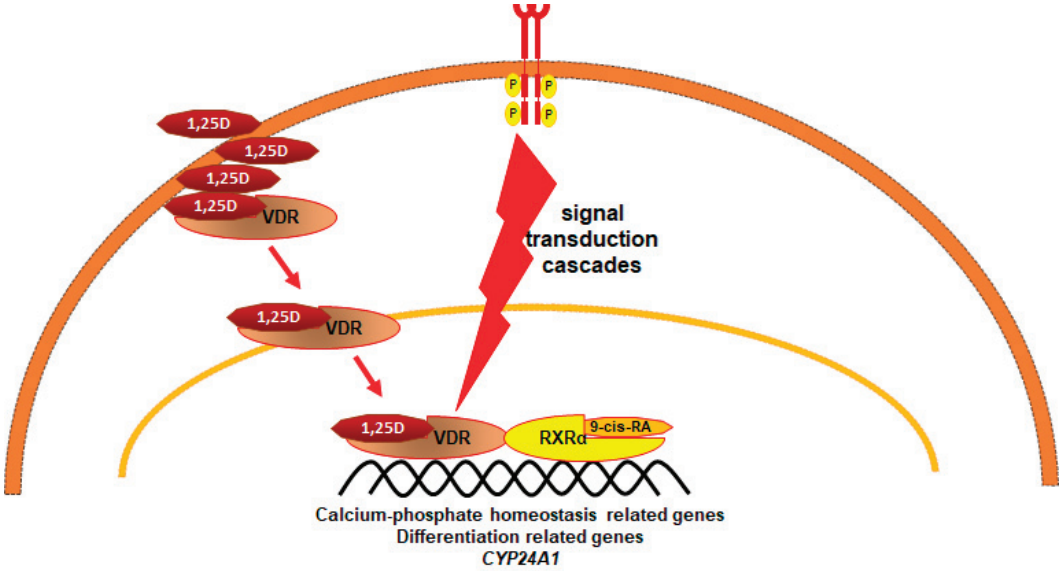

Figure 3. Vitamin D receptor (VDR) pathway. 1,25D translocates through the plasma membrane and binds to its receptor in the cytosol. Ligated VDR is transported to the cell nucleus, where it dimerizes with RXRα. VDR/RXRα complex binds to response elements in the DNA to regulate transcription of target genes. Signal transduction from membrane receptors participates in the activity and stability of VDR [47–49].

Encouraging results of in vitro and murine studies prompted some clinical trials conducted with small groups of patients with myelodysplastic syndrome (MDS) and AML [50,51]. In these trials either 1,25D or its precursor 25D were used, but results were variable and inconclusive. In general, combination treatments resulted in better outcomes than 1,25D alone [52,53]. For example, the combination of 1,25D, AraC, and hydroxyurea resulted in complete or partial responses in 79% of patients with AML [54].

4. Low-Calcemic Analogs of 1,25D

One of the problems with therapeutic uses of 1,25D is its calcemic action and possible consequences of hypercalcemia [55]. In fact, in some of the very few clinical trials in which 1,25D was used against MDS, patients suffered from hypercalcemia [56,57]. The symptoms of hypercalcemia might vary from mild to severe, such as nausea, fatigue, loss of appetite, arrhythmia, kidney failure, calcification of soft tissues, and decalcification of bones [58]. This problem may be overcome by use of low-calcemic analogs which are available from many laboratories [59].

Many analogs of 1,25D have been synthesized with intention to split its activities. The idea was to reduce calcemic actions and retain pro-differentiating activities. Despite the fact that numerous analogs have been available for over 30 years, it is still not clear how the split of these activities is obtained [60]. The most puzzling is the fact that there is only one VDR which mediates calcemic and pro-differentiating actions. It is possible, then, that different analogs activate different intracellular signaling pathways, but it is still not clear how this would be achieved [61].

Analogs of 1,25D have been modified in one or more sites of the structure of the parental compound [59]. Some modifications are minor, but some change the structure substantially [62]. It is noteworthy that not only analogs of 1,25D can be used as agonists of VDR: lithocholic acid (LCA) is a natural ligand, and a very weak agonist of VDR. Modifications of LCA structure can substantially increase the pro-differentiation potency of

LCA, without affecting calcium phosphate homeostasis [63–65]. Unfortunately, the clinical trials using analogs of 1,25D were also far from these for ATRA in APL [66].

5. The Heterogeneity of AML

The most likely source of failure in differentiation therapy using 1,25D and analogs lies in the heterogeneity of AML. There are two systems of AML classifications, the French–American–British (FAB) system from 1976 [67], and the World Health Organization (WHO) system from 2008 [68]. In the FAB system, all AMLs are divided into 8 groups, based predominantly on the cell morphology and cytochemical staining [69]. The later WHO system divided AMLs into 7 groups. This system is much more complicated because it is based on a combination of clinical characteristics, morphology, immunophenotype, cytogenetics, and molecular genetics of the blasts. It takes prognostic factors known to affect the treatment and the outcome of the leukemia into consideration [68,70]. Neither of these classifications is ideal; therefore, there are some attempts to make amendments [3]. APL is an M3 subtype according to FAB, and belongs to group 1 according to WHO (AML with recurrent genetic abnormalities). In addition to variability of driver mutations in AML, there is also intrinsic heterogeneity in each patient resulting from clonal diversification of blasts [71]. The most frequent mutations in AML have been identified and are used to guide treatment and predict outcome. These are *NPM1* mutations, DNA methyltansferase 3A (*DNMT3A*) mutations, *FLT3* mutations, isocitrate dehydrogenase (*IDH*) mutations, ten-eleven translocation 2 (*TET2*) mutations, runt-related transcription factor (*RUNX1*) mutations, CCAAT enhancer binding protein α (*CEBPA*) mutations, additional sex comb-like 1 (*ASXL1*) mutations, mixed lineage leukemia (*MLL*) mutations, protein p53 (*TP53*) mutations, c-Kit mutations, or *PML-RARA* translocation t(15,17)(q22;q12). Out of these examples, only the M3 subtype, characterized by *PML-RARA*, is susceptible to ATRA-based differentiation therapy.

6. AMLs Resistant to 1,25D

The lessons learnt from ATRA therapies prompted studies focused on identification of AML subtypes sensitive and resistant to 1,25D-induced differentiation. In one study, the majority of patient's blasts did not respond to 1,25D or to the analogs with monocytic differentiation [72]. Figure 4 shows that only about 25% of the blasts were responsive. The correlation study performed using blasts isolated from AML patients indicated that blasts carrying FLT3 mutations are resistant to 1,25D and to its analogs [73]. Surprisingly, available cell lines which carry FLT3 mutations, MV-11 and MOLM-13, are responsive in vitro to 1,25D and to analogs [74]. There are some possible explanations for this phenomenon, including that the correlation observed was not due to a causal implication, or that the cell lines grown in vitro for many years had changed their phenotype due to epigenetic changes.

The data from ALM patients indicate that *VDR* expression levels positively correlate with patients' survival. VDR controls the stemness of blast cells and promotes their differentiation [75].

The cell line which was found to be completely resistant to 1,25D-induced cell differentiation is KG1 [76]. This cell line has very low expression of *VDR* gene as compared to other AML cell lines, very low levels of VDR protein, and almost no response of VDR target *CYP24A1* [74]. KG1 cells originated from 8p11 myeloproliferative syndrome, a blood disease which rapidly develops into AML [77]. KG1 cells are characterized by a chromosomal translocation where FGFR1 oncogene partner 2 (*FOP2*)—the fibroblast growth factor receptor 1 (*FGFR1*) fusion gene—encodes a constitutively active fusion protein FOP2–FGFR1. This fusion protein constitutively activates signal transducer and activator of transcription (STAT) 1 and STAT5 [78,79]. Disruption of this fusion gene restored expression of VDR gene, and sensitivity to 1,25D-induced monocytic differentiation [80]. Whether or not a similar situation exists in patients with 8p11 myeloproliferative syndrome remains to be

elucidated. The obstacle to study this is that the mutations observed in this syndrome are not routinely tested in patients with AML [81,82].

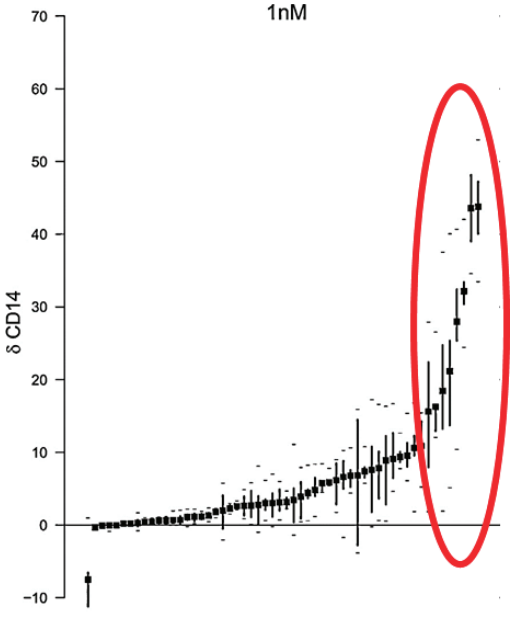

Figure 4. The monocytic differentiation of blasts from AML patients in response to 1 nM 1,25D and 1 nM analogs. The blasts of AML patients were isolated from peripheral blood and exposed to either 1 nM 1,25D or to one of the eight low-calcemic analogs at 1 nM concentration. Mean gain in expression of CD14 cell surface antigen for each patient is presented as a dot (•). Quartiles of response are marked by vertical lines, while minimum and maximum values are marked by dashes (–). Red oval surrounds the data from patients whose blasts were susceptible to 1,25D and to analogs. Adapted from [72].

7. AMLs Sensitive to 1,25D

It seems obvious that in order to benefit from immuno-stimulating activity of 1,25D in patients with AML, it is necessary to define the subtypes of the disease which are sensitive to 1,25D-induced differentiation.

An interesting observation was made about AML cases with IDH mutations. These mutations result in the production of the (R)-2-hydroxyglutarate (2-HG), which causes a hypermethylation, and dysregulates hematopoietic differentiation. One specific mutation in IDH is a R132H substitution. AML blasts with this specific mutation have been shown to have certain transcription factor genes upregulated when compared to the cells without this mutation. *CEBPA* gene and resulting protein C/EBPα were enriched in mutated cells. Interestingly, AML blasts harboring this particular mutation were more responsive to ATRA than blasts with wild-type (wt) IDH. Moreover, a cell-permeable form of 2-HG sensitized wt-IDH1 AML cells to ATRA-induced myeloid differentiation [83]. AML cells with IDH-R132H mutation also have higher levels of VDR and RXRα proteins than the cells with wt-IDH. Consequently, these cells respond better to 1,25D than wt-IDH cells, and even better to the combination of 1,25D and ATRA [84].

In fact, combination therapy using 1,25D and ATRA was postulated long ago, when VDR protein was found to be upregulated in ATRA-treated Kasumi-1 cells [85]. However, the regulation of *VDR* gene by ATRA is quite complex, and depends on the cell context [74]. This is because an abundant and unligated RARα acts as a suppressor of *VDR* transcription,

while following ligation with ATRA or with RARα agonists, starts to act as an activator [86]. This shows that patient-tailored combination therapy should be advised.

Another recent observation about the sensitivity of AML cells to 1,25D concerns the cells with overexpression of *FGFRs*. In addition to chromosomal translocations, *FGFR* genes may be affected by other mutations. Gene amplification of *FGFR1* was discovered in squamous cell lung cancers and estrogen-receptor-positive breast cancers, while *FGFR2* in some gastric cancers and in some triple-negative breast cancers [87,88]. There are data that indicate that the *FGFR1* gene is amplified in some cases of AML also [25]. In AML cell lines, overexpression of *FGFR1-3* caused enhanced sensitivity to 1,25D-induced differentiation, due to enhanced expression of *VDR* gene (Figure 5) [89]. Whether a similar regulation exists in the AML blasts of patients remains to be studied.

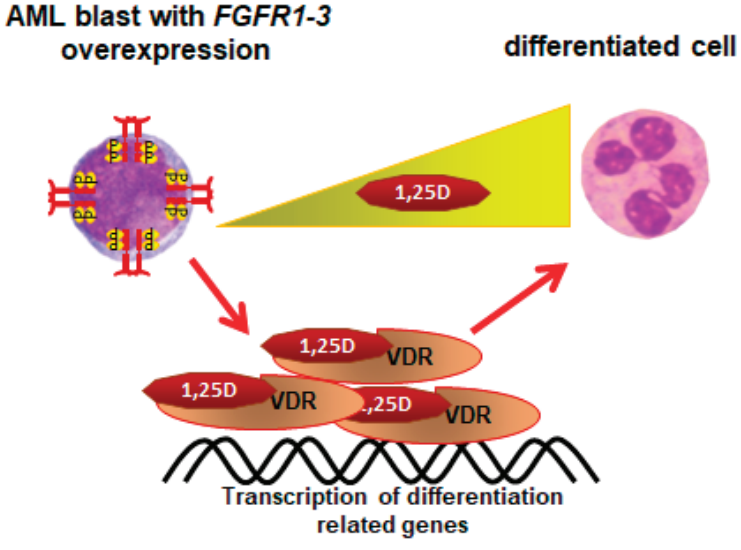

Figure 5. Differentiation of AML blasts with *FGFR 1-3* overexpression. The AML blasts with overexpression of *FGFR 1-3* produce more VDR protein than wild-type cells, and therefore are more susceptible to 1,25D-induced differentiation.

The *FGFR* family contains five genes, out of which four encode transmembrane tyrosine kinase receptors that exist in multiple splicing variants. Binding of the ligand to FGFRs results in a dimerization of these receptors and transphosphorylation of their tyrosine kinase domains [90]. As a result, FGFRs activate different signaling cascades including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase Cγ (PLCγ) [91]. It has been shown in the past that activating some of the MAPK pathways, namely JNK and Erk-1,2 pathways, enhances 1,25D-induced cell differentiation [92,93]. In contrast, constitutively active FGFRs, such as in FOP2–FGFR1 fusion kinase, cause downstream activation of signal transducer and activator of transcription (STAT) pathways [94]. Our unpublished data indicate that activation of STAT1 is responsible for low *VDR* expression.

8. Conclusions

AML is a disease of the elderly, and the proportion of older people is increasing steadily in modern societies. The current estimate of the probability of developing cancer is one in two for people born after 1960 [95], and despite the fact that AML is a relatively rare malignancy, its numbers will grow in the near future. For more than 40 years, all AML patients have been treated using standard intensive chemotherapy, but intensive chemotherapy cannot be used for elderly people. When chemotherapy is given to elderly

patients, they are often unable to tolerate it. Consequently, there is a need for gentler drugs for use alone or in a combined treatment. Differentiation therapy provides a much milder approach to treating malignancy, and should be advanced. However, the great success of ATRA-based differentiation therapy against APL has shown that this type of therapy must be targeted to molecular lesions susceptible to differentiation-inducing drugs. Recent data indicate that similarly to ATRA, 1,25D, or its analogs should be applied only to these patients who are likely to respond. Recent advances in next-generation sequencing, transcriptome analysis, immunophenotyping, and multiparameter flow cytometry will provide the means to delivering patient-tailored and tolerable differentiation therapies in the near future.

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Review

Vitamin D and the Central Nervous System: Causative and Preventative Mechanisms in Brain Disorders

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Abstract: Twenty of the last one hundred years of vitamin D research have involved investigations of the brain as a target organ for this hormone. Our group was one of the first to investigate brain outcomes resulting from primarily restricting dietary vitamin D during brain development. With the advent of new molecular and neurochemical techniques in neuroscience, there has been increasing interest in the potential neuroprotective actions of vitamin D in response to a variety of adverse exposures and how this hormone could affect brain development and function. Rather than provide an exhaustive summary of this data and a listing of neurological or psychiatric conditions that vitamin D deficiency has been associated with, here, we provide an update on the actions of this vitamin in the brain and cellular processes vitamin D may be targeting in psychiatry and neurology.

Keywords: brain; development; vitamin D deficiency; neuroprotection; disease mechanisms

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1. Introduction

The vitamin D receptor is part of the nuclear receptor super family containing members such as testosterone, estradiol, cortisol, progesterone, vitamin A derivative all-trans retinoic acid and the thyroid hormones [1]. These factors have important roles in the differentiation of all organs including the brain. It is now 21 years since we first suggested that vitamin D was a "possible" neurosteroid [2]. At that time, all we knew from existing research was that immunohistochemical evidences for the vitamin D receptor (VDR) had been shown in non-neuronal glial cells and that vitamin D may affect neurotrophic factor expression in vitro. There was very little knowledge about how vitamin D could exert its genomic effects in brain cells and a complete absence of any understanding about whether, like other neurosteroids, vitamin D would also have more rapid non-genomic actions. Over the last two decades, research on neurons, non-neuronal brain cells, in both human and animal brains, has confirmed not only does the brain possess the molecular machinery for vitamin D's actions, but also that VDRs are functional, that liganded VDRs directly regulate the expression of target genes and that vitamin D in the form of 1,25-hydroxyvitamin D_3, $1,25(OH)_2D_3$ can rapidly alter ion channel function [3]. Moreover, only very recently have new epigenetic mechanisms been revealed as gene regulatory pathways that vitamin D targets to affect gene expression in the developing brain [4,5]. As a result of all this research, vitamin D can now be considered an important steroid in brain development [6]. More recently the neuroprotective functions of vitamin D have been highlighted in models of adult brain disorders. Here we integrate these data and outline the molecules and processes vitamin D appears to target in both developing and mature brains and how such actions shape behaviour and brain function.

We are now aware of the large number of non-skeletal targets for vitamin D. In brain cells, changing vitamin D status alters cytokine regulation and has been shown to affect cell differentiation, neurotrophin expression, intracellular calcium signalling, neurotransmitter release, anti-oxidant activity, anti-inflammatory actions, stress responsivity and the expression of genes/proteins important to neuron physiology [7,8]. The purpose of

this short update is not to exhaustively summarise all such associations, but to bring the reader up to speed with recent findings using the very latest techniques in molecular manipulation/quantitation and cell visualisation.

With respect to brain disorders, there is now reliable epidemiology linking embryonic or neonatal vitamin D deficiency with an increased risk of neurodevelopmental disorders, such as schizophrenia [9,10], autism [11–14] and, more recently, attention deficit hyperactivity disorder (ADHD) [15–17]. There are also numerous studies suggesting adult vitamin D deficiency can be correlated with certain neurodegenerative conditions. This clinical epidemiological literature has been reviewed elsewhere [18,19], and we will return to this in the final section of this article. Our purpose here will be to focus on the latest preclinical studies modelling these epidemiological links and to discuss plausible biological mechanisms behind disease-relevant phenotypes.

Although we were pioneers in this area, works from a number of laboratories have firmly established the biological plausibility for how low levels of vitamin D could adversely affect how brains form and how this could lead to subtle changes in brain function and behaviour. Our task now is to discover exactly how low levels of vitamin D impair the function of specific brain cells/circuits leading to adult brain disorders and whether correcting vitamin levels and/or the processes affected by impaired vitamin D signalling can diminish phenotype/symptom severity.

2. Vitamin D Signalling in the Brain, the Basics and Controversies

Early studies reporting levels of vitamin D metabolite levels in the brain produced widely varied results. This has been to a large extent due to technical difficulties in extraction and quantification methods. The major circulatory form of vitamin D, 25-hydroxyvitamin D_3, ($25(OH)D_3$), and its active hormonal form, 1,25-hydroxyvitamin D_3, $1,25(OH)_2D_3$, have been reported to be present in brain [20,21]. Whilst the exact concentrations are debatable, they are routinely reported as far lower than blood levels. The later use of LC/MS/MS with isotope dilution provides absolute chemical identification due to its selectivity and greater sensitivity. Using this technology, one group shows $25(OH)D_3$ to be around (4 ng/g tissue) in rodent brain [22,23]. This group used atmospheric pressure, photoionisation which may avoid ion-suppression artifacts which may have affected earlier methods. Very recently, Fu and colleagues developed a method to measure vitamin D metabolites in the human brain and found $25(OH)D_3$ was measurable, but levels were far lower than those found in the blood around 0.2–0.3 ng/g tissue [24].

With respect to the VDR, despite immunohistochemical studies confirming its presence in human, chick, rat, mouse and zebrafish brains, [25–30] claims that some of the antibodies used may have been less-than-specific and have invited dispute [31,32]. Now unambiguous evidence has been provided using mass-spectrophotometry to electrophoretically resolve proteins from adult rodent brains to identify five unique VDR peptides with a confidence interval greater than 99% [33]. There is also close anatomical overlap between brain regions in rats and humans, with respect to VDR location [25–27,29,34,35]. Consistent close cross-species overlap in VDR distribution validates the use of rodents in modelling vitamin D-related brain outcomes.

Immunohistochemistry, mRNA and protein analyses all reveal a gradual expression of the VDR in the developing brain [33,36–38]. In the developing brain, the VDR is concentrated in differentiating fields, such as the ependymal surface of the lateral ventricles [39] (the greatest source of cell division in the brain), consistent with vitamin D actions as a differentiation agent. In particular, VDR expression in the nucleus of dopamine neurons which appear very early in brain development correlates tightly with the ontogeny of these neurons [35]. New genomic technologies have now allowed VDR location and activity to be assessed in animal brains. Using clustered regularly interspaced short palindromic repeats (CRISPR), Liu et al. inserted a Cre-expression sequence driven by the endogenous VDR promotor. When such animals were mated with a tdTomato reporter mouse ((Ai14; B6.Cg-Gt(ROSA)26Sor tm14 (CAG-tdTomato)Hze/J), intense tdTomato fluorescence was

detected in multiple brain regions, including caudate putamen, amygdala, reticular thalamic nucleus, cortex and less intense tdTomato fluorescence in hippocampus, hypothalamus, paraventricular thalamic nucleus (PVH), dorsal raphe and bed nucleus of the stria terminalis (BNST). RNAscope confirmed the presence of VDR RNA in these same tdTomato positive neurons. However, perhaps most importantly, $1,25(OH)_2D_3$ (5 μM) selectively depolarised the tdTomato positive but not negative neurons in the PVH, indicating vitamin D directly regulates neuronal activity, but only in VDR-expressing neurons [40].

Immunohistochemical evidences for the enzymes that convert $25(OH)D_3$ to the active hormonal form of vitamin D, $1,25(OH)_2D_3$, *CYP*27B1, and enzymes responsible for its breakdown, *CYP*24A1, have been provided in the foetal [41] and adult human brain [25,42], suggesting the active hormone can be made or removed locally in human brains. The most thorough investigation of expression of the major vitamin D metabolising enzymes *CYP*24A1, *CYP*271B and *CYP*271A in brain cell types was undertaken by Landel and colleagues [43]. Using primary cultures of neurons, astrocytes, microglia and oligodendrocytes (encompassing all major brain cell types), this group showed a broad distribution across all cell types but at a lower level than the kidney and liver. Strikingly, however, the addition of $1,25(OH)_2D_3$ induced a profound upregulation of *CYP*24A1 only in astrocytes and microglia, a finding also previously observed [44]. Again, genomic technologies have been employed to study the activity of these enzymes. Hendrix et al. created a transgenic mouse that expresses the luciferase reporter under the control of a full-length human CYP27B1 promoter. The organ distribution of CYP27B1 luciferase activity is similar to endogenous CYP27B1 with highest activity in the kidney, testis, brain, skin and bone [45]. Unfortunately, this useful model apparently has not yet been utilised to study what factors upregulate CYP27B1 brain activity.

3. Vitamin D and Normal Brain Development

A wealth of experimental evidence exists regarding the plausibility for how altering vitamin D signalling could affect critical events in brain development, such as axonal elongation, neurotransmitter synthesis, neurotrophin production and later brain function. Although there are some comprehensive reviews in this space [46,47], here, we bring this up to date.

3.1. Vitamin D and Neurite Growth

The addition of $1,25(OH)_2D_3$ to embryonic hippocampal neurons in culture increases neurite outgrowth possibly via an increased nerve growth factor (NGF) [48,49]. We also now have new unpublished data replicating the neurite-promoting potential of $1,25(OH)_2D_3$ in developing dopamine neurons differentiated from (a) a neuroblastoma cell line and (b) dopamine neurons in explant mesencephalic cultures. This action is in line with most other neurosteroids. Now, we are exploring the molecular mechanisms behind these effects. Others have chosen to lesion peripheral neurons and to show that vitamin D enhances axonal repair, myelination and functional recovery [50,51]. The $1,25(OH)_2D_3$ treatment also restores neurite outgrowth in models where it is impeded. The knockdown of the important neuronal epigenetic regulator, MeCP2, in cortical neurons blunts neurite extension potentially through reducing the activity of NFκB pathways. The $1,25(OH)_2D_3$ restores normal outgrowth in this model [52]. Vitamin D deficiency has also been shown to reduce peripheral nerve fibre density [53], and vitamin D has been considered a potential therapeutic in spinal cord repair [54].

3.2. Vitamin D and Neurotrophic Factors

The first evidence that vitamin D had any action on brain cells came from the early studies from Didier Wion's group in glioblastoma cells. Early studies showed that vitamin D promoted the expression of NT-3, NT-4 and nerve growth factor (NGF) [48,49,55–57]. Vitamin D-mediated increases in NGF were shown to be highly relevant to neuronal survival in vitro [48,58–60].

Given its prominent role in dopaminergic neuron differentiation (see below) and survival in earlier studies, there was a strong focus on vitamin D and neurotrophic factors important for dopaminergic neurons, such as glial cell line-derived neurotrophic factor (GDNF) [61,62] and now brain-derived neurotrophic factor (BDNF) [63]. Blocking vitamin D-mediated increase in GDNF synthesis prevents vitamin D's trophic effects on these neurons [64]. We have described the genomic proof that vitamin D regulates the transcription of both receptors for GDNF. The $1,25(OH)_2D_3$ suppresses GDNF family receptor alpha 1 (GFRa1), but ligand bound VDR binds to the promoter of the other major receptor for this neurotrophin, the proto-oncogene tyrosine-protein kinase receptor Ret (C-Ret), to upregulate C-Ret expression. Accordingly, the maternal absence of vitamin D decreases C-Ret expression in the developing rat mesencephalon [65].

The effect of vitamin D on neurotrophic factors in the developing brain has been far less explored. One study showed DVD-deficiency in rats reduced NGF and GDNF protein in neonatal brains [66]. Reductions in BDNF and transforming growth factor-β1 (Tgf-β1) in DVD-deficient embryonic mouse brains have also been described [67].

Most recently, there have been numerous studies showing a convincing link between hippocampal BDNF levels and vitamin D status in adult animals. Consistent with findings in development where the absence of vitamin D leads to a BDNF deficit, very recent studies show chronic treatment with $1,25(OH)_2D_3$ induces a profound upregulation in BDNF in rat hippocampus [68]. Others show stress- or drug-induced memory deficits correspond with decreased hippocampal BDNF expression that can be restored via chronic supplementation with high doses of cholecalciferol [69,70]. Another study revealed age-induced memory deficits could be reversed with cholecalciferol supplementation with corresponding increases in hippocampal BDNF and NGF expression [71]. In a model of type 2 diabetes in mice, BDNF and phosphorylation of its downstream effector CREB are reduced in the brain, and cholecalciferol restores these and associated behavioural deficits [72].

In summarising this data, $1,25(OH)_2D_3$ *increases*, and the developmental dietary absence of vitamin D *reduces* the expression of these crucial neurotrophic factors in neurons [73] and glia in developing and adult brains. Vitamin D's regulation of neurotrophic factors remains a central feature in brain ontogeny.

3.3. Vitamin D Regulates the Development of Dopamine Neurons

Since we first reported the VDR within the human substantia nigra [25], we have gone on to confirm the VDR is prominent in neuromelanin containing Tyrosine Hydroxylase (TH) positive human nigral neurons [35]. Vitamin D deficiency during development leads to a reduction in specification factors crucial for dopamine neurons [74,75], alters dopamine turnover [76] and leads to decreased lateral positioning of dopamine neurons that will form the substantia nigra [75]. Importantly, similar developmental anomalies in dopamine neuron ontogeny created by other adverse developmental exposures are rescued by maternal treatment with $1,25(OH)_2D_3$ [77]. To better understand the molecular processes involved, we have used neuroblast cells in which the VDR has been over-expressed. In such a model, we have shown $1,25(OH)_2D_3$ increases TH mRNA [78], drives cells down a dopaminergic lineage [79] and directly targets the promoter of Catechol-o-methyl transferase (COMT), an important enzyme in brain dopamine turnover [79].

One very recent study has examined microRNAs in developing dopamine neurons as epigenetic factors in neuronal maturation. This study showed that in flow cytometry-sorted E14 dopamine neurons from DVD-deficient rat embryos, the expression of a number of microRNAs was increased and that gene-ontology analysis indicated these microRNAs were involved in neuronal differentiation. These authors then examined the functional consequences of this in developing dopamine neurons and showed that some of these microRNAs decreased neurite outgrowth [4]. This represents the first evidence in the brain indicating how vitamin D may epigenetically differentiate dopamine neurons.

Taken together, the decreased expression of dopamine-promoting neurotrophic and specification agents and delayed dopamine neuron maturation in DVD-deficient embryonic brains along with the abundant in vitro evidences for $1,25(OH)_2D_3$ as a maturation agent for dopamine neurons, all confirm a central role for vitamin D in dopamine ontogeny. Given the links between DVD-deficiency and schizophrenia [9,10] and that dopamine abnormalities are perhaps causal in this disease, further studies examining this possible mechanistic link are needed.

4. Developmental Vitamin D Deficiency Effect on Brain Function and Behaviour

The long-term effects of DVD-deficiency on offspring behaviour have also been extensively studied in both rats and mice. Outcomes vary based on the species used/strain and duration and degree of vitamin D deficiency. Notably, there are alterations in critical early dam/pup interactions, such as maternal licking and grooming [80] and pup ultrasonic vocalisations [5,80], along with signs of delayed motor development [5]. As juveniles or adults, offspring have social behavioural deficits and alterations in stereotyped behavioural phenotypes of relevance to autism [5,80]. Interestingly, vitamin D deficiency leads to subtle increases in testosterone levels in maternal blood [81], a finding which has long-been considered a risk-modifying factor in autism [82]. Additionally, foetal male brains from vitamin D deficient dams have increased testosterone compared to control males. This study went on to show silencing of the major enzyme involved in testosterone breakdown, aromatase, by hypermethylation of its promoter in the brain may be the mechanism [81].

DVD-deficiency also leads to long-term changes in adult behaviours. Novelty [83], or exposure to psychomimetics, such as the N-methyl-D-aspartic acid receptor (NMDA-R) antagonist, MK-801 [84–86] or amphetamine [87], all increase locomotor activity in the adult offspring of DVD-deficient dams. Along with sensitivity to these exposures/agents that release dopamine, DVD-deficient rats have a greater response to antipsychotics that block dopamine 2 receptors [84,88]. Brain functional measures are also abnormal in these animals. Long-term potentiation, a cellular correlate of learning and memory and latent inhibition, a measure of attentional processing are also abnormal in DVD-deficient adults [89,90]. With respect to cognition, DVD-deficient offspring have impaired response inhibition, a deficit normalised by the antipsychotic clozapine [91]. Associative learning is also impaired in DVD-deficient adults [92].

Only recently have investigators begun to assess the impact of vitamin D treatment in dams exposed to either DVD-deficiency or another developmental animal model that induces brain changes, maternal immune activation. Such interventions reverse behavioural phenotypes produced by these models related to anxiety, depression cognition, stereotyped and social behaviours [93,94].

As discussed previously, given the stark differences in the effects of DVD-deficiency on brain development between rats and mice, it is not surprising that the behavioural phenotypes in adult offspring also vary widely [95]. For instance, although there are some similarities in novelty-induced hyperlocomotion [84] and perseverative responses [96], outcomes vary widely in tests of exploratory behaviour [88,97] and locomotor response to psychomimetics [98].

Whether DVD-deficiency affects cognitive performance in children is not clear. Two studies show DVD-deficient children have delayed cognitive development [99,100]. However, these findings were not replicated in much larger studies [15]. A recent meta-analysis that summarised 31 studies could find no conclusive evidence for the association between maternal or child vitamin D status and behavioural or cognitive outcomes in children and adolescents. A summary of twelve mother–child studies (n = 17,136) and five studies just in children (n = 1091) showed low maternal or child $25(OH)D_3$ levels led to impaired behavioural outcomes in children. In contrast, fifteen mother–child studies (n = 20,778) and eight studies in children (n = 7496) showed no association [101]. A large Finnish nested case-control study (1607 children with learning difficulties with matched controls) also showed no link between maternal $25(OH)D_3$ levels and behaviour [102].

One small randomised clinical trial (55 infants) in healthy term infants showed a low dose of vitamin D supplementation might benefit gross motor function compared to a higher dose of vitamin D supplementation, but this study lacked a placebo control [103]. There is also a not-insubstantial number of studies that link maternal or childhood vitamin D deficiency to neurodevelopmental psychiatric disorders such as autism, ADHD or schizophrenia [9–11,13,14,100,104,105]. This association is not universally replicated [106,107]; however, the mean population $25(OH)D_3$ levels in these later studies were much higher, making the association difficult to test. Randomised controlled trials of cholecalciferol supplementation in children with ASD or ADHD show diverse outcomes, some showed no beneficial effects and others reported some symptom relief [108–114]. In the future, trials examining vitamin D levels in mothers/children prior to supplementation are required as supplementing vitamin D-sufficient mothers or children is likely to have minimal effect, as shown in other clinical trials with vitamin D [115].

5. Vitamin D Is Neuroprotective in Neurons and Adult Brain

There are now sufficient epidemiological studies also linking low levels of $25OHD_3$ with various neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease and multiple sclerosis. Though many such studies suffer from reverse causality (i.e., low levels of vitamin D are a consequence of disease-induced behavioural changes rather than causal), there has been sufficient interest to launch numerous clinical supplementation trials [116–118]. There have also been studies linking exposure to various toxins with low $25OHD_3$ levels. Such links have stimulated researchers to initiate mechanistic studies in neuronal cell systems and animal models. Along with the wealth of studies showing vitamin D is neuroprotective via neurotrophin production, here, we have chosen not to focus on the clinical epidemiology, but rather on vitamin D's regulatory actions on calcium in the brain and its neuroprotective actions against reactive oxygen species (ROS) and inflammation as well as its actions in mitigating stress as plausible prophylactic/therapeutic mechanisms. We summarised the potential mechanisms underlying the action of vitamin D (Figure 1). Much of this work is very recent.

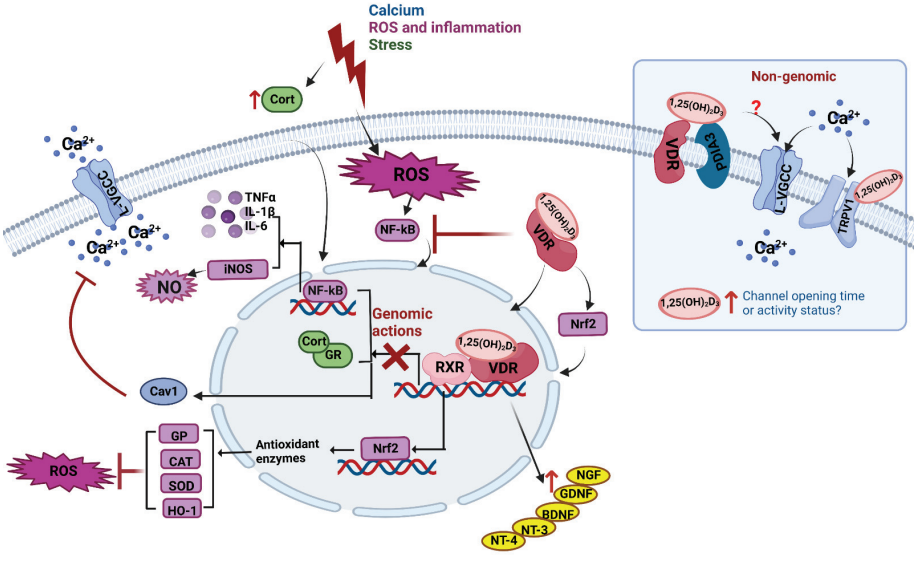

Figure 1. Neuroprotective actions of vitamin D in brain.

Calcium-related mechanisms (in blue). Non-genomic actions. The $1,25(OH)_2D_3$ acutely facilitates calcium influx via L-VGCC. The $1,25(OH)_2D_3$; can also directly bind to TRPV1 to

induce calcium influx. Both actions facilitate neuronal function. *Genomic actions.* Chronic vitamin D treatment decreases Cav1 (a L-VGCC subunit) expression which will reduce calcium influx in response to ROS/inflammation or stress.

ROS/inflammation-related mechanisms (in purple). ROS enhances NF-κB nuclear translocation to promote proinflammatory cytokine production. The $1,25(OH)_2D_3$ acts to inhibit this nuclear translocation. By also inhibiting NF-kB expression, $1,25(OH)_2D_3$ reduces iNOS expression and thus, reduces NO production. Finally, $1,25(OH)_2D_3$ increases Nrf2 to enhance the expression of anti-oxidant enzymes including GP, CAT, SOD and HO-1, thus countering ROS toxicity. The $1,25(OH)_2D_3$ also directly facilitates Nrf2 nuclear translocation.

Stress-related mechanisms (in green). Stress increases corticosterone synthesis. In the brain, corticosterone increases inflammatory cytokine production via its receptor (GR). The $1,25(OH)_2D_3$ is neuroprotective by antagonising GR expression.

Neurotrophin-related mechanisms (in yellow). A variety of neurotrophic factors have been shown to counter the effects of stress- or toxin-induced neuronal damage. Historically, $1,25(OH)_2D_3$ has been shown to increase NGF, GDNF, BDNF, NT-3 and NT-4 under such conditions.

5.1. Calcium Regulation

Calcium transients are required for normal neuronal function, but if calcium is unbuffered, it is toxic to brain cells. For more than 30 years, we have known how vitamin D regulates calcium uptake in bone cells [119,120] and similar mechanisms appear to act in neurons and the brain. In cultured neurons, $1,25(OH)_2D_3$ retards calcium influx via the downregulation of L-type voltage-sensitive calcium channels, thus potentially preventing toxic outcomes [59,121–123]. In contrast, the rapid non-genomic actions of vitamin D produce the opposite effect, increasing calcium influx in cortical slices, a process that is again dependent on L-type calcium channels [124]. In a seminal study, the non-genomic rapid actions of $1,25(OH)_2D_3$ were investigated in cortical neurons using calcium imaging, electrophysiology and molecular biological techniques. This study confirmed that physiological concentrations of $1,25(OH)_2D_3$ lead to rapid calcium influx, but only in some neurons. Somatic nucleated patch recordings revealed a rapid, $1,25(OH)_2D_3$-evoked increase in high-voltage-activated calcium currents mediated by L-type voltage-gated calcium channels [125]. Whether any of these actions are caused by the putative membrane VDR, Protein disulfide-isomerase A3 (PDIA3), in the brain remains unknown. Genetic variants in L-type voltage-gated calcium channels continue to be implicated in schizophrenia [126]. Given the epidemiological links between DVD-deficiency and schizophrenia [9,10], continued studies in this area are warranted.

Recent research also discovered that $25OHD_3$ and $1,25(OH)_2D_3$ directly bind to the transient receptor potential vanilloid subfamily member 1 (TRPV1) channel [127]. Binding to the same region as the TRPV1 agonist capsaicin, $25OHD_3$ can weakly activate TRPV1 and inhibit capsaicin-induced TRPV1 activity. TRVP1 activity modulates immune cell activation and cytokine production through regulating intracellular calcium to mediate nociceptive signals. This, therefore, may be one mechanism for how vitamin D may modulate nociceptive pain pathways, as vitamin D deficiency has been linked to chronic pain [128], although oxidative mechanisms have also been proposed [129].

5.2. ROS and Inflammation

Vitamin D increases anti-oxidants, such as glutathione and cytochrome c, to mediate anti-oxidant actions in cultured neurons [121,130] and the brain [131,132]. Along with extracellular calcium, vitamin D deficiency in the adult brain increases ROS along with producing impairments in gamma-aminobutyric acid (GABA) and glutamate release. Importantly, reintroducing dietary vitamin D normalises all deficits [133].

In the brain, microglia are the immunologically responsive cells responsible for the production of inflammatory regulators such as nitric oxide (NO). Early studies showed $1,25(OH)_2D_3$ blocks inducible nitric oxide synthetase in the rat brain in response to autoim-

mune or inflammatory factors [134–136]. Later studies suggested that oxidative stress may upregulate *CYP*27B1 in microglia to induce $1,25(OH)_2D_3$ production locally at the site of NO or ROS production to mediate vitamin D's anti-oxidant effects in the brain [133,137,138].

In primary cultured neurons, hypoxia induces apoptotic cell death and interferes with normal calcium signalling. One study, that chose to use cholecalciferol rather than calcitriol, showed that when added to cultured primary neurons exposed to hypoxia, cholecalciferol (10 nM) was anti-apoptotic and preserved calcium signalling though this effect was reversed at high doses. Upregulation of hypoxia-induced factor (HIF)-1α and/or BDNF were considered as the possible protective mechanisms [139], though conversion to calcitriol was not assessed.

DVD-deficiency would also appear to render the foetal environment more prone to oxidative stress. Ali and colleagues reported that DVD-deficient rat placenta produces more of the inflammatory cytokines IL-6 and 1L-1β upon challenge with a viral inflammatory agent [140]. Separately, when microglia were cultured from DVD-deficient mouse brains, they were increased in number, were hyperproliferative and had increased ROS production. Culturing these cells in the presence of calcitriol reversed these changes [141].

In recent years, there appears to have been intense interest in vitamin D's protective actions against ROS and inflammation in the brain induced by numerous disease models. For instance, in hypothyroid juvenile rats, cholecalciferol supplementation (100 or 500 IU/kg/day) prevented hypothyroidism-induced cognitive and learning memory impairments [142]. Plausible mechanisms included elevations in the anti-oxidant enzyme superoxide dismutase (SOD) and thiol content in hippocampus and cortical tissue and reductions in malondialdehyde (MDA), a marker of oxidative stress. Similar restorative outcomes on cognition have been achieved in models of acute inflammation and again these same anti-oxidant processes in the brain were invoked by vitamin D supplementation [143].

In spontaneously hypertensive (SH) rats, infusion of $1,25(OH)_2D_3$ into the hypothalamic paraventricular nucleus (PVN), a brain region maintaining baroreflex and autonomic function, prevented the elevation of ROS stress-related proteins such as NOX2, NOX4 and $p22^{phox}$. Chronic calcitriol infusion also attenuated microglial activation and reduced tumour necrosis factor (TNF)-α, IL-1β and IL-6 inflammatory cytokine production. Likely, mechanisms involved the inhibition of the high-mobility group box-1(HMGB1)-receptor for advanced glycation end products (RAGE)/Toll-like receptor (TLR4) and NF-κB in the PVN of SH rats [144].

The effect of vitamin D on NO is also detected in a rat model of cerebral ischemia reperfusion model. Seven days of calcitriol administration prior to ischemic surgery reduced stroke-induced elevation of MDA and NO and increased total anti-oxidant capacity. These effects could be attributed to vitamin D-increasing nuclear factor erythroid 2-related factor 2(Nrf2), a transcription factor that decreases oxidative stress following stroke and/or heme oxygenase (HO-1), a major cytoprotective enzyme with anti-oxidative, and anti-inflammatory properties [145,146].

In a traumatic brain injury model, calcitriol treatment reduced MDA production and promoted autophagic flux and activated Nrf2 pathways. Autophagy reduces oxidative stress by a timely removal of damaged substances [147]. Nrf2 is a redox-sensitive transcription factor that binds to anti-oxidant response elements to promote the expression of enzymes/proteins for detoxication and anti-oxidation [148]. Confirmation that these two processes were central to calcitriols protective actions in traumatic brain injury was shown by inhibiting the autophagy using chloroquine or deleting Nrf2 as either action blocked calcitriol's protective effects [149].

In a model of lead-induced neurotoxicity and oxidative stress, decreases in anti-oxidant molecules GSH, SOD and catalase and increased ROS production are observed in rat cortex. Cholecalciferol reversed these changes possibly via Nrf2 and/or NF-κB mechanism [150]. In a model of experimentally induced epilepsy in young male rats, cholecalciferol not only reduced seizure severity, but corrected associated memory deficits,

reduced extracellular calcium, restored anti-oxidative enzymes SOD and glutathione-related enzymes and reduced inflammatory cytokine production in the hippocampus [151].

One neurological disorder commonly associated with ROS-mediated brain damage is Parkinsons's disease (PD). The motor deficits produced by PD are believed to be caused by dopamine neurons dying selectively in the substantia nigra and the associated reduced dopamine release in the dorsal striatum. This is modelled in animals via the intracranial delivery of relatively selective dopaminergic terminal toxins, such as 6-hydroxy dopamine (6-OHDA), or nigral toxins such as 1-methyl-4-phenyltetrahydropyrine (MPTP). In a 6-OHDA-treated mouse, cholecalciferol treatment two weeks after surgical lesion attenuated 6-OHDA-induced increases in the microglia marker CD11b, IL-1β and the oxidative stress marker p47phox, a primary modulator of NADPH oxidase activity and restored some motor functional deficits [152]. Another 6-OHDA study showed that cholecalciferol prevented characteristic losses in dopamine synthetic enzymes and transporters, preserved motor function as well as reduced lipid peroxidation [153]. Another study using MPTP showed co-administration with calcitriol improved motor deficits, reduced dopamine neuron toxicity and reduced ROS production [154]. The mechanism proposed was via an interaction between the liganded VDR and poly(ADP-ribose) polymerase-1(PARP1) to reduce its contribution to ROS-induced cell death. PARP1 pathways have been proposed as one mechanism for dopamine cell death in PD.

ROS production is also linked with other degenerative processes such as Alzheimer's disease. Animal models for Alzheimer's disease frequently employ genetic models that over-express the Tau or amyloid (Aβ) proteins that are closely linked with disease pathology. In an amyloid presenilin model, 13 weeks of vitamin D deficiency exacerbated the ROS production in this model by downregulating superoxide dismutase 1 (SOD1), glutathione peroxidase 4 and enhanced the expression of IL-1β, IL-6 and TNFα, along with increased Aβ production and Tau phosphorylation [155].

In another amyloid model where rats are injected with Aβ1-40, cholecalciferol supplementation reduced Aβ-induced MDA levels, increased SOD activity and improved hippocampal neuronal survival [156]. Prolonged vitamin D hypovitaminosis in mice (from 6-weeks-old to 6-months-old) altered the expression of genes involved in amyloid precursor protein homeostasis (Snca, Nep, Psmb5), oxidative stress (Park7), inflammation (Casp4), lipid metabolism (Abca1), signal transduction (Gnb5) and neurogenesis (Plat) [157]. In another distinct amyloid protein mutant mouse model (3xtg-AD), vitamin D levels are dramatically reduced at 9 and 12 months of age. Vitamin D supplementation in this model improves memory possibly via the suppression of collapsin response mediator protein-2 (CRMP2) phosphorylation [158]. An in vitro study has also shown $1,25(OH)_2D_3$ alleviates Tau hyperphosphorylation and reduces ROS in a neuronal cell model treated with Aβ possibly via vitamin D's role in enhancing GDNF [159].

Experimentally induced autoimmune encephalitis (EAE) is a mouse model mimicking the autoimmune reaction to myelin proteins inducing multiple sclerosis-like pathology. In this model, calcitriol administration decreased the severity of EAE by attenuating inflammation and demyelination at the spinal cord [160]. This same study also showed calcitriol treatment reduces lymphocyte, macrophage and activated microglia infiltration into the brain. EAE mice have increased blood brain barrier permeability thought to be due at least in part to a reduction in endothelial tight-junction proteins such as ZO-1. This same study showed calcitriol increases this proteins expression. Calcitriol also reduced EAE-induced lipid hydroxylation and enhanced the anti-oxidant enzymes glutathione peroxidase, catalase and SOD.

The $1,25(OH)_2D_3$ not only suppresses inflammation in models of demyelination, but also enhances the differentiation or survival of oligodendrocyte progenitor cells in the spinal cord of a MOG35–55-induced EAE mouse model [161]. The promotion of oligodendrocyte maturation by $1,25(OH)_2D_3$ is also observed in a cuprizone-induced EAE mouse model [162]. Krabbe disease is an inherited leukodystrophy. This demyelinating condition is caused by a galactocerebrosidase (GALC) deficit that results in loss of oligodendrocytes

and demyelination. In an animal model of this disorder ($GALC^{twi/twi}$; twitcher mouse), supplementing the heterozygous $GALC^{+/-}$ dam from birth to weaning with cholecalciferol delayed onset of disease-induced locomotor deficits and tremors and extend the life span of offspring [163].

5.3. Glucocorticoids and Stress

Glucocorticoid release is the classic endocrine response to stress, and protracted exposure induces neuronal shrinkage then cell death [164]. The effects of $1,25(OH)_2D_3$ and glucocorticoids in the body can be considered antagonistic [165–169]. Similarly, in the brain, $1,25(OH)_2D_3$ antagonises the effects of dexamethasone (a corticosterone agonist) on hippocampal neuron differentiation and glucocorticoid receptor function [170]. Interestingly, dexamethasone can decrease $1,25(OH)_2D_3$ synthesis in the hippocampus and prefrontal cortex, indicating this process is reversible [171].

Behaviourally, vitamin D antagonises the depression-like phenotypes induced by chronic cortisol administration in animals [172–175]. Possible mechanisms include regulation of hippocampal glucocorticoid receptors or the restoration of dopamine levels in the reward centres in the brain [175]. Chronic mild stress in rats leads to increased corticosteroids, inflammatory markers and decreased anti-oxidant enzymes SOD and glutathione peroxidase and catalase in the hippocampus and prefrontal cortex. Simultaneous cholecalciferol treatment reverses these stress-mediated effects [69,176].

Chronic unpredictable stress increases immobility in a widely used test of behavioural despair (tail suspension test). Cholecalciferol treatment reduces this stress-induced immobility. Inhibiting the synthesis of the neurotransmitter serotonin abolishes cholecalciferol's actions in this test, suggesting vitamin D might act via increased serotonin synthesis [177]. Support for this idea comes from studies in glioblastoma cells showing functional VDREs are localised at −7 kb and −10 kb upstream of the serotonin, synthesising enzyme tryptophan hydroxylase [178]. Other studies chronically administering corticosterone replicate the cholecalciferol's reversal of these stress-induced behaviours and suggest either alterations to glucocorticoid signalling in the brain or reductions in stress-associated ROS production in the brain are the protective mechanisms [172,179]. Stress can be measured in animals using other behavioural paradigms such as immobility in a forced swim test. Chronic unpredictable stress increases immobility in this test. Cholecalciferol supplementation reverses this immobility as well as reduces serum corticosterone/ACTH levels and increases BDNF and NT-3/NT-4 levels in the hippocampus [174].

DVD-deficiency may also alter maternal response to stress in rats [180] and mice [181] and can adversely affect maternal care [80], which is well-known to induce permanent changes in offspring stress-response [182]. The translational potential of these later findings is enhanced given that a randomised clinical trial showed that 50,000 IU of cholecalciferol alone every 2 weeks, or in combination with Omega 3 fatty acids (1 g/day), for 8 weeks significantly reduced anxiety and improved sleep quality in women of reproductive age with pre-diabetes and hypovitaminosis D [183].

6. Hypervitaminosis D and Adverse CNS Outcomes

While the role of hypovitaminosis D in brain function has been extensively investigated, the effect of hypervitaminosis D has received less attention. This is despite an inverted U-shaped cellular response to $1,25(OH)_2D_3$ being frequently reported, i.e., both high and low levels of $1,25(OH)_2D_3$ induce adverse outcomes. Hypervitaminosis D is rare in humans, generally resulting from excess vitamin D supplementation or diseases such as sarcoidosis that produces excess $1,25(OH)_2D_3$ due to activated macrophages [184]. Though not focused on neurological conditions, an older review concluded "*There is accumulating evidence that both high and low serum calcidiol concentrations are associated with an increased risk of chronic diseases*" [185]. Hypervitaminosis D always results in hypercalcaemia, which may be toxic to brain function. Animal studies showed that high cholecalciferol intake, 25,000 IU/kg, for four consecutive days reduces brainwave activity [186]. Senescence-

accelerated-mouse-phenotype (SAMP) strain-8 mice, an animal model of accelerated human ageing, showed a progressive increase in serum $25OHD_3$, which coexists with reduced cognitive function and increased capillary permeability [187]. Deleting Fibroblast growth factor (FGF-23) in mice increased *CYP*27B1 levels, leading to increased $1,25(OH)_2D_3$ synthesis. FGF-23 null mice display early ageing, and deleting *CYP*27B1 delayed premature ageing [188]. Considering the risk of hypervitaminosis D, serum vitamin D levels should be monitored in clinical trials of vitamin D supplementation.

7. Conclusions and Future Challenges

Here we have provided an up-to-date summary of research detailing vitamin D turnover, synthesis genomic and non-genomic actions in the brain, neurons and non-neuronal cells. We advise caution when considering much of the prior literature employing either dietary restrictions which often produced hypo-calcaemia or the use of constitutive knock out models permanently reducing $1,25(OH)_2D_3$ synthesis or impairing VDR signalling, as these models produce too many non-CNS effects to make brain-related outcomes interpretable. Although there are still strain and species differences when choosing a model organism, contemporary DVD- or AVD-deficient models in rodents do not produce hypocalcaemic offspring and continue to produce findings of apparent relevance to the fields of psychiatry and neurology.

Increasingly, epidemiological studies associate low levels of vitamin D either prenatally or at birth with psychiatric conditions such as autism and schizophrenia. The epidemiology for degenerative diseases as diverse as Alzheimer's disease, Parkinson's disease and multiple sclerosis all continue to indicate a role of optimal vitamin D status throughout life. This raises the possibility of simple dietary supplementation as an adjunct to current therapies. Although not practicably possible for developmental conditions with adult onset such as schizophrenia, this could eventually be considered in early onset psychiatric disorders, such as autism or ADHD, or for maintaining neural integrity in degenerative conditions via large placebo-controlled, randomised clinical supplementation trials.

We would also like to take this opportunity to highlight design issues in many observational studies opportunistically linking early-life vitamin D deficiency with psychiatric disorders. Most of the published observational epidemiological studies linking early-life vitamin D status with a psychiatric diagnosis never address reverse causality (the condition changes behaviours that lead to less sun exposure). By way of illustration, a very high-profile recent report in the New England Journal of Medicine showed all mental illnesses were associated with an increased risk in a general medical condition [189]. In other words, patients with psychiatric conditions are generally suffering from other conditions that will curtail behaviour perhaps altering diet, exercise and exposure to sunshine. We urge all future epidemiological studies that seek to examine the relationship between vitamin D and psychiatric or neurological conditions to rigorously control for the often-poor general health of patients. This, of course, is less of an issue for gestational (DVD-deficiency) exposures in otherwise healthy mothers.

Important data have emerged from a recent mendelian randomisation study examining gene pathways related to $25(OH)D_3$ blood concentrations. This study found no evidence that genetic factors involved in the synthesis of $25(OH)D_3$ were causal for psychiatric disorders [190]. We have interpreted this to mean any link between $25(OH)D_3$ levels and brain-related outcomes are likely to be solely driven by environmental factors.

Autism is perhaps the developmental brain disorder most regularly linked with DVD-deficiency. However, well-conducted studies refuting this link are now emerging. For the studies describing an inverse relationship between maternal vitamin D levels and autism, they all had mean $25(OH)D_3$ levels of <50 nM which is considered by some authors to represent a cut off for vitamin D deficiency [11–13,191]. Two recent studies have failed to find this inverse association [106,192]. So, at face value, this appears a failure to replicate previous studies. However, it is crucial to note that in these last two studies, the mean levels of $25(OH)D_3$ were actually very high (>70–80 nM) and there were very few individuals

that were actually vitamin D-deficient, meaning the association could not be properly tested. These same six studies all used the same laboratory to analyse samples, so technical bias (so common amongst vitamin D studies in different populations) could be ruled out. This suggests a threshold effect rather than any continuous relationship between DVD-deficiency and autism. We highlight this particular relationship to illustrate some of the confusion regarding statements regarding potential causality between vitamin D and various brain-related clinical disorders.

The incidence of hypovitaminosis D in both pregnant women and their newborns and the general population remains concerning [193]. Clearly, more rigorous study design is required taking into consideration such issues to bring clarity to the future epidemiological studies. Better quality studies in the future are needed given the substantial emotional and financial burden psychiatric and neurological disorders place on the patient and community. The opportunity to use such a simple, safe and inexpensive intervention as vitamin D supplementation as treatment or as an adjunct to existing therapies in such disorders remains extremely attractive from a public health perspective.

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Abbreviations

VDR, vitamin D receptor; *PDIA3*, Protein disulfide-isomerase A3; *RXR*, retinoid X receptor; *L-VGCC*, L-type voltage-gated calcium channel; *Cav1*, L-type voltage-gated calcium subunit 1; *TRPV1*, transient receptor potential vanilloid subfamily member 1; *ROS*, reactive oxygen species; *iNOS*, inducible nitric oxide synthetase; *NO*, nitric oxide; *NF-kB*, nuclear factor kappa-B; *Nrf2*, nuclear factor erythroid 2-related factor 2; *GP*, glutathione peroxidase; *CAT*, catalase; *SOD*, superoxide dismutase; *HO-1*, heme oxygenase; *NGF*, nerve growth factor; *BDNF*, brain-derived neurotrophic factor; *GDNF*, glial cell line-derived neurotrophic factor; *NT-3*, neurotrophin 3; *NT-4*, neurotrophin 4; *GR*, glucocorticoid receptor; *CORT*, corticosterone; *TNFα*, tumour necrosis factor α; *IL-6*, interlukin-6; *IL-1B*, interlukin-1 Beta. (Figure created from BioRender.com).

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Review

Comparing the Evidence from Observational Studies and Randomized Controlled Trials for Nonskeletal Health Effects of Vitamin D

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Abstract: Although observational studies of health outcomes generally suggest beneficial effects with, or following, higher serum 25-hydroxyvitamin D [25(OH)D] concentrations, randomized controlled trials (RCTs) have generally not supported those findings. Here we review results from observational studies and RCTs regarding how vitamin D status affects several nonskeletal health outcomes, including Alzheimer's disease and dementia, autoimmune diseases, cancers, cardiovascular disease, COVID-19, major depressive disorder, type 2 diabetes, arterial hypertension, all-cause mortality, respiratory tract infections, and pregnancy outcomes. We also consider relevant findings from ecological, Mendelian randomization, and mechanistic studies. Although clear discrepancies exist between findings of observational studies and RCTs on vitamin D and human health benefits these findings should be interpreted cautiously. Bias and confounding are seen in observational studies and vitamin D RCTs have several limitations, largely due to being designed like RCTs of therapeutic drugs, thereby neglecting vitamin D's being a nutrient with a unique metabolism that requires specific consideration in trial design. Thus, RCTs of vitamin D can fail for several reasons: few participants' having low baseline 25(OH)D concentrations, relatively small vitamin D doses, participants' having other sources of vitamin D, and results being analyzed without consideration of achieved 25(OH)D concentrations. Vitamin D status and its relevance for health outcomes can usefully be examined using Hill's criteria for causality in a biological system from results of observational and other types of studies before further RCTs are considered and those findings would be useful in developing medical and public health policy, as they were for nonsmoking policies. A promising approach for future RCT design is adjustable vitamin D supplementation based on interval serum 25(OH)D concentrations to achieve target 25(OH)D levels suggested by findings from observational studies.

Keywords: breast cancer; colorectal cancer; gestational diabetes; preeclampsia; preterm birth

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1. Introduction

The year 2022 marks a century since the discovery of vitamin D [1]. The National Library of Medicine's PubMed database currently includes 97,024 vitamin D–related publications, with 73,577 having "vitamin D" in the title or abstract [accessed August 5, 2022]. Most vitamin D research before 2000 was related to skeletal effects. Interest in nonskeletal effects had become the most important topic of vitamin D research by the year 2000 when there were 69,090 publications related to vitamin D, 59,104 having "vitamin D" in the title or abstract. Despite that vast body of literature, considerable confusion remains regarding vitamin D's role in determining health outcomes; this mainly results from disagreements

between findings from observational studies of health outcome associations with serum 25-hydroxyvitamin D [25(OH)D] concentrations and those from randomized controlled trials (RCTs) of vitamin D supplementation. Findings in 2014 from the two approaches revealed widespread disagreement. Observational studies generally reported inverse correlations between serum 25(OH)D concentrations and health outcomes, whereas RCTs generally showed no significant health benefits of vitamin D supplementation [2]. Meta-analyses of prospective cohort studies of nonskeletal disorders had reported significantly reduced risk for highest versus lowest 25(OH)D concentrations for cardiovascular disease (CVD) incidence and mortality rates, diabetes incidence, colorectal cancer incidence, but not for several other cancers [2]. Because of these discrepancies, Autier and colleagues suggested that observational study findings could be the result of reverse bias—the lowering of 25(OH)D concentrations by illness—and of various biases and confounders inherent to observational study designs [2]. Some conditions do indeed reduce the level of 25(OH)D in serum. However, that conclusion ignores the fact that such reductions limit the supply of 25(OH)D to the tissues, thereby aggravating the effects of vitamin D inadequacy and might also reflect locally increased intracellular calcitriol formation following compensatory increases in intracellular 25(OH)D activating enzyme activity in some tissues [3].

By 2017, RCTs had failed to demonstrate significant nonskeletal benefits for vitamin D except for reductions in common upper respiratory tract infections (RTIs) and in asthma exacerbations [4–6]. By April 2019, however, RCTs had shown that vitamin D supplementation had beneficial effects for primary prevention of acute RTIs and reduced acute exacerbations of asthma and chronic obstructive pulmonary disease [7]. The situation deteriorated further in 2019, when results of two large vitamin D RCTs were reported, the VITamin D and OmegA-3 TriaL (*VITAL*) for cancer and CVD risks [8] and the Vitamin D and Type 2 *Diabetes* (*D2d*) trial [9]. Neither trial showed reductions of the disease of interest based on 'intention to treat' when comparing disease incidence between the treatment and placebo arms. Neither article's abstract mentioned the beneficial effects that secondary analyses later reported. Thus, the press reported that vitamin D did not prevent cancer, CVD, or diabetes and medical and public health bodies, media, and the public thought vitamin D's beneficial effects for nonskeletal health outcomes had been disproven.

Observational studies related to vitamin D are generally prospective cohort or nested case–control studies of health outcomes in relation to baseline serum 25(OH)D concentrations. Participants are enrolled in cohorts or studies and provide blood samples, other biological data, and information on lifestyle upon recruitment. Serum 25(OH)D concentrations are measured later in standardized assays using deep-frozen serum or plasma samples. Generally, no further information (beyond details on health status) is gathered from participants during the follow-up period, which can last from weeks or months to twenty years. At the end of follow-up, changes in health status are correlated with baseline serum 25(OH)D concentrations, with adjustments for data on other relevant factors at enrollment. In nested case–control studies, control subjects may or may not be carefully matched with cases. Case–control studies also can be done with 25(OH)D concentrations measured near the time of health events. However, such studies are unreliable for pre-disease serum 25(OH)D concentrations since disease can affect them; for example, severe infections can reduce 25(OH)D concentrations, an effect that is strongest for acute inflammatory diseases such as acute RTI [10] and is a concern with COVID-19 [10].

Several observational studies now exist based on vitamin D supplementation with measurements of 25(OH)D concentrations at baseline and at follow-up. Conditions studied include breast cancer [11], arterial hypertension [12], myocardial infarction (MI), all-cause mortality rate [13], and preterm birth [14]. According to a recent review [15], such observational studies require careful interpretation in light of their inherent limitations, as discussed later in this review.

Meta-analyses of prospective observational studies of cancer incidence showed significant inverse correlations between baseline serum 25(OH)D concentrations and incidence of 10 different types of cancers between 2016 and 2021 (including bladder, breast, colorectal,

head and neck, liver, lung, ovarian, pancreatic, renal, and thyroid cancer) and a direct correlation for prostate cancer (Table 5 in [16]). However, without support from RCTs, such results from observational studies are generally overlooked or ignored.

Data from observational studies of vitamin D obtained from diet have been limited. The most important was that by Garland and colleagues, associating dietary vitamin D and calcium with reduced risk of colorectal cancer [17]. The main problem with using dietary supply values is that dietary sources generally account for only 10–20% of total vitamin D supply. Solar UVB exposure contributes most, as seen from the seasonal variation of serum 25(OH)D concentrations [18]. Meat is also an important source of vitamin D, due to its 25(OH)D content, [19] but 25(OH)D has only recently been included in food frequency tables.

Classically, RCTs are used to evaluate drugs for treatment of disease. People who might benefit from taking the drug of interest are invited to participate and, when enrolled, are randomly assigned to either the treatment or placebo arm. A basic assumption is that no participants obtain the drug outside the RCT. Drug efficacy and adverse effects are the investigated outcomes. Pharmacokinetics of new drugs are also characterized to identify optimum circulating levels of the drug for efficacy and for safety. Safety has often been studied for vitamin D, but the 25(OH) D concentrations needed for efficacy in different disorders have been largely ignored for vitamin D RCT designs until recently [20,21]. Moreover, even in the placebo group in vitamin D RCTs, there are no participants without any vitamin D intake, so that such trials can only compare groups with higher versus lower vitamin D supply.

Uncontrolled intervention studies of vitamin D to prevent dental caries were conducted on adolescents from 1924 to 1945 [22]. Vitamin D RCTs were first conducted in about 1973 for treatment of epileptic patients taking anticonvulsant drugs [23] and became more popular in the early 21st century for studies of disease prevention, being designed in accordance with the guidelines for trials of therapeutic agents, as discussed.

Most vitamin D RCTs for nonskeletal disorders report no benefits using intention-to-treat analyses [4]. However, problems with such RCTs have included that few participants had any degree of vitamin D deficiency [25(OH)D concentrations < 20 ng/mL] and that moderate, or even unspecified, vitamin D supplementation was permitted in the control or in both study arms. In addition, vitamin D doses were not optimized to achieve any specific target 25(OH)D concentration even though specific thresholds have been identified for many health benefits [15]. Thus, few nonskeletal benefits have been revealed to date from 'intention to treat' analyses. However, significant benefits identified have included reduced cancer mortality rates [24], acute RTI risks [25] and autoimmune disease risks [26], as mentioned, along with reductions in several adverse pregnancy outcomes, discussed later.

Heaney outlined guidelines for optimizing design and analysis of clinical studies of nutrients in 2014 [27]:

1. Basal nutrient status must be measured, used as an inclusion criterion for entry into study, and recorded in the report of the trial.
2. The intervention (change in nutrient exposure or intake) must be large enough to change nutrient status and must be quantified.
3. The change in nutrient status produced in trial participants must be measured by validated laboratory analyses and recorded in the report of the trial.
4. The hypothesis to be tested must be that a change in nutrient status (not just a change in diet) produces the sought effect.
5. Conutrient status must be optimized to ensure that the test nutrient is the only nutrition-related, limiting factor in the response.

A version of those guidelines specifically for vitamin D also has been published [28]. No large vitamin D RCTs reported to date for prevention of chronic and infectious diseases have followed these guidelines, partly because most of the trials were designed before 2014. Most RCTs still fail to use 25(OH)D concentrations as a criterion for participation. If they did, enrolling the desired number of participants would be hard. Many RCTs do

not measure baseline or achieved 25(OH)D concentrations of any, let alone all, participants. Vitamin D_3 doses generally range from <1000 to 2000 IU/d and up to ~100,000 IU/month, with some trials using 4000 IU/d. No large vitamin D RCTs have yet optimized co-nutrient status. Magnesium concentration, for example, plays an important role in vitamin D metabolism and affects serum 25(OH)D concentrations upon vitamin D supplementation [29], and magnesium deficiency is common [30]. By contrast, some vitamin D RCTs gave the treatment arm calcium supplements which do not affect 25(OH)D concentrations but can affect health outcomes [31]. For example, calcium supplements but not high dietary calcium intakes may increase the risk of CVD [32].

Pilz and colleagues have also discussed secondary outcomes and subgroup analyses, noting that those analyses reported some beneficial effects of vitamin D supplementation but that they should be considered "explorative outcome" analyses [15]. The case could be made that even though the researchers did not propose looking for such outcomes in the trial design, if good mechanistic data exist to suggest that such results could be expected, they should be considered useful trial findings, especially since, given the large cost and effort needed to conduct large-scale vitamin D RCTs, it is doubtful that any further such trials will be carried out, as discussed later.

Mendelian randomization (MR) studies examine how genetic variation—typically single-nucleotide polymorphisms (SNPs)—affects health outcomes through the vitamin D pathways. Polymorphisms affecting serum 25(OH)D are used as proxies for long-term vitamin D provision [33]. SNPs are not thought to change in response to behavior or disease experiences, so that they should not be affected by confounding factors and are deemed suitable for assessing causality. However, whether epigenetic effects can modulate the effects of genetic variants throughout adulthood is the subject of ongoing research [34–36]. A total of 143 genetic variants associated with vitamin D have been identified in the UK Biobank dataset [37], and recent MR studies have used up to 77 SNPs [38]. Another recent study used 35 SNPs, accounting for 2.8% of the variation in 25(OH)D in the UK Biobank dataset [39]. MR analyses generally require many thousands, often ~100,000 participants, to obtain sufficient statistical power. Whereas linear MR analyses have indicated some significant effects of vitamin D, nonlinear analyses [for different levels of participant vitamin D status] may be more appropriate, since vitamin D effects are non-linear, particularly when J- or U-shaped curves are expected a priori [39,40].

Geographical and temporal ecological studies have been used to identify diseases affected by solar UVB exposure, an index for vitamin D production. Annual solar radiation was shown to be inversely correlated with U.S. colon cancer mortality rates by the brothers Garland in 1980 [41], who hypothesized vitamin D production as the mechanism. Many more ecological studies of cancer incidence and/or mortality rates with respect to indices of solar UVB doses have since been reported [16].

Ecological studies are often considered hypothesis generating and, indeed, have led to many further studies exploring vitamin D's role in reducing cancer risks [16]. Many other diseases also have incidence and/or mortality rates inversely correlated with solar UVB doses: anaphylaxis/food allergy, atopic dermatitis and eczema, attention deficit–hyperactivity disorder, autism, back pain, cancer, dental caries, type 1 diabetes, hypertension, inflammatory bowel disease, lupus, mononucleosis, multiple sclerosis (MS), Parkinson disease, pneumonia, rheumatoid arthritis, and sepsis. [42]; all those diseases have also been linked to low 25(OH)D concentrations. Unfortunately, geographical ecological studies are considered weak, not just being hypothesis generating, but because unmodeled confounders (residual confounding) might explain the findings. However, two ecological studies have looked at cancer mortality rates for whites in the United States with respect to summertime solar UVB doses. The findings with UVB alone [43] were later found to be virtually unchanged after including other cancer risk–modifying factors such as alcohol consumption, Hispanic heritage, lung cancer (as a proxy for smoking and diet), poverty, and degree of urbanization [44]. For diseases where solar UVB doses have been inversely correlated with risk, no non–vitamin D effects are apparent from UVB exposure. However,

for diseases with pronounced seasonal variations, such as CVD, hypertension, and RTIs, it appears that UVA-induced increases in serum nitric oxide (NO) as well as changes in temperature could also affect risk [45], though those diseases show no significant inverse correlations with annual UVB doses.

Reconciling differences between observational studies and RCTs regarding vitamin D is important for several reasons. One is that observational studies are generally not considered able to establish causality because some degrees of bias and confounding can never be totally excluded. For example, 'associations' could be due to unmodeled factors such as non–vitamin D health benefits of UV exposure [45]. RCTs are generally considered able to show causality and are relied on in medicine and public health policies for guidance but major vitamin D RCTs for nonskeletal effects have not shown any significant effects in primary outcome analyses. By contrast, MR studies are now reporting significant inverse correlations between genetically determined 25(OH)D concentrations and the risk of diseases such as CVD [40]. If observational studies can be shown to determine 25(OH)D concentration–health outcome relationships reliably, at least for some important diseases or outcomes, those studies could provide a basis for public health policies instead of waiting for "proof" of causality from RCTs that may never be carried out, as was the case for lung cancer due to smoking [46].

This review aims to present findings from observational studies of 25(OH)D concentrations and from RCTs of vitamin D supplementation for major health outcomes and to evaluate each approach's strengths and weaknesses together with brief discussions of results from other approaches, such as MR studies, the mechanisms of vitamin D known to be relevant to each outcome, and ecological studies relevant to possible health effects of vitamin D. The primary health outcomes considered are Alzheimer's disease (AD) and dementia; autoimmune diseases; cancers; CVD; COVID-19; major depressive disorder (MDD), type 2 diabetes mellitus (T2DM); hypertension; mortality (all-cause); and RTIs, as well as pregnancy and birth outcomes. This review is a narrative rather than a systematic review. It aims to summarize the existing literature regarding some common health outcomes and focuses on comparing findings from different study designs and discussing their strengths and limitations.

2. Results

2.1. Diseases and Outcomes

2.1.1. Autoimmune Diseases

In autoimmune diseases, an aberrant immune response is directed against normal human proteins [47]. Calcitriol modulates the immune system through effects on B cells, $CD4^+$ and $CD8^+$ cells, dendritic cells, innate lymphoid cells, macrophages, and unconventional T cells [48].

By March 2019, observational studies reported an inverse association between vitamin D status and developing autoimmune diseases, such as systematic lupus erythematosus, thyrotoxicosis, type 1 diabetes, MS, iridocyclitis, Crohn's disease, ulcerative colitis, psoriasis vulgaris, seropositive rheumatoid arthritis, and polymyalgia rheumatica [47].

In the VITAL trial, vitamin D supplementation reduced the risk of incident autoimmune diseases [26]. In the vitamin D arm, 123 participants in the treatment group and 155 in the placebo group developed a confirmed autoimmune disease (hazard ratio [HR] = 0.78 [95% confidence interval (CI), 0.61–0.99; $p = 0.05$]). The incident autoimmune diseases that differ in the raw numbers in the vitamin D versus placebo group were unspecified autoimmune disease (40 vs. 56), polymyalgia rheumatica (31 vs. 43), autoimmune thyroid disease (21 vs. 11), rheumatoid arthritis (15 vs. 24), and psoriasis (15 vs. 23). In this context, viral or bacterial infections, through increasing inflammation, are risk factors for autoimmune thyroid disease [49], rheumatoid arthritis [50], and psoriasis [51]. The finding that vitamin D supplementation reduces autoimmune disease risks can be supported logically, by the ability of vitamin D to reduce the risk of many infections through inducing the secretion of cathelicidin (LL-37) [52] and by reducing inflammation per se [53].

2.1.2. Cancers

Cancers generally arise from genetic mutations of cells leading to tumor formation. The immune system maintains active cell surveillance to evaluate whether they belong in the organs or tissues where they are located. Vitamin D plays an important role in that process, regulating cellular differentiation, progression and apoptosis. Vitamin D also reduces angiogenesis around tumors and reduces the development of metastasis [16,54].

The first observational report of serum 25(OH)D and cancer outcome was a 1989 U.S. study [55] over 8 years in Maryland, involving 34 colon cancer cases diagnosed between August 1975 and January 1983 and 67 matched controls from a pool of 25,620 volunteers. The risk of colon cancer decreased with 25(OH)D concentrations above 20 ng/mL: for ranges of 4–19, 20–26, 27–32, 33–41, and 42–91 ng/mL, odds ratios (ORs) were 1.00, 0.48, 0.25, 0.21, and 0.73, respectively. The higher OR for 42–91 ng/mL was probably due to participants' taking vitamin D supplements, perhaps on medical advice to address concerns about osteoporosis [56]. A recent meta-analysis of colorectal cancer incidence with respect to serum 25(OH)D concentration in prospective studies found an insignificant increase in OR for 25(OH)D above 100 ng/mL compared to 87.5–100 ng/mL [57].

Many similar observational studies have been conducted, generally for colorectal cancer. A 2019 meta-analysis included 12 studies for men and 13 for women. Muñoz and Grant [16] point out that the authors of that meta-analysis did not consider or adjust the results for follow-up time. Thus, the reduced risk for higher 25(OH)D concentrations would have been underestimated, leading to the conclusion that men who developed colorectal cancer derived no benefit [57]. As shown in Figure 1 of [16], a linear regression fit to OR versus follow-up time had the equation OR = 0.74 + 0.031x years (r = 0.79) for men and OR = 0.77 + 0.081x years (r = 0.25) for women. Thus, adjusting the reported values for study duration could have yielded similar beneficial effects for men as for women because the apparent reductions decline with increasing follow-up time, probably due to changes in serum 25(OH)D concentrations [58–60]. The 2015 analysis showed that the regression fitted to the prospective observational studies of breast cancer incidence over time had an association for time zero, by extrapolation, that corresponded well with results from case–control studies in which 25(OH)D had been measured near the time of diagnosis [60].

An observational study of breast cancer incidence based on analysis of individual participant data from 3325 participants in two vitamin D RCTs [31,61] and 1713 participants in the GrassrootsHealth (GRH) prospective cohort study was reported in 2018 [11]. Serum 25(OH)D concentrations were measured at baseline and after a year of follow-up in the RCTs and every 6 months in the GRH cohort. Participants in the treatment arms of the RCTs took 1100 IU/d of vitamin D_3 plus 1450 mg/d of calcium in the first Lappe study and 2000 IU/d of vitamin D_3 plus 1500 mg/d of calcium in the second Lappe study while participants in the GRH study chose their own vitamin D supplemental intake. A total of 77 women developed breast cancer during the study periods, of whom 14 were from the GRH cohort. Women who achieved a 25(OH)D concentration >60 ng/mL compared with those achieving <20 ng/mL had a large risk reduction: HR = 0.20 (95% CI, 0.05–0.82, p = 0.03; p_{trend} = 0.04) after adjustment for age, body mass index (BMI), smoking status, and calcium supplement intake. That study's strengths included that many participants were from vitamin D RCTs, that all had 25(OH)D concentrations measured at baseline and follow-up, and that 25(OH)D concentrations ranged from 10 to 75 ng/mL.

Cancers—RCTs

The *VITAL* study was an ambitious project aiming to determine whether vitamin D and omega-3 supplementation reduced cancer rates, CVD incidence, and mortality rates [8]. VITAL enrolled 25,871 participants, including 18,046 white people, 5106 African Americans, and 2719 people of other or unknown race or ethnic group. Mean (SD) age was 67 ± 7 years, mean (SD) BMI was 28 ± 6 kg/m^2, and mean (SD) baseline 25(OH)D concentrations in the vitamin D treatment group were 28 ng/mL for 395 males and 32 ng/mL for 441 females as determined from using blood samples collected mainly

in winter or spring. Participants were recruited between November 2011 and March 2014, and the intervention ended December 31, 2017, yielding a median follow-up period of 5.3 years. Participants in the vitamin D treatment arm took 2000 IU/d of vitamin D_3. However, all participants up to 70 years of age were permitted to take additional amounts of up to 600 IU/d of vitamin D and up to 800 IU/d if aged over 70 years.

The VITAL study did not show that supplementing with 2000 IU/d of vitamin D_3 reduced risk of incident cancer according to intention-to-treat analyses [8]. However, in secondary analyses, people with BMI < 25 kg/m^2 had a reduced risk (HR = 0.76 [95% CI, 0.63–0.90]). The baseline and achieved 25(OH)D concentrations for those participants with such data gave values of 33.3 and 45.9 ng/mL, respectively. The difference between baseline and achieved 25(OH)D concentration was 12 ng/mL for three BMI categories (healthy weight, <25; overweight, 25 to <30; and obese, $\geq$30 kg/m^2). Evidently, the vitamin D supplementation used did not counter obesity-related inflammation, because obesity increases vitamin D requirements [62]. In a 26-week RCT involving 52 participants aged 18–50 years given 7000 IU/d of vitamin D_3, mean serum 25(OH)D concentrations increased from 13 to 44 ng/mL, but inflammatory markers including high-sensitivity C-reactive protein (hsCRP) were unaffected [63]. Chronic inflammation is an important cancer risk factor and meta-analyses haves associated CRP with breast cancer (HR = 1.14 [95% CI, 1.01–1.28]; OR = 1.23 [95% CI, 1.05–1.43]); colorectal cancer (OR = 1.34 [95% CI, 1.11–1.59]); and lung cancer (HR = 2.03 [95% CI, 1.59–2.50]) [64].

Why have RCTs not shown that vitamin D supplementation reduces risk of cancer incidence? That could be explained in three ways:

1. With findings based on vitamin D dose rather than achieved 25(OH)D concentration, enrolled participants would include those with relatively high 25(OH)D concentrations, lowering the chances of detecting reduced cancer incidence.
2. Higher 25(OH)D concentration lower cancer mortality risks more strongly than it reduces cancer incidence rates [24].
3. Vitamin D simply has no significant effect on cancer incidence.

Table 1 shows that vitamin D's effect was always stronger for cancer mortality rate than incidence rate in studies comparing those outcomes.

One narrative review includes 25 papers (8 RCTs on cancer patients, 8 population RCTs, and 9 observational studies) found through March 2021, published between 2003 and 2020. That review revealed some evidence that vitamin D supplementation in cancer patients could improve cancer survival, but no significant effect was reported in RCTs [68]. Some observational studies reported evidence associating vitamin D supplementation with increased survival among cancer patients, and only one study indicated an opposite effect. Those findings, therefore, were inconclusive.

Prospective or retrospective cohort studies evaluating the association between blood 25(OH)D level and survival outcomes in women with breast cancer were included in another review [69]. Outcome measures included overall survival, breast cancer–specific survival, and disease-free survival. Twelve studies involving 8574 female breast cancer patients were identified and analyzed. In comparing the lowest with highest category of baseline 25(OH)D level, the pooled adjusted HR was 1.57 (95% CI, 1.35–1.83) for overall survival, 1.98 (95% CI, 1.55–2.53) for disease-free survival, and 1.44 (95% CI, 1.14–1.81) for breast cancer–specific survival.

Table 1. Findings regarding cancer incidence and mortality rates with respect to 25(OH)D concentrations or vitamin D supplementation.

Study	Change in 25(OH)D	Incidence, RR or HR (95% CI)	Mortality, RR or HR (95% CI)	Author
Observational Studies				
Harvard Health Professionals Follow-Up Study, all cancer	10 ng/mL	RR = 0.83 (0.74–0.92)	RR = 0.71 (0.60–0.83)	Giovannucci et al. [65]
Adult patients living in Olmsted County, Minnesota, all less skin cancer	<12 ng/mL	HR = 1.56 (1.03–2.36)	HR = 2.35 (1.01–5.48)	Johnson et al. [66]
Meta-analysis, breast cancer	High vs. low	RR = 0.92 (0.83–1.02)	RR = 0.58 (0.40–0.85)	Kim et al. [67]
VITAL, exclude first 2 years	Vitamin D treatment vs. placebo	HR = 0.94 (0.83–1.06)	HR = 0.75 (0.59–0.96)	Manson et al. [8]
RCTs				
RCTs, meta-analysis	All participants	(12 RCTs) SRR = 0.99 (0.04–1.03)	(6 RCT) SRR = 0.92 (0.82–1.03)	Keum et al. [24]
RCTs, meta-analysis	Normal-weight individuals	(1 RCT) SRR = 0.76 (0.60–0.94)		Keum et al. [24]

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 95% CI, 95% confidence interval; HR, hazard ratio; RCT, randomized controlled trial; RR, relative risk; SRR, summary RR; VITAL, VITamin D and OmegA-3 TriaL.

Cancers—Geographical Ecological Studies

Geographical ecological studies strongly support the UVB–vitamin D–cancer hypothesis as proposed in 1980 by the brothers Garland after comparing the U.S. colon cancer mortality rate map with the map of annual solar radiation [41]. In the latest review, four single-country or region ecological studies published between 2006 and 2012 reported inverse correlations between indices of solar UVB doses for incidence rates for 21 cancers. Furthermore, five single-country ecological studies published between 2006 and 2011 reported inverse correlations between indices of solar UVB doses and mortality rates for 24 different types of cancer [16].

The Centers for Disease Control and Prevention continues to post maps of cancer incidence rates averaged by state (https://gis.cdc.gov/Cancer/USCS/#/AtAGlance/, accessed 21 June 2022). The most recent maps are for 2019 and are similar to those in the *Atlas of Cancer Mortality Rates in the United States*, *1950–94* [70]. A comparison of the lung cancer incidence maps with maps of U.S. obesity prevalence shows a high correlation between states with the highest obesity rates and states with the highest lung cancer rates. That finding is consistent with the finding that inflammation is an important risk factor for lung cancer [64] and that vitamin D does not reduce the raised CRP associated with obesity [63]. Similar findings are reported for colorectal cancer but not for female breast cancer [64]. Recent MR studies using nonlinear methodology do show significant effects of higher genetically determined vitamin D status in reducing CRP in deficient subjects [40].

Geographical ecological studies are based on indices of solar UVB doses. A concern is that solar UVB could be an index for more than one mechanism of solar UV, though that is considered unlikely. In support, a recent review examined the role of three UV mechanisms related to the seasonality of CVD, hypertension, and infectious diseases [45]. Reasonable evidence exists that solar UVB has non–vitamin D effects and that solar UVA increases serum NO concentrations, both of which can contribute to the seasonality of several diseases. However, cancers have little seasonal variation [71], and the mechanisms

by which vitamin D reduces cancer risk are well known [16]. Thus, geographical ecological studies should be considered a strong support for the UVB–vitamin D–cancer hypothesis.

Such ecological studies are useful for several reasons:

- They are easy to conduct because they can be based on publicly available data.
- They include many participants.
- No participants are omitted.
- The analysis can include many other cancer risk–modifying factors averaged at the population level.
- They can be used to locate cancer hot spots globally.
- Analyses can be performed for different ethnicities and races and can be repeated for different periods.

Cancer—Mendelian Randomization Study

A 2021 MR study of genetically predicted cancer incidence used up to 77 independent SNPs for 25(OH)D, representing about 4% of the normal phenotypic variation in serum 25(OH)D, based on analysis of more than 400,000 UK Biobank participants [38]. Various cancer datasets with a genome-wide association study dataset were used. The total number of cancer cases included 122,977 for breast cancer, 25,509 for epithelial ovarian cancer, 12,906 for endometrial cancer, 79,148 for prostate cancer, 12,874 for melanoma, and 10,279 for esophageal cancer. The only cancer with a statistically significant result for increased genetically determined 25(OH)D was epithelial ovarian cancer, with an adjusted odds ratio (aOR) of 0.89 (95% CI, 0.82–0.96). Findings for endometrial, lung, mucinous, neuroblastoma, and pancreatic cancers showed aORs < 1.00 but were not statistically significant. The aORs for skin cancers and melanoma were in the expected direction: melanoma, 1.05 (95% CI, 0.90–1.23); squamous cell carcinoma of the skin, 1.02 (95% CI, 0.88–1.19); and basal cell carcinoma of the skin, 1.16 (95% CI, 1.04–1.28). The aOR for prostate cancer also was in the same direction, 1.11 (95% CI, 0.93 to 1.33). However, that MR study probably failed to detect significant associations between genetically determined 25(OH)D concentrations and cancer risk, having too few cancer cases, and since the genetic variant size effect on serum 25(OH)D values was no larger than the variance of the 25(OH)D assays used at time of measurement.

2.1.3. Cardiovascular Disease

Vitamin D could reduce CVD risk by reducing risks of vascular inflammation, endothelial dysfunction, proliferation of smooth muscle cells, hypertension, and secondary hyperparathyroidism [72]. Vitamin D also has several mechanisms to lower risk of metabolic syndrome [72], T2DM [73], seasonal influenza [74], and periodontal disease [75], that are all risk factors for CVD.

In the Framingham Offspring Study [76], serum 25(OH)D concentrations were measured in 1739 eligible participants between 1996 and 2001 followed up for a mean of 5.4 years. For the 688 hypertensive participants who developed a first CVD event, the fully adjusted HRs, compared with participants with 25(OH)D $\geq$ 15 ng/mL, were 2.07 (95% CI, 1.19–3.67) for 25(OH)D from 10 to <15 ng/mL and 2.43 (95% CI, 1.23–4.80) for those with 25(OH)D < 10 ng/mL; $p_{\text{trend}} = 0.003$. For those without hypertension, no significant differences were found.

A 2017 meta-analysis looked at total CVD events with respect to baseline 25(OH)D concentrations among 180,667 participants across 34 publications between 2008 and 2015 [77]. The RRs for an increase of 25(OH)D of 10 ng/mL varied as a function of follow-up time: for <5 years, RR = 0.84 (95% CI, 0.78–0.90); for 5 to <10 years, RR = 0.88 (95% CI, 0.77–1.01); and for $\geq$10 years, RR = 0.92 (95% CI, 0.89–0.96). The linear fit to the RR is RR = 0.81 + 0.0084x years. Again, the longer the follow-up, the lower the apparent beneficial effect of higher baseline 25(OH)D concentration. Figure 2 in that review showed that the RR varied from 1.00 at 0 ng/mL to 0.8 $\pm$ 0.02 at ~20 ng/mL.

Data from four prospective studies of CVD mortality rate versus baseline serum 25(OH)D concentrations were used to generate a graphical meta-analysis of HR versus 25(OH)D concentration for CVD mortality rate (Table 2 and Figure 1). The HR values were adjusted so that the aHR adjusted value at the highest 25(OH)D concentration for each study fell on the second-order fit to the data [78]. Thus, vitamin D is apparently more effective at reducing risks of CVD mortality than at reducing incidence rates, in general agreement with the finding for all-cancer incidence and mortality rates.

Table 2. Data on mean 25(OH)D concentration and aHR for CVD mortality extracted from four research articles as further adjusted as just discussed [78–81].

Mean 25(OH)D Concentration (ng/mL)	aHR	aHR Adjusted	aHR Adjusted, 95% CI Low	aHR Adjusted, 95% CI High	Author
12.5	1.20	1.39	1.01	1.90	Melamed et al. [79]
21.0	0.88	1.02	0.80	1.32	
28.3	0.83	0.96	0.75	1.24	
36.0	1.00	1.16	1.16	1.16	
7.00		2.36	1.17	4.75	Ginde et al. [78]
15.0		1.54	1.01	2.34	
25.0		1.26	0.85	1.88	
35.0		1.20	0.79	1.81	
45.0		1.00	1.00	1.00	
7.00	2.64	3.55	2.27	2.96	Semba et al. [80]
13.0	1.68	2.26	1.03	5.02	
21.0	2.19	2.95	1.42	6.20	
30.0	1.00	1.35	1.35	1.35	
15.0	1.52	1.79	1.52	2.11	Liu et al. [81]
25.0	1.09	1.29	1.11	1.51	
35.0	1.00	1.18	1.18	1.18	

Abbreviations: 95% CI, 95% confidence interval; 25(OH)D, 25-hydroxyvitamin D; aHR, adjusted hazard ratio.

A recent analysis of data from the UK Biobank dataset reported a linear inverse relationship between adjusted 25(OH)D concentration and odds of an incident CVD event [40]. The odds were 1.08 $\pm$ 0.03 at 4 ng/mL, 1.0 at 20 ng/mL, and 0.88 $\pm$ 0.03 at 52 ng/mL of 25(OH)D. Figure 3 in that article suggests that correcting 25(OH)D to above 20 and 40 ng/mL could reduce CVD incidence rates in the UK by 4% $\pm$ 2% and 6% $\pm$ 4%, respectively.

A long-term follow-up study of patients treated at the U.S. Veterans Health Administration from 1999 to 2018 showed reduced risk of MI for people supplementing with vitamin D [13]. The patients followed up were those with 25(OH)D concentration < 20 ng/mL at baseline. Many were counseled to take vitamin D supplements. For patients who achieved >30 ng/mL, the HR for MI was 0.65 (95% CI, 0.49–0.85) compared with those who achieved 20–30 ng/mL and 0.73 (95% CI, 0.55–0.96) as compared with those with 25(OH)D < 20 ng/mL. That effect is higher than that found in the UK Biobank study but only applied to MI. That study used propensity scores to correct for potential systematic differences between comparison groups. Included covariates were age, sex, BMI, hypertension, diabetes, coronary artery disease, congestive heart failure, peripheral arterial disease, chronic kidney disease, chronic obstructive pulmonary disease, smoking, concomitant therapies (aspirin, statin, and beta-blockers), and low-density lipoprotein cholesterol levels. The researchers also used propensity score–weighted, stabilized inverse probability of treatment weights to obtain unbiased estimates of treatment effects; hence strengthening

the design and analysis. The propensity score is the probability of treatment assignment conditional on observed baseline characteristics and allows designing and analyzing an observational (nonrandomized) study so that it mimics some particular characteristics of an RCT [82].

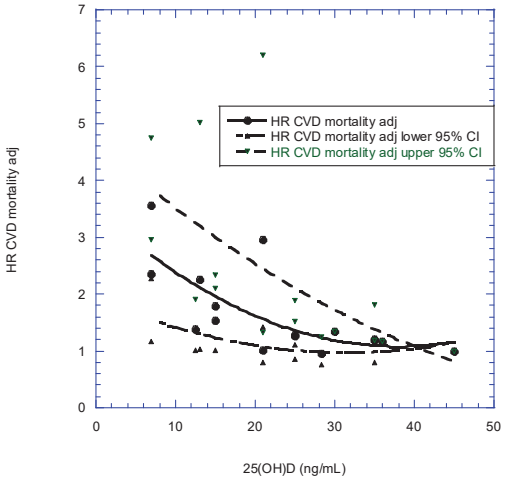

Figure 1. Hazard ratio (HR) and 95% confidence interval (CI) for CVD mortality according to mean serum 25-hydroxyvitamin D [25(OH)D] concentrations for data from four prospective observational studies.

Parathyroid hormone (PTH) plays an important role in CVD risk. Several observational studies report independent increases in risk for CVD for low 25(OH)D concentrations and high PTH concentrations [83–85]. Elevated PTH but not vitamin D deficiency has been associated with increased risk of heart failure [86]. An explanation for the difference in the relative contributions of 25(OH)D deficiency and higher PTH concentrations is that the PTH–25(OH)D relationship changes over time, as this ratio changes with age [87]. Figure 2 in that article shows median PTH values as a function of 25(OH)D concentration for ages < 20, 20–40, 40–60, and >60 years. When the PTH value of 65 pg/mL is used as the threshold concentration for significantly increased CVD events [84], the value is exceeded, with 25(OH)D concentrations of 7 ng/mL for those aged 40–60 years and of ~14 ng/mL for those > 60 years. This PTH effect may also help explain why CVD rates increase with age as well as why they are higher in winter than in summer [45].

A meta-analysis of observational studies of risk of atrial fibrillation with respect to serum 25(OH)D concentration as a continuous variable reported higher 25(OH)D concentrations associated with lower risk: OR = 0.96 (95% CI, 0.83–1.00; $p = 0.04$) from five cohort studies and 0.85 (95% CI, 0.79–0.92; $p < 0.0001$) from four case–control studies [88].

Cardiovascular Disease—RCTs

The VITAL study did not find that vitamin D supplementation reduced overall CVD risks [8], most likely because few participants had baseline 25(OH)D concentrations below 20 ng/mL (only 68/502 in the vitamin D treatment and placebo arms combined). A recent MR study however, clearly indicated that CVD risk decreases rapidly with increasing 25(OH)D concentration in those with baseline vitamin D inadequacy, [40] though the outcomes were not analyzed with respect to achieved 25(OH)D, PTH or season of the CVD event. However, half the participants had hypertension treated with medication and treating hypertension can significantly reduce CVD burden [89] and overall mortality rate [90].

Cardiovascular Disease—Mendelian Randomization

The MR study just mentioned showed that genetically predicted 25(OH)D concentrations correlated inversely with CVD risk [40], based on 44,510 CVD cases and 251,269 controls from the UK Biobank; it succeeded because the researchers calculated genetically instrumented 25(OH)D concentrations (as estimated using the 40 principal genetic factors affecting serum 25(OH)D) for each of 100 strata of baseline serum 25(OH)D concentration. Individual participants with 25(OH)D of 10 ng/mL had an OR of 1.11 (95% CI, 1.05–1.18) vs. 20 ng/mL. Individual participants with 25(OH)D of 30 ng/mL had an OR of 0.98 (95% CI, 0.97–0.99) vs. those at 20 ng/mL. The OR for the lowest genetically instrumented 25(OH)D concentration (4 ng/mL) was ~2.3 (95% CI, 1.4–3.5); their reductions in CVD risk reached 6% ± 3% as baseline 25(OH)D concentrations rose to 40 ng/mL.

The mechanisms by which vitamin D might reduce CVD risks are well understood. A recent meta-analysis reported inverse associations of vitamin D status with risks of metabolic syndrome and obesity, BMI, dyslipidemia, blood pressure (BP), insulin resistance, and dysglycemia. Meta-analysis of data from seven RCTs reported that supplementation reduced BP, abdominal obesity, and insulin resistance—all recognized CVD risk markers [63]. Mechanistic evidence shows that vitamin D reduces inflammation, an important factor in the progression of atherosclerosis. Vitamin D also lowers serum triglyceride levels and reduces the secretion of matrix metalloproteinase enzymes 2 and 9, which macrophages release when infiltrating arterial plaque, a process causing the plaque disruption that leads to acute arterial events through overlying clot formation [91]. Such findings are supported by the adverse effects of vitamin D deficiency seen experimentally on the vasculature [92].

2.1.4. COVID-19

Grant and colleagues suggested (April 2020) that higher 25(OH)D concentrations should reduce risk of COVID-19 incidence and death [93]. As of 16 June 2022, more than 2100 publications were found at pubmed.gov by searching for "vitamin D, COVID-19". Most observational reports were case–control studies with 25(OH)D measured around the time of a SARS-CoV-2–positive PCR test or COVID-19 symptoms.

Vitamin D's mechanisms to reduce risk of SARS-CoV-2 infection and COVID-19 incidence, severity, and death are now well known. These include reduced viral replication through inducing cathelicidin (LL-37) and reduced risk of the cytokine storm [93,94].

A large database of test results for SARS-CoV-2 positivity for patients who had serum 25(OH)D concentrations measured during the 12 months preceding the positive test by Quest Diagnostics between 9 March and 19 June 2020 was examined [95]. The 25(OH)D concentrations were seasonally adjusted, with a value of 32 ± 12 (mean ± SD) ng/mL. As analyzed by race/ethnicity, for white, non-Hispanic patients, SARS-CoV-2 positivity declined from 9% for 25(OH)D < 20 ng/mL to 5% for 60 ng/mL; for Hispanic patients, from 16% for <20 ng/mL to 8% at 50 ng/mL and from 19% for <20 ng/mL to 10% at 25(OH)D > 55 ng/mL in Black non-Hispanic subjects. However, such studies can be confounded by factors linked to both low circulating 25(OH)D concentrations and more severe COVID-19 [96].

More recently, two observational studies reported COVID-19 risk for people using vitamin D supplementation. One was from Barcelona, using data for vitamin D prescriptions and risk of SARS-CoV-2 or COVID-19 [97]. Most identifiable vitamin D use is by prescription rather than over-the-counter, though current usage is probably mainly over-the-counter. Patients on cholecalciferol treatment achieving 25(OH) D concentrations ≥30 ng/mL had lower risk of SARS-CoV2 infection, lower risk of severe COVID-19, and lower COVID-19 mortality than supplemented 25(OH)D–deficient patients (HR = 0.66 [95% CI, 0.46–0.93]; $p = 0.02$). Patients on calcifediol treatment achieving serum 25(OH)D concentrations ≥ 30 ng/mL had lower risks of SARS-CoV2 infection, of severe COVID-19, and of COVID-19 mortality than 25(OH)D–deficient patients not supplementing with vitamin D (HR = 0.56 [95% CI, 0.42–0.76]; $p < 0.001$).

The second study was based on 4599 veterans in U.S. Department of Veteran Affairs health care facilities. Participants received a positive SARS-CoV-2 test and a blood 25(OH)D test between 20 February 2020, and 8 November 2020, and were monitored for up to 60 days. After adjustment for all covariates, including race/ethnicity and poverty, a significant independent inverse dose–response relationship was evident between increasing 25(OH)D concentrations (from 15 to 60 ng/mL as a continuous variable) and decreasing probability of COVID-19–related hospitalization (from 24.1% to 18.7%; $p = 0.009$) and mortality (from 10.4% to 5.7%; $p = 0.001$) [98].

COVID-19 outcomes with respect to serum 25(OH)D concentrations from meta-analyses based on 72 observational studies published through 30 May 2021, were reported recently [99]; vitamin D deficiency/insufficiency increased odds of developing COVID-19, severe COVID-19, and death. Mean 25(OH)D concentrations were 4–5 ng/mL lower in people with COVID-19 than in controls for all outcomes. Associations between vitamin D deficiency/insufficiency and death were insignificant when studies with high risk of bias or reporting unadjusted effect estimates were excluded but bias and heterogeneity risks were high across all analyses. Discrepancies in timing of vitamin D testing, definitions of severe COVID-19, and of vitamin D deficiency/insufficiency contributed to that heterogeneity while serum 25(OH)D concentrations fall with severe COVID-19 illness [10], though whether that effect might increase the health benefits of adequate supplementation is a postulate urgently needing to be tested.

A meta-analysis of vitamin D supplementation on COVID-19–related outcomes from publications through January 2022 reported significantly reduced risk of admission to intensive care units (RR = 0.35 [95% CI, 0.20–0.62]) and reduced mortality (RR = 0.46 [95% CI, 0.30–0.70]) [100], though vitamin D status had no significant independent effect on COVID-19 incidence. Recently, positive effects of vitamin D supplementation on hospitalized COVID-19 patients were reported [101], adjuvant supplementation reducing hospital stay and duration of oxygen requirement.

Given that observational studies on the potential effectiveness of vitamin D supplementation for the prevention and treatment of COVID-9 are justifiably criticized for their limitations, inherent to observational study designs, readers should note that several public health recommendations during the COVID-19 pandemic, such as for the fourth SARS-CoV-2 vaccine dose, were also based solely on observational data and risk to benefit estimates but not on RCTs. Results from vitamin D RCTs are limited and inconsistent for COVID-19, so that no final conclusion can be drawn to date on the value of vitamin D supplementation regarding COVID-19.

2.1.5. Diabetes Mellitus Type 2

T2DM usually develops after a long period of increased insulin resistance (IR) where increased insulin concentrations become necessary to activate insulin effects in tissues such as liver and muscle. The demand for increased insulin secretion leads to islet beta cell damage and eventual inadequacy of insulin secretion with resultant hyperglycemia. Calcitriol is essential for normal insulin secretory responses to glucose and reduces the abnormal hepatic production of glucose and triglycerides seen in IR. Vitamin D effects also suppress inflammatory processes active in IR that contribute to the increased risks of both T2DM, and CVD seen with IR [102–105]. Vitamin D reduces inflammation and regulates intracellular Ca^{2+} level in many cell types, including islet beta cells and hepatocytes, contributing to reduced IR as reviewed by Szymczak-Pajor and colleagues [106].

Observational studies have long reported that 25(OH)D concentrations are inversely correlated with T2DM and with features of the metabolic syndrome [107]. A 2007 meta-analysis noted that for prevalence of T2DM in non-black people, the OR for highest versus lowest 25(OH)D concentration was 0.71 (95% CI, 0.57–0.89) [108]. A 2013 meta-analysis of 21 prospective studies involving 76,220 participants and 4996 incident T2DM cases showed that for each 4-ng/mL increase in 25(OH)D concentration, risk of T2DM decreased by 4% (95% CI, 3–6%), with the lowest risk near 60 ng/mL [109]. That analysis was updated by

adding studies published up to 31 August 2016 [110]. With data from 31 (nested) case–control and cohort studies comparing participants with 25(OH)D values of approximately 20–30 ng/mL with those in the lowest category, the OR was 0.77 (95% CI, 0.72–0.82). With 23 (nested) case–control and cohort studies comparing participants with the highest 25(OH)D concentrations with those with the lowest 25(OH)D category, the OR was 0.66 (95% CI, 0.61–0.73). Again, reduced T2DM risks were seen up to 25(OH)D concentrations of ~50–60 ng/mL.

Diabetes Mellitus Type 2—RCTs

The *D2d* trial evaluated whether vitamin D supplementation could reduce risk of progressing from prediabetes to T2DM [9] and enrolled 2423 prediabetic participants, mean (SD) age 60 ± 10 years. Participants mean (SD) 25(OH)D concentration was 28 ± 10 ng/mL. Half were randomized to take 4000 IU/d of vitamin D_3 and half to take a placebo during a mean time of 2.5 years. On the basis of intention to treat, the HR for vitamin D compared with placebo was 0.88 (95% CI, 0.75–1.04; $p = 0.12$). In secondary analyses of this dataset, participants with BMI < 30 kg/m^2 had reduced T2DM risk, HR = 0.71 (95% CI, 0.53–0.95) as did those not given calcium supplements, HR = 0.81 (85% CI, 0.66–0.98).

A further secondary analysis of D2d data was then made, based on intratrial 25(OH)D concentrations [111]. For participants in the vitamin D treatment arm, each 10-ng/mL increase in 25(OH)D concentration above 20–30 ng/mL up to >50 ng/mL was associated with a significant HR of 0.75 (95% CI, 0.68–0.82) for progression to diabetes. No effect was seen in the placebo arm. Those findings strongly support the Heaney guidelines for basing analyses of vitamin D RCTs on achieved 25(OH)D concentrations, and show the importance of assessing vitamin D's effects on health by vitamin D status, not by dosages [27].

Diabetes Mellitus Type 2—Mendelian Randomization

Two MR analyses found that genetic variants of 25(OH)D were causally linked to risk of T2DM. The first one was reported in 2018 [112]. It used data from the China Kadoorie Biobank as well as other studies. A 10-ng/mL higher biochemically measured 25(OH)D was associated with a 9% (95% CI: 0–18%) lower risk of diabetes in the China Kadoorie Biobank. In a meta-analysis of all studies, a 10-ng/mL higher genetically instrumented 25(OH)D concentration was associated with a 14% (95% CI: 3–23%) lower risk of diabetes ($p = 0.01$) using two synthesis SNPs. An equivalent difference in 25(OH)D using a genetic score with 4 SNPs was not significantly associated with diabetes (odds ratio 8%, 95% CI: −1% to 16%, lower risk, $p = 0.07$), but had some evidence of pleiotropy.

A second MR analysis was performed using data on genetic variants for 25(OH)D from a genome-wide association study on UK Biobank subjects [n = 329,247] of European ancestry. A higher genetically instrumented 25(OH)D was causally linked to reduced risk of T2DM (OR per standard deviation increase in 25(OH)D = 0.95 [95% CI, 0.91–0.99]; $p = 0.01$) [113]. That study also confirmed vitamin D's causal role by studying two SNPs of the vitamin D–activating enzyme, where the HR for each 1-SD increase in 25(OH)D = 0.89 (95% CI, 0.82–0.98; $p = 0.02$).

2.1.6. Hypertension

Hypertension is generally defined by a systolic BP (SBP) >140 mmHg and a diastolic BP (DBP) > 90 mmHg. One risk causal factor for hypertension is endothelial dysfunction [114] that is related to lower availability of NO, an important vasodilator. Vitamin D can reduce endothelial dysfunction by suppressing renin production, which reduces activity of the renin–angiotensin–aldosterone system and increases expression of endothelial NO synthase (eNOS) [115]. Vitamin D also reduces production of reactive oxygen species and cyclooxygenase 1 (COX-1) mRNA and protein expression [116], thereby reducing production of endothelium-derived vasoconstrictive factors [117].

Researchers have found inversely correlated serum 25(OH)D concentrations with BP, starting with cross-sectional studies in 2005 [118] and nested case–control studies

in 2007 [119]. Forman and colleagues included 613 men from the Health Professionals Follow-Up study and 1198 women from the Nurses' Health Study with measured baseline 25(OH)D concentrations who were followed up for 4–8 years. During the 4-year period of follow-up, 61 men and 129 women developed hypertension. The multivariable RRs comparing lowest with highest deciles of 25(OH)D were 2.31 (95% CI, 2.03–2.63) in men and 1.57 (95% CI, 1.44–1.72) in women.

A meta-analysis including 10 prospective studies from 2007 to 2015 reported that a linear increase of each 10 ng/mL in 25(OH)D resulted in an RR of 0.95 (95% CI, 0.90–1.00) [120].

Another meta-analysis of dose–response curves for hypertension versus 25(OH)D concentrations from 11 prospective cohort studies showed that the RR at 6 ng/mL versus 29 ng/mL = 1.37 (95% CI, 1.13–1.65). The RR decreased quasi-linearly up to 29 ng/mL before plateauing with 25(OH)Ds of ~50 ng/mL, where the RR was approximately 0.9 (95% CI, 0.8–1.0) [121]. The same meta-analysis found no effect for vitamin D supplementation on BP from the analysis of 27 RCTs.

A Canadian open-label vitamin D supplementation study looked at how raising serum 25(OH)D concentrations affected BP and hypertension [12]; the 8155 participants were given free vitamin D_3 and other supplements and counseled on how to achieve a 25(OH)D concentration > 40 ng/mL, with medically supervised dose adjustments. Of 592 participants hypertensive at enrollment, 71% were no longer hypertensive 12 ± 3 months later. Their mean (SD) SBP dropped by 18 ± 19 mmHg (95% CI, −24 to −12 mmHg) if not taking BP medications or by 14 ± 21 mmHg (95% CI, −18 to −9 mmHg) if taking BP medications after joining the program. Decreases in DBP were similar. However, for patients not hypertensive at enrollment, decreases in both SBP and DBP were insignificant. Though an observational study, the quality appears to be higher than that of many vitamin D RCTs. For example, many parameters were evaluated at baseline and tabulated by 25(OH)D concentration in 20-ng/mL increments. Values for most parameters, including SBP and DBP, did not vary significantly with baseline 25(OH)D concentration < 60 or 80 ng/mL. At the end of 1 year, mean (SD) serum 25(OH)D concentration had increased from 35 ± 15 ng/mL to 45 ± 16 ng/mL, but mean SBP, DBP, and pulse pressure were unchanged. After correction for possible confounding factors, only participants who were vitamin D insufficient (20–30 ng/mL) at baseline and achieved 25(OH)Ds > 40 ng/mL at follow-up had a lower risk of hypertension (OR = 0.10, 95% CI, 0.01–0.87; $p = 0.03$).

An unconsidered factor for BP risk is that solar UVA induces release of NO into the circulation, which can significantly lower BP as reported in 2014 by a team led by Weller [122]. The researchers showed that UVA irradiation of the skin lowers BP, that UVA increases nitrite production and reduces circulating nitrate, and that UVA increases blood flow in the forearm, the vasodilator NO being released by photolysis of nitrite to nitrate.

Hypertension—RCTs

Several vitamin D RCTs have looked at BP and hypertension. One studied black patients during the winters of 2008 and 2010 [123]. A total of 283 black people with median age 51 years were enrolled with a median 25(OH)D concentration of 16 ng/mL (interquartile range [IQR], 11–23 ng/mL), median SBP of 122 mmHg (IQR, 110–136 mmHg), and median DBP of 78 mmHg (IQR, 71–86 mmHg). Participants were assigned to four groups for 3 months of treatment in ~equal numbers for placebo, 1000, 2000, or 4000 IU/d of vitamin D_3 daily. The 3-month change in SBP per 1000 IU/d of vitamin D_3 given was −1.4 ± 0.7 mmHg (mean ± SE); $p = 0.04$, whereas that for DBP was −0.5 ± 0.5 mmHg.

Another vitamin D RCT regarding BP was conducted in Austria between June 2011 and August 2014 [124]. It enrolled 200 hypertensive participants with mean (SD) age 60 ± 11 years and 25(OH)D concentrations below 30 ng/mL. Half of the patients were assigned to the vitamin D treatment arm and received 2800 IU/d of vitamin D_3 for 8 weeks, whereas the other half received a placebo. The mean (SD) change in SBP was −0.4 (95% CI, −2.8 to 1.9) mmHg ($p = 0.71$). Data in this study [124] were reanalyzed for achieved 25(OH)D concentration [125], suggesting reduced SBP for high versus low

25(OH)D concentrations between 5 and 55 ng/mL (threshold of ~23 ng/mL). However, changes in this very short trial were not statistically significant.

Hypertension—Mendelian Randomization

An MR study regarding vitamin D and BP and hypertension was published in 2014 [126]. In a meta-analysis of data from 142,255 participants, the synthesis score for genetic increases in 25(OH)D was associated with a reduced risk of hypertension (OR per allele = 0.98 [95% CI, 0.96–0.99]; $p = 0.001$). In instrumental variable analyses, each 10% increase in genetically instrumented 25(OH)D concentration was associated with a change in SBP of −0.37 mmHg (95% CI, −0.73 to 0.003; $p = 0.052$), in DBP of −0.29 mmHg (95% CI, −0.52 to −0.07; $p = 0.01$), and an 8.1% decrease in hypertension (OR = 0.92 [95% CI, 0.87–0.97]; $p = 0.002$).

2.1.7. Mortality, All-Cause

All-cause mortality rate is mainly due to deaths from CVD, T2DM, respiratory disease, AD and other dementias, autoimmune diseases, and cancer, all of whose mortality rates are inversely correlated with serum 25(OH)D concentrations. Meta-analyses of prospective observational studies also report significant inverse correlations with all-cause mortality rates. One review involved 13 studies published between 2006 and 2010, including 5562 deaths among 62,548 participants [127]. That study showed a significant inverse relationship for 25(OH)D concentrations and mortality below 20 ng/mL. A second meta-analysis based on 32 studies published pre-2013 reported a similar cutoff value of 30 ng/mL [128]. A European individual participant data meta-analysis with 26,916 participants, of whom 6802 died during the median follow-up of 10.5 years, indicated a significant inverse correlation between baseline 25(OH)D concentration and all-cause mortality rates for values < 20 ng/mL [129]. The observational study based on veterans treated by the U.S. Veterans Administration Health System reported a HR of 0.61 (95% CI, 0.56–0.67) for all-cause mortality rate for participants who achieved 25(OH)Ds >30 ng/mL vs. those remaining at <20 ng/mL during 20 years of follow-up [13]. An MR study showed significant inverse correlations between genetically determined 25(OH)D concentrations and all-cause mortality rate [130], as a more recent study also reports [131].

2.1.8. Respiratory Tract Infections

Vitamin D reduces the risk of RTIs by increasing antimicrobial peptide gene expression, thereby increasing serum concentrations of human cathelicidin (LL-37) and defensins [52]; it also shifts the cytokine balance from proinflammatory T-helper (Th1) cell cytokine production to the production of anti-inflammatory Th2 cell cytokines [132].

Interest in vitamin D's role in RTIs began before the antibiotic era with a finding of reduced tuberculous illness with exposure to sunshine. That interest reignited with publication of the hypothesis that seasonal variations in solar UVB doses might explain seasonal variations in epidemic influenza, with peak rates in winter, from Cannell and colleagues [133]. That hypothesis was later shown to be only partially correct since temperature and absolute humidity also play important roles [134], while UV has a minor role [135]. However, that hypothesis did lead to further studies, e.g., showing that vitamin D supplementation reduced the risk of influenza A but not influenza B [136]. A meta-analysis of vitamin D RCTs for acute RTIs showed that daily or weekly supplementation with vitamin D significantly reduced risk of acute RTIs [6]. A later meta-analysis reported that maximal protection from vitamin D supplementation was achieved with daily doses of <400 IU for up to 12 months in younger participants (aged 1–16 years) with baseline 25(OH)D concentrations < 10 ng/mL [25].

2.1.9. Alzheimer's Disease and Other Dementias

The mechanisms by which vitamin D reduces risk of AD and dementia are reasonably well understood. Vitamin D triggers several neural pathways that may protect against

those neurodegenerative mechanisms, including the deposition of amyloid plaques, inflammatory processes, neurofibrillary degeneration, glutamatergic excitotoxicity, excessive intraneuronal calcium influx, and oxidative stress [137].

Two recent meta-analyses looked at dementia and AD risks [138,139]. Jayedi and colleagues calculated the reduction in risk of dementia or AD for a 10-ng/mL increase in 25(OH)D for each study. Table 3 presents the data used in their meta-analysis. Values for mean 25(OH)D concentration were obtained from each article (except Littlejohns et al.). Mean 25(OH)D concentration was taken as the midpoint of 25(OH)D associated with a change in the HR in their Figure 2 and they found that higher 25(OH)D concentrations (up to 32 ng/mL) significantly reduced risk of dementia when Swedish study data [140] were omitted. Figure 2 shows the results for dementia (regression fit equation; HR = 3.3 − 0.16 × [25(OH)D] + 0.0027 × $[25(OH)D]^2$, r = 0.95). Figure 3 shows the same equation for AD; HR = 3.3 − 0.17 × [25(OH)D] + 0.0032 × $[25(OH)D]^2$, r = 0.75. This analysis highlights the value of considering different ways of doing meta-analyses of observational study data with vitamin D status. Graphs for follow-up time were also generated, but proved less informative than those based on 25(OH)D concentrations.

Table 3. Data associated with the observational studies in the meta-analysis by Jayedi and colleagues [138].

Country	Mean 25(OH)D (ng/mL)	Follow-Up (yrs)	Vascular Dementia, HR (95% CI) for 10 ng/mL Increase	Alzheimer's, HR (95% CI) for 10 ng/mL Increase	Author
US	12	5.6	0.57 (0.34–0.97)	0.61 (0.41–0.93)	Littlejohns et al. [141]
France	14	11.4	0.60 (0.47–0.78)	0.60 (0.47–0.78)	Feart et al. [142]
Finland	16	17.0	0.77 (0.62–0.92)		Knekt et al. [143]
Denmark	16	21.0		0.91 (0.82–1.02)	Afzal et al. [130]
Netherlands	20	13.3	0.77 (0.63–0.95)	0.73 (0.59–0.93)	Licher et al. [144]
US	22	16.6	0.93 (0.79–1.07)		Schneider et al. [145]
US	25	9.0	1.01 (0.88–1.14)	1.09 (0.95–1.12)	Karakis et al. [146]
Sweden	28	12.0	1.04 (0.93–1.17)	0.95 (0.81–1.12)	Olsson et al. [140]

Abbreviations: 95% CI, 95% confidence interval; 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio.

MR studies [147–149] have reported genetically predicted 25(OH)D concentrations correlating inversely with AD risk, as do vitamin D protein binding levels [150] with dementia risks [39].

2.1.10. Major Depressive Disorder

Neuroinflammation appears to be the key factor in onset and progression of MDD [151]. Mechanisms likely to underlie the beneficial effects reported for vitamin D in treating MDD [152] reviewed were, in particular, its antioxidant, anti-inflammatory, proneurogenic, and neuromodulatory properties. Vitamin D also modulates concentrations of gut microbiota, reducing bacterially induced activation of NF-κB in the intestine, thereby reducing remote inflammation further [153].

A meta-analysis of 29 RCTs with 4504 participants concluded that vitamin D supplementation reduced MDD incidence (standardized mean difference: −0.23) and improved responses to the treatment of depression (standardized mean difference: −0.92) [154]. The effects of 2800 IU/d vitamin D over >8 weeks were significant for both prevention and treatment. A meta-analysis of data from seven prospective observational studies of 16,287 older adults with 1157 cases of incident depression reported a pooled HR for depression per 10-ng/mL increase in 25(OH)D of 0.88 (95% CI, 0. 78–0.99) [155].

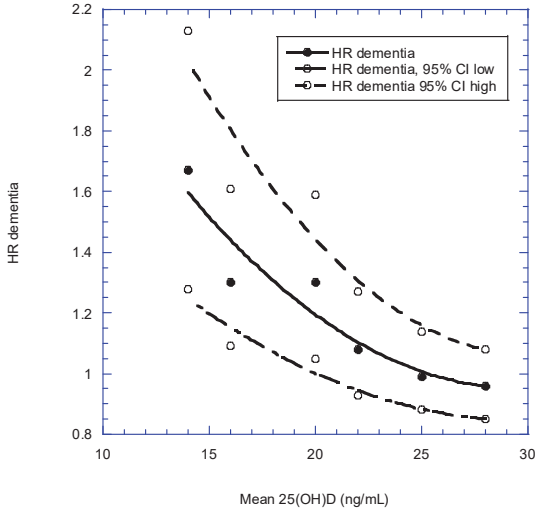

Figure 2. Hazard ratio (HR; mean and 95% confidence interval [CI]) for dementia according to mean 25-hydroxyvitamin D [25(OH)D] concentration in each of the six studies included in the meta-analysis [138].

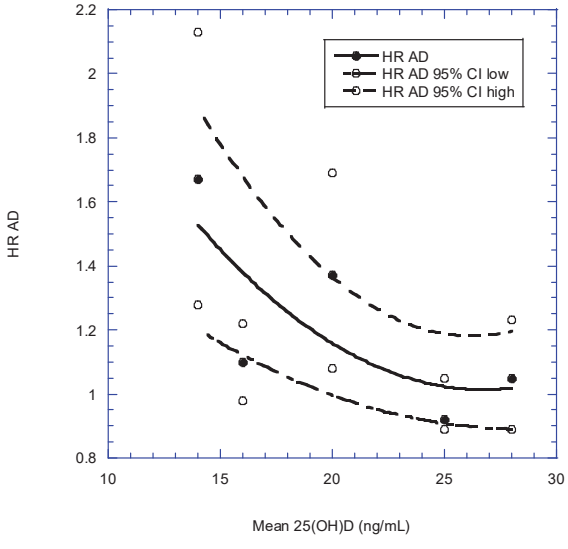

Figure 3. Hazard ratios (HR; mean and 95% confidence interval [CI]) for Alzheimer's disease (AD) according to mean 25-hydroxyvitamin D [25(OH)D] concentrations for each of the six studies included in the meta-analysis [138].

Another meta-analysis including 41 RCTs with 53,235 participants reported that vitamin D supplementation alleviated depressive symptoms (Hedges's $g = -0.32$ [95% CI, -0.41 to -0.23]; $p < 0.001$; $I^2 = 88\%$; grade, very low certainty) [156]. Vitamin D supplementation > 2000 IU/d reduced depressive symptoms ($g = -0.41$ [95% CI, -0.56 to -0.26]) much better than <2000 IU/d ($g = -0.18$ [95% CI, -0.29 to -0.08]). A ~linear decrease in

HR was seen with increases in serum 25(OH)D concentration, HR falling to 0.43 (95% CI, 0.20–0.92) at 65 ng/mL.

A nested case–control study conducted in Taiwan indicated that although moderate UVB exposure reduced risk of depression, high UVB exposure increased this risk [157]. The adjusted incidence rate ratio for moderate versus low UVB exposure was 0.89 (95% CI, 0.84–0.95); for high UVB, 1.12 (95% CI, 1.02–1.26); for very high UVB, 1.71 (95% CI, 1.51–1.95); and for extreme UVB, 2.79 (95% CI, 2.44–3.18). The authors proposed that high UVB exposure increased production of reactive oxygen species, thereby increasing inflammation. Those results also suggested that observational studies of risk of depression with changes in serum 25(OH)D concentration might be affected by whether 25(OH)D concentrations are raised by solar UVB exposure or by vitamin D supplementation in participants with higher rather than deficient baseline 25(OH)D concentrations.

A study based on the UK Biobank and two other databases showed that serum 25(OH)D concentrations were lower in participants with depression. However, MR analysis reported nonsignificant correlations between genetically determined 25(OH)D concentrations and risk of depression [158]. Thus, depression may lower 25(OH)D concentrations, perhaps by reducing sunlight exposure.

2.1.11. Pregnancy Disorders and Neonatal Outcomes

Vitamin D status during pregnancy is important for both mother and fetus. Wagner and Hollis published a comprehensive review on this topic in mid-2018 [159]. Observational studies reported significant inverse correlations between maternal 25(OH)D concentrations and risk of maternal problems, including preeclampsia [160], altered placental vascular pathology [161], cesarean delivery rates [162,163] gestational diabetes [164,165], and preterm birth rates [166,167]. Such studies also report similar associations for infant health outcomes, including brain dysfunction [168,169] and respiratory disorders [170]. Table 4 shows representative pregnancy outcomes with respect to maternal serum 25(OH)D concentrations from several observational studies [165–180].

Table 4. Representative pregnancy and infant outcomes with respect to maternal serum 25(OH)D concentration in observational studies.

Outcome	Setting	Outcome	Finding	Author
Birth weight				
Cesarean delivery, primary		Maternal 25(OH)D < 15 vs. >15 ng/mL	aOR = 3.8 (95% CI, 1.7–8.6)	Merewood et al. [162]
Cesarean delivery, primary		Maternal 25(OH)D < 15 vs. >15 ng/mL	aOR = 2.0 (95% CI, 1.2–3.3)	Scholl et al. [163]
Gestational diabetes	Meta-analysis, 29 studies	<20 vs. >20 ng/mL	OR = 1.39 (95% CI, 1.20–1.60)	Hu et al. [164]
Gestational diabetes	Meta-analysis, 27 studies	>20 vs. >30 ng/mL	OR = 1.26 (95% CI, 1.13–1.41)	Milajerdi et al. [165]
Preeclampsia	Hospital study	Early-onset severe preeclampsia, 10-ng/mL increase in 25(OH)D	aOR = 0.37 (95% CI, 0.22–0.62)	Robinson et al. [171]
Preeclampsia	Meta-analysis, 13 studies	Comparison of 25(OH)D	OR = 0.57 (95% CI, 0.51–0.65)	Serrano-Diaz et al. [172]
Preeclampsia	Meta-analysis, 11 studies	25(OH)D < 30 vs. > 30 ng/mL	OR = 1.44 (95% CI, 1.26–1.64)	Aguilar-Cordero et al. [160]
Preterm delivery	Hospital study	25(OH)D < 20 vs. > 40 ng/mL, <16 wks	OR = 3.8 (95% CI, 1.4–10.7)	Wagner et al. [166]

Table 4. *Cont.*

Outcome	Setting	Outcome	Finding	Author
Preterm delivery	Meta-analysis, 16 studies	25(OH)D < 20 vs. > 20 ng/mL	OR = 1.25 (95% CI, 1.13–1.38)	Zhou et al. [167]
Preterm delivery	Open-label vitamin D supplementation	25(OH)D > 40 vs. > 20 ng/mL	SES adjusted OR = 0.41 (95% CI, 0.24–0.72)	McDonnell et al. [14]
Infant outcomes				
Brain dysfunction	Language impairment in childhood vs. maternal 25(OH)D at 18 weeks pregnancy	6–18 vs. 29–62 ng/mL	aOR = 1.97 (95% CI, 1.00–3.93, $p < 0.05$)	Whitehouse et al. [168]
Brain dysfunction	Risk of ADHD, meta-analysis, 5 studies	High vs. low 25(OH)D	OR/RR = 0.72 (95% CI, 0.59–0.89)	Garcia-Serna et al. [169]
Respiratory dysfunction	Risk of asthma vs. maternal 25(OH)D, 11 studies	High vs. low 25(OH)D	OR = 0.78 (95% CI, 0.69–0.89)	Shi et al. [170]
Respiratory dysfunction	Risk of wheeze vs. maternal 25(OH)D, 14 studies	High vs. low 25(OH)D	OR = 0.65 (95% CI, 0.54–0.79)	Shi et al. [170]

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 95% CI, 95% confidence interval; ADHD, attention deficit–hyperactivity disorder; aOR, adjusted odds ratio; OR, odds ratio; SES socioeconomic status.

Pregnancy Outcomes in Interventional Studies

An open-label vitamin D supplementation observational study involved 1064 women delivering singleton births at the Medical University of South Carolina between September 2015 and December 2016 [14]. Women were given free bottles of 5000-IU vitamin D_3 and counseled on how to achieve a 25(OH)D concentration >40 ng/mL. Achieved 25(OH)D concentration was also measured. The goal was to see whether raising serum 25(OH)D concentration >40 ng/mL could reduce risk of preterm birth (<37 weeks of gestation). The socioeconomic status aOR was 0.41 (95% CI, 0.24–0.72). The gestation period increased from 36.8 ± 0.3 weeks with 25(OH)Ds~5 ng/mL to 38.3 ± 0.2 weeks at 25 ng/mL after supplementation

Meta-analyses of RCTs have largely, but not consistently, reported benefits of vitamin D supplementation in reducing risks of adverse pregnancy outcomes including low birth weight [173,174], cesarean delivery rates [175], gestational diabetes [174], preeclampsia [176], and preterm delivery [177] (see Table 5).

Table 5. Meta-analyses of vitamin D supplementation RCTs, comparing vitamin D supplementation with placebo.

Outcome	Setting	Finding	Author
Birth weight, low	Review of 5 RCTs	RR = 0.55 (95% CI, 0.35–0.87)	Palacios et al. [174]
Birth weight	Review of 11 RCTs	Increased weight, mean difference = 114 g (95% CI, 63–165 g)	Gallo et al. [173]
Cesarean delivery, primary	Review of 6 RCTs	OR = 0.9 (95% CI, 0.7–1.2)	Gallo et al. [173]
Cesarean delivery in Iran	Review of 5 RCTs	RR = 0.61 (95% CI, 0.44–0.83)	Saha and Saha, [175]
Gestational diabetes	Review of 4 RCTs	RR = 0.51 (95% CI, 0.27–0.97)	Palacios et al. [174]
Preeclampsia	Review of 27 RCTs	RR = 0.37 (95% CI, 0.26–0.52)	Fogacci et al. [176]
Preterm delivery	Review of 17 RCTs	RR = 0.70 (95% CI, 0.49–1.00)	Kinshella et al. [177]

Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio; RCT, randomized controlled trial; RR, relative risk.

Maternal vitamin D deficiency leads to epigenetic changes in offspring and is associated with increased risks to bone health in childhood and to increased childhood obesity, those problems appearing to persist into later life. These changes serve as a further reason to ensure vitamin D adequacy in pregnancy [178,179].

3. Discussion

Why most vitamin D RCTs used small vitamin D doses—generally at or below 2000 IU/d—is puzzling. One reason may be the Institute of Medicine's 2011 report setting 4000 IU/d of vitamin D as the upper limit and recommending 600 IU/d for those aged up to 70 years and 800 IU/d for those aged above 70 years—intakes aiming to achieve 25(OH)D of at least 20 ng/mL [180]. The committee was concerned about reports of U-shaped 25(OH)D concentration–health outcome relationships at that time because of a National Cancer Institute review [181] of findings from prospective studies with respect to baseline 25(OH)D concentrations for breast, esophageal, pancreatic, and prostate cancer. The cited studies had drawbacks such as long follow-up times up to 15 years, which reduce the apparent benefit of higher baseline 25(OH)D concentration [16]; not evaluating whether participants changed vitamin D supplementation practices before or during the follow-up periods; and not evaluating vitamin D status during the study or at study completion. Some U-shaped 25(OH)D concentration–health outcome relationships seen have been proposed to be due to participants' starting vitamin D supplementation shortly before enrolling in prospective studies, for example, after recommendations by doctors over concerns for bone health [56]. Meta-analyses of prospective studies now report inverse relationships with 25(OH)D concentrations for breast cancer incidence from case–control and nested case–control studies, nonsignificant relationships for pancreatic cancer incidence, and direct relationships for prostate cancer incidence [16]. Prostate cancer is unique in that increased risk of mild prostate cancer is due to increased absorption of calcium and phosphorus [16]. However, a number of observational studies have reported U-shaped or reversed J-shaped relations for mortality rates with respect to serum 25(OH)D concentrations such as one that found a significantly increased risk of mortality rate for 25(OH)D concentrations > 120 ng/mL [182].

Several recent vitamin D supplementation observational studies showed that higher daily-dose vitamin D_3 supplementation was relatively safe and effective. One such study involving 19 lactating women reported that 6400 IU/d of vitamin D_3 supplementation safely raised 25(OH)D concentrations from 32 ± 4 to 59 ± 7 ng/mL [183]. That group of researchers provided concrete evidence about the safety of 6400 IU/d even for pregnant women to the NIH. A study in Canada had several thousand participants taking vitamin D_3 doses of their choice. A total of 2229 participants achieved a 25(OH)D concentration > 40 ng/mL by taking 2600 ± 2800 IU/d (for those with mean (SD) value of 47 ± 5 ng/mL) to 6300 ± 500 IU/d, for a mean (SD) value of 117 ± 15 ng/mL [12].

More recently, McCullough and colleagues [57] reported that supplementing over 400 hospital inpatients with 5000–50,000 IU/d of vitamin D_3 for 30 months was safe and significantly raised serum 25(OH)D concentrations and lowered PTH concentrations. Thus, future vitamin D RCTs should use higher doses of vitamin D_3 where necessary, for example, to achieve target status. However, although we have solid safety data on vitamin D supplementation, we should nevertheless be cautious, particularly with using high doses in older and/or ill people who may be more prone to vitamin D toxicity.

Hill's criteria for causality in a biological system.

Long before vitamin D RCTs were conducted, Sir Austin Bradford Hill outlined the criteria for causality in a biological system in an address to the Royal Society of Medicine in 1965 [184]. The criteria important for vitamin D include:

1. Strength of association
2. Consistency in findings
3. Temporality, that is, the risk factor must be experienced before the event
4. Biological gradient, that is, dose–response relationship.

5. Plausibility, for example, mechanisms that can explain the relationship
6. Coherence with known biological facts
7. Experiment, for example, RCT
8. Analogy with related associations

Added later [185]:

9. Accounting for confounding factors
10. Accounting for bias such as publication bias
11. Quality of study design

Hill stated that not all criteria need be satisfied to claim causality, but the more that are, the better.

Temporality seems to be interpreted as meaning that prospective studies after measurement of 25(OH)D should be used; however, as discussed, that approach can lead to underestimating the effect of higher 25(OH)D concentration. As long as the disease state does not affect serum 25(OH)D concentration, case–control studies with 25(OH)D measured near time of diagnosis should be acceptable.

Hill's criteria have been evaluated for vitamin D's role in several diseases, mainly using observational studies. Those studies reported beneficial effects of vitamin D on the basis of serum 25(OH)D concentrations, finding that nearly all criteria are satisfied except, in some cases, experimental verification for BP [12], cancer [186–189], CVD [190,191], COVID-19 [94,192], dementia [193], diabetes and pancreatic cancer [194], type 1 diabetes [195], MS [196,197], oral health [198], and periodontal disease [199].

4. Conclusions

Observational studies consistently report significant inverse correlations between serum 25(OH)D concentrations and health outcomes, but residual confounding cannot be completely excluded. Prospective studies are preferred over case–control studies on the basis of concern that the disease state could affect serum 25(OH)D concentrations. However, that concern appears to be mainly relevant for inflammatory and infectious diseases such as acute respiratory infections and not other outcomes. The same concern, however, exists when diseases are diagnosed at an advanced stage in their development, as with cancer. One major problem with prospective observational studies is that serum 25(OH)D concentrations change not only with season but also with respect to time, hence resulting in a potential underestimation of the effect of higher 25(OH)D concentrations as discussed earlier.

Another issue regarding observational studies is whether the cases and controls are well matched. Propensity score matching can help ensure good matches, given a sufficiently large pool of prospective controls. Another less-recognized limitation is that if cases and controls are not matched closely with respect to when blood was drawn to measure 25(OH)D concentration, additional bias may be introduced.

Another concern is whether all pertinent confounding factors were considered in the study's design and analysis. For causality, it is proposed that results of observational studies be included in the broader context of what is known about vitamin D in the health outcome of interest—for example, in relation to mechanistic data or on evaluation using Hill's criteria for causality [184].

RCTs have largely failed to support vitamin D's role in reducing risk of adverse health outcomes [15]. The main reasons appear to be that few vitamin D–deficient participants are enrolled, that low vitamin D doses are used, and that outcomes are evaluated by vitamin D dose rather than by achieved 25(OH)D concentrations. Moreover, secondary analyses not proposed in the trial protocols are generally ignored. Those analyses seem to be disregarded based on the concern that if many secondary analyses are conducted, some might accidentally find a significant result, in particular if multiple testing issues are not adequately considered. However, if the secondary outcome is one that researchers forgot to include in the protocol but which reasonably makes sense to include based on other evidence or on mechanistic data, such as the effect of race/ethnicity and BMI on cancer

risk in the VITAL study [8]—then such evidence should rather be accepted. Given the time, effort and expense required for major vitamin D RCTs, it seems unlikely that many more will be conducted soon, or ever. Thus, it is imperative to learn to use findings from completed trials and from other research approaches as efficiently as possible.

Examining the role of genetic factors might also be helpful in understanding the inconsistency of results between observational studies and RCTs since supplements might interact differently according to specific genotypes and variants [200]. Unfortunately, such potential genotype/supplement interactions have rarely been examined in RCT settings.

Observational studies using variable vitamin D dose supplementation and frequent (annual or semiannual) measurement of 25(OH)D concentrations and other pertinent data have several apparent advantages over traditional observational studies and RCTs. Permitting variable vitamin D doses allows large 25(OH)D ranges to be covered, which should make any significant health benefits more likely to become apparent [11,12].

MR studies that establish vitamin D's causal role for several health outcomes are now being reported. By using genetically predicted 25(OH)D concentrations for many participants combined with reported health outcomes, MR studies of large cohorts average out lifestyle factors in ways that match the effects of randomization in RCTs. Recently, stratifying subjects into ~100 subgroups by baseline 25(OH)D concentrations for separate MR analyses (which allows analysis of nonlinear data) provides an excellent approach to examining the effects of genetic increases in serum 25(OH)D at low baseline 25(OH)D concentrations [40], especially when those effects cannot be adequately considered when all the data are included in a single analysis.

Ecological studies have historically been useful in highlighting that UVB radiation reduces risk of diseases such as MS and cancers [42]. The advantages of ecological studies include the fact that many participants are studied and that data for many population-level confounding factors can be used. Geographical ecological studies surpass temporal ecological studies because the multiple factors involved in seasonal variations are hard to untangle, including UVB production of vitamin D, UVB non–vitamin D mechanisms, UVA-induced increases in serum NO concentrations, and temperature [45].

Knowledge of the mechanisms whereby vitamin D reduces particular adverse health outcomes is fundamental to understanding the problems of deficiency and should be routinely considered in designing future trials. The mechanisms by which correcting deficiencies can reduce tissue dysfunction in each of the common disorders we have discussed are all reasonably well understood which supports the case for ensuring better vitamin D provision in populations commonly afflicted with deficiency.

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Review

Genetic Determinants of 25-Hydroxyvitamin D Concentrations and Their Relevance to Public Health

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Abstract: Twin studies suggest a considerable genetic contribution to the variability in 25-hydroxyvitamin D (25(OH)D) concentrations, reporting heritability estimates up to 80% in some studies. While genome-wide association studies (GWAS) suggest notably lower rates (13–16%), they have identified many independent variants that associate with serum 25(OH)D concentrations. These discoveries have provided some novel insight into the metabolic pathway, and in this review we outline findings from GWAS studies to date with a particular focus on 35 variants which have provided replicating evidence for an association with 25(OH)D across independent large-scale analyses. Some of the 25(OH)D associating variants are linked directly to the vitamin D metabolic pathway, while others may reflect differences in storage capacity, lipid metabolism, and pathways reflecting skin properties. By constructing a genetic score including these 25(OH)D associated variants we show that genetic differences in 25(OH)D concentrations persist across the seasons, and the odds of having low concentrations (<50 nmol/L) are about halved for individuals in the highest 20% of vitamin D genetic score compared to the lowest quintile, an impact which may have notable influences on retaining adequate levels. We also discuss recent studies on personalized approaches to vitamin D supplementation and show how Mendelian randomization studies can help inform public health strategies to reduce adverse health impacts of vitamin D deficiency.

Keywords: 25-hydroxyvitamin D; vitamin D; genetic risk; heritability; personalized supplementation; genome-wide association study; Mendelian randomization

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1. Introduction

Interest in the genetic architecture of 25-hydroxyvitamin D (25(OH)D) has been active during the past couple of decades, promoted by heritability estimates from twin studies which suggest that up to 80% of the variability in 25(OH)D concentrations might be explained by genetic variation in some populations [1–5]. Also an evolutionary perspective provides strong cues about the importance of genetic variation for vitamin D metabolism [6], as differences in skin colour are believed to have evolved at least in part as an adaptation to ultraviolet B radiation exposure during migration to more northern latitudes, where reduction in skin pigmentation became critical to vitamin D synthesis. Important insights into the genetic architecture of 25(OH)D concentrations have been obtained from genome-wide association studies (GWASs) which have used information across thousands of genomes to find polymorphisms which are statistically associated with 25(OH)D. For this paper, we systematically looked through the GWAS literature for genes that influence serum 25(OH)D levels. We describe key variants for which evidence has been provided from several independent studies and address the public health importance and some of the uses of this information.

2. Materials and Methods

For the systematic search of GWASs, we searched the MEDLINE, Embase, Cochrane, CINAHL and NHGRI-EBI GWAS catalogue [7] databases for original studies and meta-analyses of studies performed in humans and published in English from inception to February, 2022. The search terms used were ("vitamin D" OR "calcidiol" OR "25-hydroxyvitamin D" OR "25(OH)D") AND ("genome-wide association study" OR "genome-wide association scan" OR "genome-wide association analysis") along with the expanded MeSH search terms (in titles, abstracts, or keywords). The search identified 791 publications in total. After excluding duplicate entries, 522 publications remained, of which 29 were relevant. These were further scrutinized to identify sample overlap. We also scrutinized references within the selected articles, and from studies otherwise known to the authors, with evidence on gene function queried using the gene ontology (GO) resource (http://geneontology.org/, accessed on 27 September 2022), KEGG (https://www.genome.jp/kegg/, accessed on 27 September 2022), ConsensusPathDB (http://cpdb.molgen.mpg.de/, accessed on 27 September 2022) and other NCBI (https://www.ncbi.nlm.nih.gov/guide/all/, accessed on 27 September 2022) databases.

3. Results

3.1. Genome-Wide Association Studies on 25(OH)D

Our literature search identified 29 published GWASs that looked for SNPs associated with 25(OH)D [8–36], although many of these analyses were conducted using overlapping samples. Most of the studies only include adult participants of white European ancestry (n = 6722 to 443,374) [8,10,14,16,19,20,22,24,26,29,30,32–34,36]. There were seven studies including data from transethnic analyses [15] and studies with small to modest sample sizes (n = 697 to 9823) which had been conducted using data from African Americans (n = 697 in the discovery sample) [25], African descent in the UK (n = 9354) [35], Hispanic (n = 1190) [9] and Asian populations (n = 1387 to 9823) [13,23,28,35]. There were also five small GWASs on children/toddlers/new-borns [11,12,18,21,27].

Three loci, including *DHCR7, CYP2R1*, and *GC* were consistently reported across European [8,10,14,16,19,20,22,24,26,34] and several non-European GWASs [11,13,15,18,21,23,25,27,28]. These loci were also confirmed in GWAS conducted in children/toddlers/newborns [11,12,18,21,27]. These genes fit with existing evidence of the involvement of their corresponding proteins in the vitamin D metabolic pathway. The *DHCR7* gene encodes the 7-dehydrocholesterol reductase, which is an enzyme that converts dehydrocholesterol to cholesterol in the skin, and affects the substrate availability for vitamin D3 synthesis, which is a precursor of 25(OH)D [37] (Figure 1). The *CYP2R1* gene encodes the enzyme in the cytochrome P-450 family 2R1, and is the primary 25-hydroxylase in the liver, converting vitamin D to 25(OH)D. In the circulation, vitamin D metabolites including 25(OH)D are mainly found bound to vitamin D binding protein (encoded by *GC*), which in most of the GWASs to date, have come up with the strongest signal for 25(OH)D. GWAS on non-European cohorts have also reported some novel loci (e.g., *FOXA2/SSTR4* [13], *HSPG2* [25], *TINK* [25] and *KIF4B* [15]) which have not been identified in GWAS studies on white European ancestry. Independent replication is lacking with respect to most of these novel loci, and many have no clear link with the vitamin D metabolic pathway. One exception is *CYP2J2*, discovered in a multi-ethnic cohort of 942 pregnant women of Malay, Indian and Chinese ancestry [27]. *CYP2J2* encodes an enzyme (Cytochrome P-450 family 2, subfamily J, polypeptide 2) shown in vitro to act as a vitamin D hydroxylase [38]. There is thus a strong biological basis for this association. It is notable that variants coding the α1-hydroxylase (*CYP27B1*) or the vitamin D receptor (*VDR*) have not been identified by the GWASs on 25(OH)D conducted to date. Concentrations of active $1,25(OH)_2D$ in the circulation are ~1000 times lower than those of 25(OH)D. Therefore, it is possible that differences arising from the conversion of 25(OH)D to $1,25(OH)_2D$, or those reflecting *VDR* related differences in the 'usage' of $1,25(OH)_2D$, may simply be too small to detect.

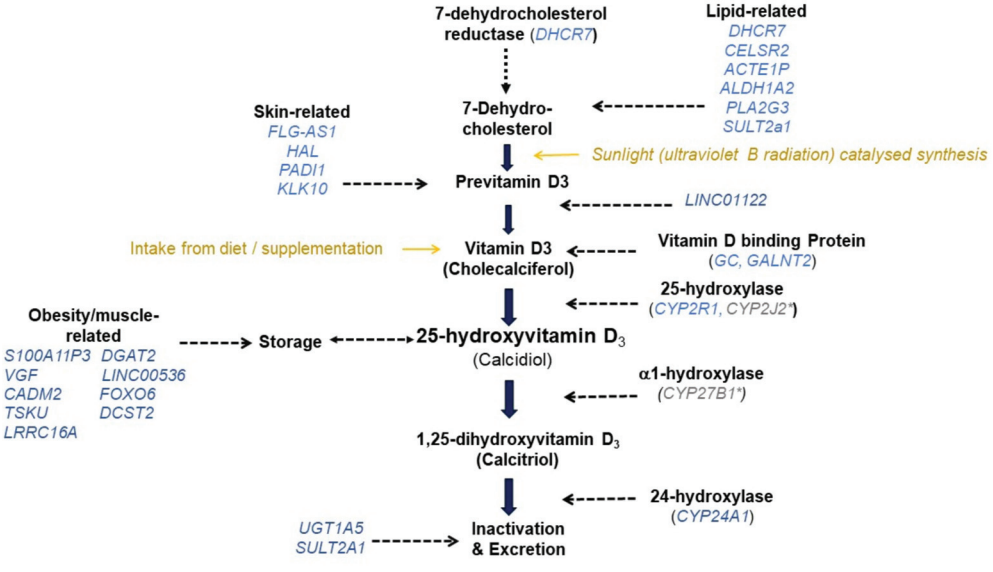

Figure 1. Possible role of selected replicated variants in the vitamin D pathway. * Indicates candidate genes with a confirmed role in vitamin D metabolism but which were not among the replicating variants. For the full list of single nucleotide polymorphism relating to each gene, please refer to Table 1.

Variants with Evidence for Replicated Association with 25(OH)D

In the largest GWAS published to date, identified variants for 25(OH)D concentrations were enriched in genes in the metabolic vitamin D pathway, lipid and lipoprotein pathways, and pathways related to skin properties. The liver, the brain and the skin were identified as the top three locations where 25(OH)D-associated loci may exert their actions [20]. In Table 1 we present more detailed information for the 35 common autosomal SNPs which were identified as top hits in the GWAS conducted in the UK Biobank [20], and which were associated in a consistent direction with serum 25(OH)D concentrations in the SUNLIGHT consortium meta-analyses [16,20,39]. In addition, there were 70 common variants that could not be replicated in the SUNLIGHT consortium meta-analyses. These included variants that are known to be pleiotropic (i.e., affect multiple traits) and/or affect cholesterol metabolism (e.g., *PCSK9*, *LIPC*, *ABCA1*, *CETP*, *APOE*, *APOB*, *APOC1*, *LIPG* and *LDLR*). It is possible that some of the differences between the UK Biobank findings and the SUNLIGHT consortium meta-analysis relates to the body mass index adjustment by the SUNLIGHT, which reduces the likelihood of adipose tissue related variants (reflecting differences in storage capacity) being detected. It should be noted, however, that replication does not imply causality, and the possible connection which we have identified with vitamin D metabolism/function is only based on literature available to date and may not fully describe the connection with 25(OH)D concentrations.

Table 1. Genetic variants with replicating evidence for an association with 25-hydroxyvitamin D concentrations.

Gene (SNP)	CHR	
PEX10 (rs6671730)	1	*PEX10* encodes a protein involved in import of peroxisomal matrix proteins. Mutations in *PEX10* gene have led to Zellweger syndrome [40] and osteopenia [41], for which vitamin D supplementation has been the treatment.
PADI1 (rs35408430)	1	*PADI1* encodes an enzyme, which catalyses the post-translational deimination of proteins by converting arginine residues into citrullines in the presence of calcium ions [42]. Deimination by PADIs occurs during epidermal differentiation [43], with possible influence on skin properties [20].
FOXO6 (rs7522116)	1	*FOXO6* encodes a protein that has been predicted to enable DNA-binding transcription factor activity, and RNA polymerase related DNA binding activity [44]. FoxO6 expression is downregulated in the brain of dietary obese mice [45].
CELSR2 (rs7528419)	1	*CELSR2* encodes the cadherin EGF LAG seven-pass G-type receptor 2 that is involved in contact-mediated communication, with cadherin domains acting as homophilic binding regions and the EGF-like domains involved in cell adhesion and receptor-ligand interactions [46].
FLG-AS1 (rs1933064)	1	FLG antisense RNA 1 (*FLG-AS1*) is an RNA Gene that is affiliated with the long non-coding RNA class. Skin pigmentation-related diseases such as Ichthyosis Vulgaris [47] and Peeling Skin Syndrome 6 [48] have been shown to be associated with FLG-AS1.
DCST2 (rs76798800)	1	*DCST2* gene encodes the DC-STAMP domain containing 2 protein that has been shown to be an important regulator of osteoclast cell-fusion in bone homeostasis [49]. *DCST2* gene is associated with early length and adult height [50].
GALNT2 (rs6672758)	1	*GALNT2* gene encodes the polypeptide N-acetylgalactosaminyltransferase 2 which is a member of the glycosyltransferase 2 protein family and which has been linked to post-translational modification of vitamin D-binding protein [51].
LINC01122 (rs727857)	2	*LINC01122* gene is an RNA gene that is affiliated with the lncRNA class [52]. *LINC01122* was one of the 989 differentially expressed genes which was significantly enriched in vitamin D3 biosynthesis [53].
CPS1 (rs1047891)	2	*CPS1* gene encodes the carbamoyl-phosphate synthase 1 which is a mitochondrial enzyme that catalyses synthesis of carbamoyl phosphate from ammonia and bicarbonate [54].
UGT1A5 * (rs2012736)	2	*UGT1A5* gene encodes the UDP glucuronosyltransferase family 1 member A5 which has been shown to transform small lipophilic molecules, into water-soluble, excretable metabolites [55]. Related isoenzymes have been identified as catalysts for 25(OH)D3 glucuronidation in the human liver [56].
CADM2 (rs6782190)	3	*CADM2* gene encodes the cell adhesion molecule 2 which is a member of the synaptic cell adhesion molecule 1 family [57]. In animal studies, *CADM2* is associated with metabolic traits [58], suggesting possible influence on vitamin D concentrations through its effect on obesity and storage capacity of 25(OH)D.
GC (rs705117, rs1352846)	4	*GC* gene encodes the vitamin D binding protein which binds to vitamin D and its plasma metabolites and transports them to target tissues [59].
CARMIL1/LRRC16A (rs78151190)	6	*CARMIL1* gene encodes the capping protein regulator and myosin 1 linker 1 with a role in actin filament network formation [60]. Approximately 10% of muscle tissue consists of actin, providing a possible link with 25(OH)D through storage capacity.
VGF (rs75741381)	7	*VGF* gene encodes a protein that is expressed in neuroendocrine cells and is upregulated by nerve growth factor [61]. *VGF* has been linked with appetite control [62], and diet-induced obesity [63], with a possible link through storage capacity.
LINC00536 (rs12056768)	8	*LINC00536* gene interacts with Wnt3a/β-Catenin signalling [64]. Wnt/β -Catenin signalling is an important signalling pathway in regulating adipose tissue lipogenesis with a possible link with 25(O)D through storage capacity.
GRID1 (rs77532868)	10	*GRID1* gene encodes the glutamate ionotropic receptor delta type subunit 1 which is a subunit of glutamate receptor channels that mediate the fast excitatory synaptic transmission in the central nervous system [65].
CYP2R1 (rs12794714)	11	*CYP2R1* gene encodes the cytochrome P450 family 2 subfamily R member 1 which acts as 25-hydroxylase of vitamin D [66].
TMEM151A (rs61891388)	11	*TMEM151A* has been predicted to be an integral component of membrane and *CD248* enables extracellular matrix binding activity and regulates endothelial cell apoptotic process.
AP002387.1/ACTE1P (rs1660839, rs12803256)	11	*ACTE1P* gene is an RNA gene. ACTE1P [67] and vitamin D [68] are both involved in adolescent idiopathic scoliosis (abnormal curvature of the spine), suggesting a possible role of ACTE1P in bone health.
S100A11P3 (rs12798050)	11	*S100A11P3* gene encodes the S100 calcium binding protein A11 pseudogene 3. It has multiple roles in buffering calcium ion concentration, participating in energy metabolism, regulating cell proliferation and differentiation [69].

Table 1. *Cont.*

Gene (SNP)	CHR	
DGAT2 (rs72997623)	11	*DGAT2* encodes the diacylglycerol O-acyltransferase 2, catalysing the synthesis of triglycerides [70]. Affects adipose tissue formation [71] with possible link to 25(OH)D storage.
GUCY2EP/TSKU (rs1149605)	11	*GUCY2EP* gene encodes guanylate cyclase 2E that is involved in chemosensation and *TSKU* gene encodes tsukushi, small leucine rich proteoglycan that has been predicted to act upstream/within several processes, including negative regulation of Wnt signaling pathway.
HAL (rs10859995)	12	*HAL* gene is upregulated during the differentiation of keratinocytes [72]. HAL deaminates L-histidine to trans-uronic acid [73], which in the stratum corneum absorbs UVB [74] and reduce the production 25(OH)D [75].
SEC23A (rs8018720)	14	*SEC23A* gene encodes the Sec23 homolog A, coat complex II component which plays a role in the ER-Golgi protein trafficking.
ALDH1A2 (rs261291)	15	*ALDH1A2* gene encodes aldehyde dehydrogenase 1 family member A2 which catalyses the synthesis of retinoic acid (RA) from retinaldehyde [76].
PDILT (rs77924615)	16	*PDILT*/*PDIA7* gene encodes the protein disulphide isomerase like, testis expressed which catalyses protein folding and thiol-disulphide interchange reactions [77].
SULT2A1 (rs212100)	19	*SULT2A1* gene encodes a liver- and intestine-expressed sulpho-conjugating enzyme that is responsible for the inactivation by sulphonation of 25(OH)D [78,79].
KLK10 (rs10426)	19	*KLK10* gene encodes the kallikrein related peptidase 10 that has been shown to play a role in dermal integrity [80].
CYP24A1 †	20	*CYP24A1* gene encodes cytochrome P450 family 24 subfamily A member 1 which is an important candidate for vitamin D metabolic pathway given that it initiates the degradation of 1,25-dihydroxyvitamin D3 by hydroxylation of the side chain [81]. In addition, this enzyme also plays a role in calcium homeostasis and vitamin D endocrine system [82].
PLA2G3 (rs2074735)	22	*PLA2G3* gene encodes the phospholipase A2 group III which functions in lipid metabolism and catalyses the calcium-dependent hydrolysis of the sn-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids [83].

* *UGT1A5*, *UGT1A6*, *UGT1A7*, *UGT1A8*, *UGT1A9*, *UGT1A10*. † rs6123359, rs17216707, rs2585442, rs2762943.

For many of the variants which have a replicated association with 25(OH)D we found some evidence that was compatible with a role in the vitamin D pathway (Figure 1, Table 1). Several of the variants were related to lipid levels, while others had been linked with skin integrity, suggesting a possible link with substrate availability for conversion to the circulating 25(OH)D metabolite. Despite body mass index adjustment in the SUNLIGHT consortium meta-analyses, for several of the replicating variants we observed suggested links with adiposity or muscle mass, which may be because these tissues serve as storage sites for 25(OH)D. In addition to confirming the role of 24-hydroxylation in the inactivation and removal of vitamin D metabolites, GWAS identified variants in *UGT1A5* and *SULT2A1* as potentially relevant. This suggests that sulphonation and glucuronidation pathways, which conjugate sulphur and glucuronide with vitamin D metabolites, are relevant for 25(OH)D excretion and/or recycling.

3.2. Heritability and the Genetic Contribution to the Prevalence of Deficiency

25(OH)D is a commonly used indicator of 'vitamin D status' with much of the concentrations determined based on availability of sunlight induced skin synthesis, with contributions from supplement intake and diet. According to twin- and family-based studies, there is great variability in the heritability of 25(OH)D, with overall estimates ranging from 28% to 80% [1–4]. As 'heritability' merely reflects the proportion of total variance that can be explained by genetic factors, it will be the higher with lower environmental contributions (total variance = genetic variance + environmental variance). Indeed, the highest heritability rates are seen in populations measured during winter, when contributions from sunlight synthesis (and hence, the environmental effects) are at their lowest. This may explain why a study on 510 middle-aged male twins found heritability estimates to be ~70% when assessed in winter compared to negligible (~0%) during summer [84]. Also a twin study of Hispanics and African Americans, reported heritabilities of 23%, 28% and 41% for 25(OH)D levels from data taken from California, Texas, and Colorado (n = 1530 individuals from 130 families), respectively [85]. These differences were broadly reflective of geographical location, such that the higher the latitude (and the less sunlight exposure) the higher the estimated heritability. However, seasonal differences in heritability have not been consistently observed, and for example in the recent 25(OH)D GWAS [20], heritability was higher in summer than in winter (0.19 vs. 0.10, respectively). Indeed, another way to estimate heritability is to use information from unrelated individuals using GWAS data (i.e., SNP heritability) and in the large UK Biobank GWAS on white Europeans, SNP heritability for serum 25(OH)D concentrations was estimated to be 13–16% [19,20]. Of practical relevance is the extent to which these genetic variants affect the circulating 25(OH)D levels and the prevalence of vitamin D deficiency. In Table 2**,** we show the distribution of 25(OH)D concentrations based on data from the 35 replicating variants in the UK Biobank. The odds of low concentrations are about halved for individuals in the highest 20% of vitamin D genetic risk score compared to the lowest quintile. Again, these genetic associations appear to be slightly stronger during summer compared to winter, possibly reflecting genetic differences in vitamin D skin synthesis. The overall difference in the mean 25(OH)D between individuals in the highest vs. lowest quartile of the GRS is about 9 nmol/L [40], which is similar to the association seen with self-reported use of vitamin D supplementation in the UK Biobank during winter (9.7 nmol/L) [84]. This suggests that if the supply from sunlight or diet is limited, differences in 25(OH)D concentrations by higher genetic burden may be of clinical relevance.

Table 2. Average 25-hydroxyvitamin D level and the odds of low concentrations by quintiles in vitamin D genetic risk score in the UK Biobank.

	Vitamin D Winter (*n* = 176,577)			Vitamin D Summer (*n* = 130,855)		
	25(OH)D Mean (SD)	<25 nmol/L OR (95% CI)	<50 nmol/L OR (95% CI)	25(OH)D Mean (SD)	<25 nmol/L OR (95% CI)	<50 nmol/L OR (95% CI)
Quintile 1 (Lowest 20%)	39.16 (17.41)	Reference	Reference	52.13 (17.37)	Reference	Reference
Quintile 2	41.84 (18.47)	0.79 (0.76–0.82)	0.75 (0.73–0.78)	56.16 (18.51)	0.72 (0.66–0.79)	0.68 (0.66–0.71)
Quintile 3	43.73 (19.36)	0.68 (0.66–0.71)	0.64 (0.61–0.66)	58.50 (19.20)	0.63 (0.57–0.69)	0.56 (0.54–0.58)
Quintile 4	45.40 (20.14)	0.60 (0.58–0.62)	0.54 (0.53–0.56)	60.65 (20.05)	0.53 (0.48–0.58)	0.49 (0.47–0.51)
Quintile 5	47.51 (21.25)	0.52 (0.50–0.54)	0.47 (0.45–0.48)	64.05 (21.17)	0.42 (0.38–0.47)	0.39 (0.37–0.40)

Genetic risk score calculated using 35 variants with replicated association with 25(OH)D concentrations. Adjusted for age, sex, month in which blood sample was taken, fasting time before blood sample was taken, sample aliquots for measurement, assessment centres, SNP array, and top 40 genetic principal components. Vitamin D winter classified as November to May and vitamin D summer as June to October, based on distribution of 25(OH)D concentrations in the UK biobank [86].

3.3. Genetic Differences in Response to Supplementation and the Need for Personalized Approaches

There were 25 independent loci which were suggestive of gene–environment (GxE) interaction in the recent GWAS [20], suggesting that the size of the genetic association with 25(OH)D can vary by environmental factors influencing serum 25(OH)D concentrations. For five loci, including *CYP2R1* and *SEC23A* there was a genome-wide significant interaction with season [19,20], where the carriers of 25(OH)D-lowering alleles appeared to be less responsive to season compared to non-carriers. This could suggest that some individuals may be more prone to low serum 25(OH)D levels regardless of the season of measurement [19]. In an earlier genome-wide GxE analysis, carriers of 25(OH)D-lowering allele at the *CYP2R1* locus were less responsive to dietary vitamin D intake [16]. A similar interaction with vitamin D lowering alleles has also been observed in the context of the *GC* locus and vitamin D supplementation [87], of vitamin D3-fortified bread and milk consumption [86,87] and UVB treatment [88,89].

There has been recent interest in genetic risk scores (GRS) that combine variants according to their vitamin D lowering alleles, and which look into whether individuals with genetically low 25(OH)D are less or more responsive to treatments for correcting low vitamin D status [87–89]. One study used a GRS combining variants in the *CYP2R1* and *GC* loci, and reported a somewhat more modest (~23%) increase in serum 25(OH)D concentrations in response to UVB treatment for individuals carrying four risk alleles compared to the 54% increase for those carrying no risk alleles [88]. They also found that individuals with four risk alleles benefitted the least from the consumption of vitamin D3–fortified bread and milk during this 6-month study [88]. GRS for 25(OH)D has been suggested to be useful for guiding the screening and treatment for vitamin D deficiency. This was tested in a recent study [26], where participants with serum 25(OH)D < 50 nmol/L were recommended to take vitamin D supplements, adjusting the dosage according to their genetic risk. Again, this study used a simple GRS (two SNPs only, taken from *GC* and *CYP2R1)*, and the individuals with three or four 25(OH)D-lowering alleles were instructed to take 50 μg (2000IU) per day, those with one to two risk alleles to take 20–30 μg/day and those with no risk alleles, 10–20 μg per day. In their study, recommendation to take 50 μg (2000IU) per day over 4 months was enough to reduce the gap between individuals carrying three or four risk alleles and those with no risk alleles both with respect to serum 25(OH)D concentration and the prevalence of 25(OH)D < 50 nmol/L. However, the prevalence of 25(OH)D < 50 nmol/L remained elevated for those with two risk alleles compared to no risk alleles. While these results are very interesting and even promising, they are tentative, as the higher vitamin D intakes were achieved by recommendations, and not by testing in a placebo controlled, and randomized context. This was also a relatively small study (*n* = 10 to

n = 36 per treatment group), so further trials with appropriate controls and a larger sample are required to examine possible benefits and effective approaches for personalized vitamin D supplementation. Given more profound genetic adaptations to differences in vitamin D intakes ('vitamin D scarcity') are possible, more research is also needed to establish target levels reflecting 'optimal' 25(OH)D concentrations and supplementation approaches for specific population groups, including indigenous Arctic and Tropical peoples [90].

3.4. Mendelian Randomization to Establish Evidence for Causal Effects of 25(OH)D

With the identification of genetic variants associated with serum 25(OH)D concentrations, it has become possible to use Mendelian randomization (MR) to examine evidence for the causal effect of vitamin D on other traits. This method is sometimes called the "natures controlled trial", as assuming random allocation of genetic variants during the gamete formation, individuals are randomized on different exposure groups based on the genetic variants they carry. Reliable causal inference based on MR studies is conditional to some key method assumptions, and where these hold, this method can help avoid bias due to confounding and reverse causation which more strongly affect other types of observational studies [91]. MR analyses on 25(OH)D have used several strategies, and many of the studies have restricted the variants used to those in the actual vitamin D pathway (including *DHCR7*, *CYP2R1*, *GC* and *CYP24A1*). With additional loci being discovered for serum 25(OH)D [19,20], MR studies now commonly incorporate these new loci into the analyses. While the inclusion of additional loci can improve statistical power, it is also important to keep in mind the potential for pleiotropic effects that these variants could bring into the models and which could bias the MR analysis [92].

MR studies on 25(OH)D have been conducted across a wide range of outcomes, with evidence supportive of a causal effect seen for multiple sclerosis [93], type 2 diabetes [94] and hypertension [95]. However, many of the newly discovered variants do not have clear or known function with respect to vitamin D metabolism, and some appear pleiotropic, with associations with other traits, such as BMI, and lipid measures. One approach to alleviate concerns relating to pleiotropy and residual genetic confounding affecting variant selection, is to restrict the analyses to variants which have consistent replicating association with 25(OH)D concentrations [39], as would be the case if we use the 35 SNPs described above. However, even there, pleiotropy is likely to remain a concern, and sensitivity analyses using different sets of variants and different analytical approaches will be required to help to assess the robustness of the findings. A multivariable MR approach, which directly accounts for pleiotropic effects by modelling the genetic effects on 25(OH)D simultaneously with pleiotropy related indicators, may also be helpful. However, to allow for the use of this approach, the relevant pleiotropic pathways will need to be hypothesized and relevant information must be available for the analyses. In the context of threshold effects, rigorously conducted MR studies that take into account non-linearity can increase the value of the genetic approach for vitamin D research, as evidence for an effect may only be seen at very low or high levels [96,97]. Recruiting people with vitamin D deficiency to supplementation trials is an important challenge, and often studies test the effects of supplementation in individuals who already have adequate concentrations, and who are typically allowed to take over-the-counter supplements [96,97]. Evidence for benefits with rectifying vitamin D deficiency with respect to outcomes such as mortality [98,99], cardiovascular disease [39] and dementia [100] has been obtained from recent studies using non-linear or stratified MR approaches. For dementia, evidence for a causal effect of vitamin D had already been provided by linear MR studies [101–103], while effects on mortality had been supported by RCT meta-analyses [104], but the non-linear studies in both contexts suggest that the benefits of increasing levels may be largely confined to the correction of clinical deficiency. These findings provide important insight into strategies that are likely to provide the greatest benefits, suggesting that large-dose supplementation is unlikely to be required, but population level strategies such as food fortification, which can ensure at least minimal

intakes and eradicate severe deficiency across the range of population groups, is likely to work.

4. Conclusions

Vitamin D status is in part determined by genetic variation and GWAS studies have identified a large number of variants that are associated with circulating 25(OH)D concentrations. Some of them are linked to the actual vitamin D metabolic pathway, and others to lipid metabolism and skin properties. In terms of methodology, they may provide MR studies with the means to measure the various determinants of serum 25(OH)D concentrations. Further research is needed to understand how such genetic information may be used to personalize vitamin D supplementation and prevent vitamin D deficiency.

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Data Availability Statement: Original data for Table 2 is available from the UK Biobank upon application.

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