Chapter

Vitamin D Metabolism

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Abstract

Vitamin D plays an important role in bone metabolism. Vitamin D is a group of biologically inactive, fat-soluble prohormones that exist in two major forms: ergocalciferol (vitamin D2) produced by plants in response to ultraviolet irradiation and cholecalciferol (vitamin D3) derived from animal tissues or 7-dehydrocholesterol in human skin by the action of ultraviolet rays present in sunlight. Vitamin D, which is biologically inactive, needs two-step hydroxylation for activation. All of these steps are of crucial for Vitamin D to show its effect properly. In this section, we will present vitamin D synthesis and its action steps in detail.

Keywords: Vitamin D, Vitamin D characteristics

1. Introduction

Vitamin D plays an important role in calcium and phosphorus metabolism, which are essential for bone health and various biological functions. In vitamin D deficiency, clinical and biochemical rickets characterized by hypocalcemia (irritability, fatigue, muscle cramps, seizures), hypophosphatemia and skeletal manifestations (delayed closure of fontanelles, craniotabes, frontal bossing, bowed legs, enlarged wrists, bone pain, and short stature) in children and adolescents or osteomalacia in adults may occur. Over the past several decades, it has been reported that the efficiency of vitamin D is not limited only to maintaining bone health by managing the calcium homeostasis, but also seems to have anti-inflamatory, immunemodulating and pro-apopitothic properties [1]. There are two different precursor molecules of vitamin D. The first is vitamin D3, or cholecalciferol, which is the main source of vitamin D in the body and is synthesized from the skin by exposure to sun. Vitamin D3 can also be obtained from dietary animal foods (fish, egg yolks) or medicines (vitamin supplements). The second precursor is vitamin D2, or ergocalciferol, which can be used as a source of vitamin D via oral medication or through enriched foods. Vitamin D3 differs in molecular structure from vitamin D2 in that it has a double bond between the 22nd and 23rd carbon atoms and a methyl group on the 24th carbon atom [2]. These structural differences in vitamin D2 affect its catabolism. Compared to vitamin D3, vitamin D2 has a lower affinity for vitamin D-binding protein (VDBP), which leads to its easy removal from the circulation, a reduced formation of 25-hydroxy vitamin D2 (25OHD2) by the 25-hydroxylase enzyme, and increased inactivation by the action of 24-hydroxylase [3–5]. Although both vitamin D2 and D3 are used as drugs, studies have shown that a higher serum 25OHD2 vitamin level is obtained when vitamin D3 is used in treatment compared to vitamin D2 [6]. In addition, it has been shown that active vitamin D obtained from vitamin D3 has a higher affinity for the vitamin D receptor (VDR) [4]. Despite these differences, vitamins D2 and D3 are both metabolized in substantially the

same way and are commonly referred to as vitamin D. Vitamin D is a prohormone and inactive, and to be activated, it must go through a series of enzymatic and non-enzymatic steps.

2. Vitamin D synthesis

2.1 The synthesis of vitamin D3 from the skin and the factors affecting this synthesis

Formation of vitamin D3, which is the first step of vitamin D synthesis, takes place in the epidermis by a non-enzymatic process (**Figure 1**). Vitamin D3 is the most important source of vitamin D in the body. 90–95% of vitamin D3 in the human body is produced from the skin with the effect of sunlight. Therefore, sunlight is the main source of vitamin D synthesis, and if there is sufficient exposure to sunlight, there is no need to take additional vitamin D. The mechanism of non-enzymatic photolysis of vitamin D by ultraviolet B (UVB) rays with wavelengths in the range of 290–315 nm involves the breaking of a bond in the B ring of 7-dehydrocholesterol (pro-vitamin D3), resulting in pre-vitamin D3 formation in the epidermis. Subsequently, two different double bonds are formed between the broken carbon atoms in the B ring by thermo-sensitive non-enzymatic process, and the formation of vitamin D3 from pre-vitamin D3 is completed (**Figure 2**) [7].

The synthesis of vitamin D3 from pro-vitamin D3 in the skin is adjusted according to the needs of the organism. In a period of just fifteen minutes, pre-vitamin D3 is synthesized from pro-vitamin D3 with the effect of ultraviolet light. Conversion from pre-vitamin D3 to vitamin D3 occurs by isomerization in a rather slow and thermo-sensitive manner. In the case of exposure to UV rays or solar radiation for a long period, pre-vitamin D3 converts to a number of photolyzed inactive by-products, such as lumisterol (irreversible) or tachysterol (which can be converted back to pre-vitamin D3). These by-products have no biological effects (**Figure 2**). In other words, once pre-vitamin D3 is formed in the skin, it turns into either vitamin D3 or inactive metabolites. This is a physiological control mechanism that protects the body from vitamin D intoxication by preventing unnecessary vitamin D synthesis [8, 9].

Some conditions that prevent UVB rays from reaching the skin cause a decrease in vitamin D production. One of these reasons is the ozone (O3) layer surrounding the atmosphere, which reflects some of the sun's rays, preventing them from reaching the Earth and their harmful carcinogenic effects on the skin.

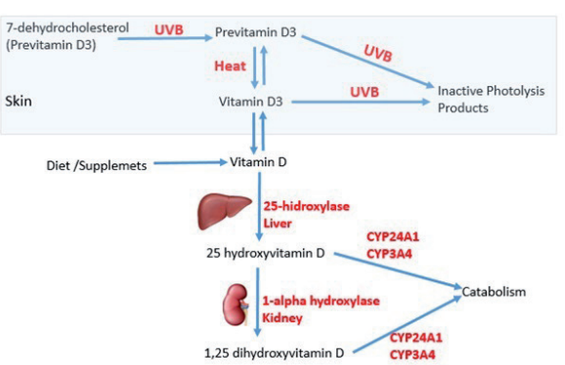

Figure 1.
Vitamin D metabolism.

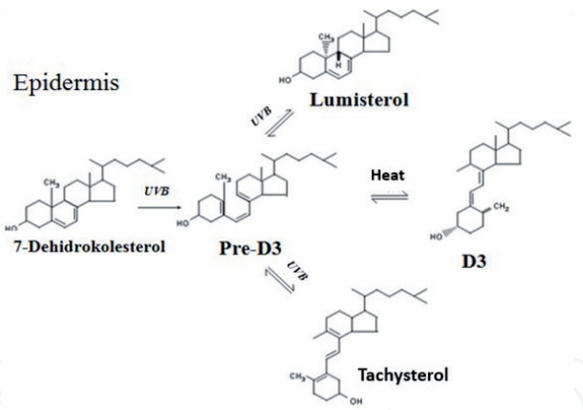

Figure 2.
Vitamin D3 synthesis from 7-dehydrocholesterol in the epidermis.

The peak UVB wavelength required for optimal vitamin D synthesis from the skin is 297 (290–315) nm [1, 8]. In addition, air pollution, aerosols, water vapors, and increased nitrogens in the air also play a role in preventing sunlight reaching the Earth, and consequently result in a potential reduced synthesis of vitamin D [8]. Another factor affecting the effectiveness of UVB rays in the synthesis of vitamin D in the skin is the solar zenith angle, which affects how UVB rays reach the world quantifiably. When the sun moves in a path closer to the horizon, which occurs in the northern latitudes in the winter season, vitamin D synthesis is more adversely affected (or reduced). In the summer time in the northern latitudes, a normal biosynthesis is more propitious or favorable. The narrowing of this angle indicates that the sun rays reach the Earth more steeply and intensely. The solar zenith angle is closely related to sunbathing time during the day, the seasons and the geographic region (latitude). Sunlight reaches the Earth most intensely in the "mid-day" when it is summer in the northern latitudes and the weather is clear. Finally, it is thought that sunlight exposure is sufficient for vitamin D synthesis in all geographic regions below 35 degrees north or south latitude all year round. In regions beyond this latitude toward the poles, especially in winter, sunlight is not sufficient for vitamin D synthesis. For example, UVB rays are not sufficient for vitamin D synthesis between October and April in Rome, which is located on 41.9 degrees north latitude, and between November and February in Berlin and Amsterdam, which are located on 52 degrees north latitude. For the reasons mentioned above, it is difficult to predict how much UVB rays reach the skin and how much of this increases serum vitamin D levels. In experimental studies, it has been reported that UVB rays that will cause minimal erythema in 25% of the skin are equivalent to 1000 units of oral vitamin D intake [2, 3, 8].

UVB rays are also affected by the individual's clothing style, use of sunscreen, and skin colour determined by pigmentation with melanin. In dressing style, especially the type of the clothing fabric used is of great importance [10]. Non-synthetic, light-colored, and linen garments play a less preventive role in UV rays reaching the skin than do garments made of silk, nylon, polyester, and wools. For example, black-dyed cotton clothing prevents 98.6% of UVB rays from reaching the skin compared to white (undyed) cotton clothing, which blocks 47.7% of UVB. Topical sunscreens also prevent UVB rays from reaching the skin by absorbing, reflecting or dispersing them. Topical creams with a sun protection factor of 8 or higher block vitamin D synthesis above 95% [11]. Melanin is a large, opaque polymer synthesized by melanocytes in the skin through the stimulus of exposure to UVB rays. Melanin competes with dehydrocholesterol 7 in the skin to absorb UVB photons and thus inhibits vitamin D synthesis [12]. Individuals with dark skin

colour have more melanin pigment in their epidermis than light-skinned individuals and require higher concentrations of sunlight for the same amount of vitamin D synthesis [12]. In addition, the 7-dehydrocholesterol level (provitamin D) in the epidermis can also affect the serum vitamin D concentration. For example, 7-dehydrocholesterol levels in scar tissue caused by the burn are reduced by 42.5% of normal. In these cases, progressive vitamin D deficiency develops, especially if supplemental dietary vitamin D is not provided. Moreover, the content of pro-vitamin D in the skin decreases with age. Skin temperature is also important for vitamin D synthesis. Vitamin D from pre-vitamin D by isomerization whose rate of formation is temperature- dependent. The rate decreases as the skin temperature decreases. In a healthy person, the skin temperature is lower than the central body temperature and varies between 29 and 35 degrees Celcius. When the skin temperature is 37 degrees Celcius, the isomerization of vitamin D from pre-vitamin D occurs within 2.5 hours [13, 14].

2.1.1 Biosynthesis of 25OHD3 (25-hydroxylase) in liver

Vitamin D3 synthesized in the skin is released into the systemic circulation and all forms are transported by binding to VDBP in serum. A portion of vitamin D, a fat-soluble vitamin, is stored in adipose tissue for use when necessary. The ability of vitamin D to be stored in adipose tissue extends its total half-life in the body up to approximately 2 months. When vitamin D3 is transported to the liver, it is first converted into 25OHD3 by the cytochrome P450 25-hydroxylase enzyme. 25OHD3 is the main circulating form of vitamin D, and it is the parameter that provides the best estimation about the body's vitamin D pool [15]. Various enzymes that show 25-hydroxylase properties have been described in the body. Among these, the first one is CYP27A1 located in mitochondria, and the second is microsomally located CYP2R1 [1, 6, 16]. CYP27A1 also exerts 27-hydroxylase effect and is involved in bile acid synthesis. Although CYP27A1 is expressed in different tissues of the body, the tissues where it is most commonly found are liver and skeletal muscle tissues [1, 2]. In experimental studies, it was reported that the serum 25OHD3 levels were increased in mice which possess an inactivated CYP27A1 gene, and that rickets did not occur in these mice [17]. Interestingly, in this study, it was shown that CYP2R1 expression increased after CYP27A1 gene inactivation, and consequently 25-hydroxylation activity increased [17]. In addition, individuals with a CYP27A1-inactivating mutation develop a cerebrotendinous xanthomatosis disease with bile and cholesterol synthesis disorders, but without rickets manifestation [18]. Besides CYP27A1, different CYP-450 enzymes with 25-hydroxylase activity (CYP2D25, CYP2J2, CYP2J3, and CYP2C11) have been identified in humans and animals, with the most important one in human being CYP2R1. It is assumed that enzymes other than CYP2R1 have effects only on serum 25OHD3 levels [2].

Studies have suggested that CYP2R1 is the major enzyme responsible for 25-hydroxylation in the human body. This enzyme is expressed in many tissues, mainly liver, skin, and testis [1, 2, 17]. The 25-hydroxylase encoded by the CYP2R1 gene was first described by Cheng et al. [19]. It was first reported by Chen et al. [20] that homozygous inactivating mutations of this gene lead to clinically observed rickets (vitamin D-dependent rickets type IB) in Nigerian families. It has been reported that these cases gave suboptimal response to standard vitamin D (inactive vitamin D2 or D3 forms) treatment [21]. The CYP2R1 enzyme has equal affinity for the different forms of vitamin D precursors (D2 or D3) [19]. Studies have shown that 25-hydroxylase effect increased in male rats given estrogen, whereas this activity decreased in female rats given testosterone [21]. Despite experimental studies, the effect of sex steroids on 25-hydroxylase enzyme activity in humans is unknown.

It has been shown that in CYP2R1-null mice, the level of 25OHD3 decreases by 50%, when both CYP2R1 and CYP27A1 are inactivated, and that serum 25OHD3 levels decrease by 70%, and serum 25OHD3 level remains at a measurable level in both cases [2, 17]. This supports the view that serum vitamin D level is compensated by other enzymes with recruitable 25-hydroxylase enzyme activity.

2.1.2 Formation of active vitamin D [1,25 (OH) 2D3] by 1-alpha hydroxylase (CYP27B1) in the kidney

The final step of active vitamin D formation takes place in the proximal tubules of the kidney, led by the enzyme 1-alpha hydroxylase. 25OHD3, which is bound to VDBP, is taken into tubule cells and metabolized (1-alpha hydroxylation) through megalin and cubilin, which are transmembrane proteins located in renal tubules and act as surface receptors for VDBP in tubules. 25OHD3, which then undergoes 1-alpha hydroxylation [1, 2]. The 1-alpha hydroxylase enzyme hydroxylates the first carbon atom in the A ring of 25OHD3, resulting in the formation of 1,25 (OH) 2D3 [1]. CYP27B1 is the only enzyme that has 1-alpha hydroxylase activity. This enzyme, which belongs to the cytochrome P-450 enzyme system, is located in the inner mitochondrial membrane and carries out electron transport to NADPH via ferrodoxin-ferrodoxin reductase [1, 2]. The gene for the enzyme consists of nine exons and is located 12q14.1 chromosomal region. Four different groups reported the cloning and sequencing of the gene from rats, mice and humans [22–26]. In biallelic inactivating mutations of this enzyme, which is highly homologous to some mitochondria located cytochrome P-450 enzymes (CYP27A1 and CYP24A1), 25OHD3 cannot be converted to 1.25 (OH) 2D3, which is the active vitamin D form. In this case, the clinical picture of vitamin D-dependent rickets type 1A (also called pseudo-vitamin D deficiency rickets) occurs [23]. This disease is typically characterized by rickets, with clinically observed very low 1.25 (OH) 2D3, low serum calcium/phosphorus, and high parathyroid hormone (PTH) levels. CYP27B1 is expressed mainly in the renal proximal tubules and in the placenta during pregnancy [27]. While the expression of the gene encoding this enzyme increases with the effect of PTH, it decreases with FGF23 (fibroblast growth factor 23) and 1.25 (OH) 2D3. CYP27B1 gene is also expressed in lung, brain, breast and intestinal system epithelial cells, immune system cells (macrophage, T/B lymphocytes and dendritic cells), osteoblasts, chondrocytes, and some tumor cell types [1, 2]. The regulation of the extra-renal localized 1-alpha hydroxylase enzyme differs. In some granulomatous diseases where monocyte/macrophage cells play an important role (sarcoidosis, tuberculosis, Chron's disease, etc.), with the effect of IL-1, TNF-α, IFN-γ, 1-alpha hydroxylase enzyme activity increases and 1,25 (OH) 2D3 is synthesized in greater quantities than normal, and consequently, hypercalcemia and hypercalciuria emerge [28–30]. Additionally, since cells in these tissues do not have PTH receptors, it is not yet understood how PTH exerts its enhancing effect on the 1-alpha hydroxylase enzyme activity in these cells. In one study, it has been suggested that this enhancing effect of PTH may have occurred through post-transcriptional effects [31]. Moreover, 1-alpha hydroxylase enzyme in these cells is not inhibited by 1,25 (OH) 2D3 or hypercalcemia, unlike the renal tubules.

2.1.3 Inactivation of vitamin D by 24-hydroxylase (CYP24A1)

The 24-hydroxylase enzyme is located in the mitochondrial inner membrane of the cells located in the proximal kidney and, like CYP27B1, uses the electron transport system that enables electron transport to NADPH via ferrodoxine-ferrodoxin reductase. It is known that CYP24A1, which is the only enzyme showing

24-hydroxylase enzyme activity in humans, can also exhibit 23-hydroxylase enzyme activity [2]. Which enzyme will be more prominent varies according to the species [32]. The 23-hydroxylase, another enzyme that degrades vitamin D, is the first step activity in the conversion of 1,25 (OH) 2D3 to 1,25 (OH) 2D3-23,26-lactone.

The CYP24A1 enzyme, encoded in 20q13 chromosomal region and having 24-hydroxylase enzyme activity, initiates catabolic processes that lead to the inactivation of vitamin D by hydroxylating the 24th carbon atom. This enzyme can use both 25OHD3 and 1.25 (OH) 2D3 as substrates, but has a higher affinity for 1.25 (OH) 2D3. As a result of a series of enzymatic reactions, calcitroic acid is formed, which becomes biologically inactive. On the other hand, it has been suggested that the 1,25 (OH) 2D3-23,26-lactone, which is formed in the 23-hydroxylase pathway, lowers serum calcium level, inhibits bone resorption induced by 1.25 (OH) 2D3, and stimulates the formation of collagen tissue in bone tissue [33]. In addition, it has been suggested that 24,25 (OH) 2D3 is not only a degradation product, but has an important role in bone metabolism, especially in endochondral bone formation [34].

There are two vitamin D response elements (VDRE) in the promoter region of the CYP24A1 gene [35]. When active vitamin D is bound to the these one of VDRE after heterodimerization with various molecules, thus initiates the inactivation process of vitamin D. In addition, it has been shown that CYP24A1 gene expression decreases with the effect of PTH, whereas it increases with increased FGF23 concentrations [1, 32, 36, 37]. Inactivating mutations in CYP24A1 lead to an idiopathic infantile hypercalcemia clinic characterized by hypercalcemia, hypercalciuria, nephrocalcinosis, low PTH, low 24.25 (OH) 2D3 and high 1.25 (OH) 2D3 levels [37]. As a result, CYP24A1 is a critical enzyme that protects the body from excessive accumulation and possible intoxication of vitamin D.

2.1.4 3-epimerization of Vitamin D

3-epimerase activity was first demonstrated in 2001, with the detection of the 3-epi form of 1,25 (OH) 2D3 in keratinocytes [38]. In the following years, epimer forms of 25OHD3 and other vitamin D metabolites were discovered. However, the enzyme or enzymes involved in epimerization has not yet been identifiedpurified or cloned. This enzyme changes the hydroxyl group in the 3rd carbon of the A ring from the alpha orientation to the beta orientation, causing the three-dimensional structure to change and consequently alter the activity of CYP27B1 and CYP24A1 enzymes on vitamin D metabolism. These epimers can be detected by special liquid chromatography-mass spectroscopy (LC-MC) measurement methods [2]. C-3 epimer forms of 25OHD3 and 1,25 (OH) 2D3 have been shown to have lower affinity for VDR and VDBP compared to non-epimer forms [38]. The C-3 epimer form of 1,25 (OH) 2D3 has been shown to cause PTH suppression similar to the non-epimer form, but its effects on bone tissue are not clear. In addition, epimer forms have also been shown to have non-calcium effects (anti-proliferative effect, surfactant synthesis) [39]. It has been shown that the serum levels of vitamin C-3 epimer forms are found to be 60% higher in the period between the neonatal period and one year old, and decrease after one year of age and decrease to very low levels in adulthood [2, 38]. The reason why epimer forms with limited biological activity are important is that they cause interference and false high results in serum 25OHD3 and 25OHD2 measurement. Therefore, it is important to prefer the method (especially LC–MS / MS) that can exclude this effect of epimer forms that cause serum vitamin D measurement interference. However, the use of LC–MS/MS method in the measurement of vitamin D has not become widespread in the world, and the use of this method is only recommended in selected cases.

2.1.5 Transport of Vitamin D

The largest part of the circulating vitamin D is in the form of 25OHD3, and its serum concentration is in equilibrium with the level of vitamin D stored in muscle and adipose tissues. The parameter that gives the best information about the whole vitamin D pool in the body is 25OHD3 and its known half-life of 15–20 days. Most of all forms of vitamin D in circulation (85–88%) are transported by binding to VDBP and the remaining part (12–15%) to albumin [2, 40]. The serum concentration of VDBP is 4–8 nM and only 2% of it is bound with vitamin D metabolites [2]. Moreover, the affinity of VDBP to 25OHD3 is 20 times higher than 1.25 (OH) 2D3 [3]. 0.03% of 25OHD3 and 0.4% of 1.25 (OH) 2D3 are in free form [2]. In chronic liver disease or nephrotic syndrome, VDBP and albumin levels and thus total serum 25OHD3 and 1.25 (OH) 2D3 levels decrease, but the levels of free forms are not affected [41]. Likewise, since the VDBP level may decrease during the acute disease period, evaluating the body's vitamin D pool by measuring the serum 25OHD3 level with standard immunoassays may lead to misinterpretations [42]. In conclusion, while the total levels of vitamin D forms are affected by the VDBP level, there is no relationship between VDBP and free vitamin D forms, which are essential for biological activity. It was shown that both 25OHD3 and 1.25 (OH) 2D3 levels in VDBP-null mice were lower than wild type mice, but serum PTH and calcium levels were similarly normal in both groups [43]. This supports the view that serum vitamin D level measured by the standard method may not be an indicator of biologically active vitamin D pool. In addition, the predisposition of VDBP-null mice to the development of osteomalacia after a vitamin D-restricted diet suggests that VDBP may play a role in maintaining the existing vitamin D pool [44]. In addition, some single nucleotide polymorphisms (GC1F, GC1S, GC2) in the *VDBP* gene have been shown to impact the affinity of VDBP on vitamin D metabolites [1, 45, 46].

3. The mechanism of Vitamin D actions

Vitamin D provides its biological effect in two different ways. The first is by directly affecting gene transcription (genomic effect) as other steroid hormones. This effect is relatively slow and usually occurs within hours or days. The second is the non-genomic pathway whose biological effect is relatively faster (within minutes). Vitamin D exerts its non-genomic effect by directly altering the transmembrane passage of some ions (Ca, Cl) or by affecting intracellular signaling pathway activities (cAMP, PKA, PLC, PI-3 kinase and MAP kinase) [1, 2]. Genetic studies on vitamin D support that active vitamin D directly or indirectly regulates 0.8–5% of the total genome, suggesting the role of active vitamin D in many actions such as regulation of cellular growth, DNA repair, differentiation, apoptosis, membrane transport, cellular metabolism, adhesion and oxidative stress [1–3, 47].

3.1 Genomic effect of Vitamin D

The active form of vitamin D displays this effect through the vitamin D receptor (VDR). VDR is a member of the nuclear hormone receptor superfamily, which includes steroid, thyroid hormone, and retinoic acid receptors [48]. The VDR gene located on chromosome 12 consists of 427 amino acids encoded by. The structure of the VDR consists of a relatively short N-terminal domain compared to other nuclear receptors, two zinc-fingers that allow the receptor to bind to DNA, and a highly

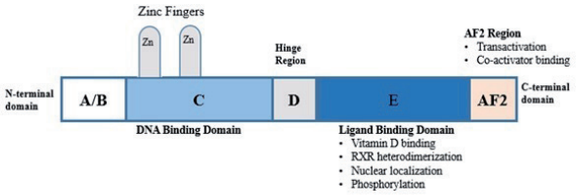

Figure 3.
The structure of the Vitamin D receptor (VDR).

variable C-terminal region, and the hinge region connecting binding these domains (**Figure 3**) [2]. The DNA-binding region of the receptor is rich in cysteine, and the sequence of this region is largely conserved between species. The zinc-finger structure close to the C-terminal part of VDR determines the specificity for the VDRE (vitamin D response element), which is the binding site on the DNA. The other zinc-finger structure is involved in the heterodimerization of VDR with RXR (retinoid X receptor) [1, 2]. The ligand-binding part of the receptor consists of 12 α-helix structures (H1-12; the H12 part is also called AF2) and 3 β-sheet structures (S1-3) [49]. The AF-2 region located at the end of the C-terminal is the binding site of co-activator complex structures such as SRC (steroid receptor coactivator) and DRIP (vitamin D receptor interacting protein). Transcription is initiated by binding co-activators to this region [50]. Apart from these functional domains, there are NLS (nuclear localization signal) regions within the DNA binding region of VDR, which are necessary for maintaining transcriptional activity [2]. In addition, there is a hinge region between the ligand-binding and DNA-binding domains of the VDR that ensures molecule stabilization.

After active vitamin D crosses the target cell membrane, it interacts with the ligand-binding domain of its own receptor (VDR) in the cytoplasm of the cell. Vitamin D is embedded in the ligand-binding domain, and subsequently, in the H12 alpha-helix H12 (AF-2) region, which is located at the end of the ligand binding part [51]. This critical conformational change of AF-2 facilitates the binding of co-activators in later stages [52]. In the next step, vitamin D-bound VDR binds to RXRα to form a VDR/RXR heterodimer structure that binds to cognate VDR elements (VDRE) in the promoter region in the target genes with a high affinity to initiate gene activation or inhibition. There are many gene-specific VDREs associated with bone metabolism, xenobiotic detoxification, drug resistance, cell growth and differentiation, angiogenesis, mammalian hair growth cycle, lipid synthesis regulation, apoptosis, and immune functions, suggesting that vitamin D has numerous regulatory roles in various organs or tissues in the body [53].

After active vitamin D-VDR-RXR-VDRE interaction, the progression of transcription is controlled by co-activator and co-repressors. The best known co-activators are the p160 co-activator family (eg CBP/p300 and p/CAF) and SRC 1,2,3. Both bind to the AF-2 part and have histone acetyl transferase (HAT) activity, which enables the opening of the histone structure and thus facilitates gene expression [54]. The SRC complex has three NR regions that facilitate binding and contain LxxLL (L, leucine; x, any amino acid) motifs. Likewise, the DRIP complex (Mediator) also has NR regions with LxxLL motifs consisting of 15 or more amino acids [55]. Unlike SRC, DRIP complex does not have HAT activity. This suggests the fact that both protein complexes play a complementary role in the initiation of transcription. The mediator multi-protein complex DRIP205/MED1 (also known as MED1) accumulates around RNA polymerase 2of the initiation complex. This complex then interacts with the TATA region in the promoter

region and enables transcription to be initiated [56]. Co-repressors (eg SMRT and NCoR) have histone de-acetylase activity and inhibit transcription by preventing unfolding of the histone core.

3.2 Non-genomic effects of vitamin D

Some of the hormones that act on the nuclear hormone receptor can also exert their biological effects on the membrane receptor without the need for additional gene regulation [2]. The non-genomic effect occurs through messenger-mediated pathways. Estrogen, progesterone, testosterone, corticosteroids and thyroid hormones have been reported to exert their effects by using both genomic and non-genomic pathways [2]. Vitamin D has been shown to directly regulate the activation or distribution of various ion-transport channel proteins (for calcium and chloride) and of enzymes (protein kinase C and phospholipase C) through the membrane receptor in osteoblast, liver, muscle, and intestinal cells (**Figure 4**) [57–62]. In order to demonstrate the non-genomic effect of vitamin D, many studies have been conducted on intestinal calcium absorption. Rapid vesicular calcium absorption (also called transcaltachia) has been shown in the chick intestinal tract [63]. Further experimental studies have shown that intestinal calcium transport cannot be blocked by the administration of actinomycin D (which inhibits the genomic effect) [64], whereas calcium absorption can be blocked by inhibition of voltage-gated L-type calcium channel proteins [65] or by protein kinase C [66].

Apart from the intestinal system, it has been suggested that the non-genomic effect also occurs in chondrocytes in the growth plate and keratinocytes in the skin [67, 68]. Vitamin D is believed to exert its non-genomic effects through VDR analog and MARRS (also known as ERp57/GRp58/ERp60) receptors located on the cell membrane [69, 70]. These membrane receptors are located within the caveolar lipid layer [71]. In addition, research findings indicate that VDR is also necessary for the expression of membrane receptors that involve in the emergence of non-genomic effect [1, 2]. In studies evaluating the effects of vitamin D analogs (6-s-cis or 6-s-trans conformations), the 6-s-cis form can activate intestinal rapid calcium entry even though the VDR affinity is very low, whereas the 6-s-trans form has been shown to be ineffective in calcium metabolism [67].

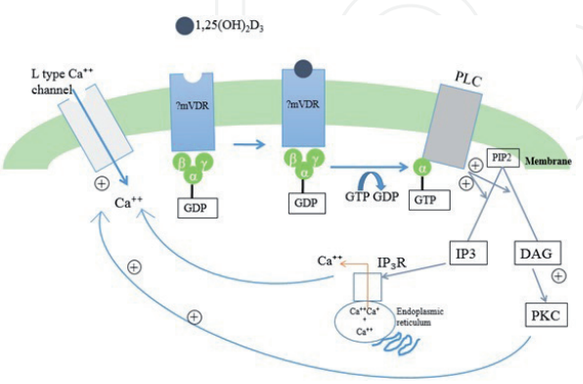

Figure 4.
Representation of the signal transduction pathways where Vitamin D has its non-genomic effect (2). After vitamin D binds to the membrane receptor, GDP in the G protein α-subunit turns into GTP and activation occurs. The α-subunit of the G protein is separated from other subunits and binds to phospholipase C (PLC). The PLC is then activated to convert phosphoinositol bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). Calcium release from the endoplasmic reticulum via the IP3 receptor (IP3R); DAG activates PKC. PKC, on the other hand, provides calcium entry into the cell via the L-type calcium channel in the membrane.

4. Effects of Vitamin D on calcium and phosphorus

4.1 Intestinal calcium absorption

One of the most important functions of vitamin D is to increase calcium absorption from the intestines. Calcium absorption from the intestinal tract occurs trans-cellular and para-cellular processes mediated through genomic and non-genomic effects. Among these, the trans-cellular pathway largely utilized by the intestinal system, which is regulated by vitamin D [2]. The absorption effect of vitamin D with non-genomic effect of calcium occurs directly on the membrane (transcaltachia). The channel-mediated calcium absorption effect of vitamin D occurs more slowly [2].

Calcium enters the epithelian cell by the effect of an electrical and chemical gradient via calcium channel protein TRPV6 (which has significant sequence homology to TRPV5 in the kidney), the transmembrane protein at the lumenal brush border edge of the intestinal epithelial cell. The expression of TRPV6 is activated by vitamin D [72]. Reduced intestinal calcium transport is observed in TRPV6 null mice [73]. Calcium entering the cell binds to calmodulin (CaM), which is bound with myosin 1A (also known as brush border myosin I). This formed complex allows calcium to be transported across the microvilli. Subsequently, the transport of calcium up to the basolateral membrane occurs inside the vesicle via calbindin-D9k (CaBP). The affinity of calcium for calbindinin is greater than for calmodulin, and better facilitates calcium transport inside the cell [74]. The calcium reaching the basolateral membrane is pumped out of the cell to systemic circulation via the Ca-ATPase (PMCA1b) pump located on the membrane [1, 2]. In addition, although it is less important, NCX (sodium/calcium exchanger), located in the basolateral region, also plays a role in excretion of calcium [2, 75]. Vitamin D shows its increasing effect on intestinal calcium absorption by inducing expression of TRPV6, CaBP and PMCAb and increasing the binding affinity of CaM to myosin 1A [1, 2].

Intestinal calcium absorption, serum calcium level and bone mineral content in Kalbindin D9k null mice (regardless of dietary calcium level) have been shown to be similar to normal mice [76]. Intestinal calcium absorption was found to be normal in calbindin D9k and TRPV6 null mice when a diet containing the daily requirement for calcium was given [77]. These findings indicate there is a mechanism other than the genomic effect through which vitamin D exerts its action (a non-genomic effect) in calcium absorption in the intestines when the amount of calcium in the diet is sufficient.

While trans-cellular calcium absorption is effective in compensating for a low-calcium diet, para-cellular calcium transport becomes important with the increase in calcium content in the diet [1]. Paracellular transport occurs through the extracellular space between the layer of the epithelial cells in the intestine. Although it was previously thought that vitamin D does not affect para-cellular calcium absorption, studies conducted in recent years indicate otherwise, with vitamin D still affecting calcium absorption by increasing levels of various transmembrane and adhesion proteins that control the extracellular space between cells [78, 79]. However, it is not clear at what stage of the paracellular pathway these proteins are involved.

Phosphate, another important molecule for bone mineralization, is actively absorbed mostly in the jejunum, with absorption influenced by vitamin D [2]. This absorption is provided by sodium-phosphate co-transporter IIb (NaPi IIb). In experimental studies, it has been shown that phosphate absorption is blocked when cycloheximide, which inhibits protein synthesis, is given [80]. This situation supports that phosphate absorption occurs by genomic effect. Vitamin D increases NaPi-IIb expression and thus phosphate absorption [2].

4.2 The effect of vitamin D on the kidneys

Most of the calcium that reaches the kidney tubules is absorbed from the proximal and distal tubules and approximately 1–2% of it is excreted through urine. Approximately 65% of calcium absorption in the kidney is passively absorbed paracellularly from the proximal tubules with the sodium gradient and independent of vitamin D direct action [1]. The rest of the calcium is absorbed from the ascending limb of the loop of Henle (20%), the distal tubules (15–20%), and the collecting ducts (5%) [81]. Vitamin D plays an important role in calcium absorption in the distal tubules and provides active calcium absorption via the trans-cellular pathway with the help of an electrochemical gradient [1]. Calcium is taken into the cell by TRPV5 channel on the surface of the tubular cell and is transported inside the cell by calbindin-D9k and D28k. Transported to the basolateral part of the cell, calcium is released into the systemic circulation by NCX1 (sodium/calcium exchanger) and PMCA1b. This mechanism is similar to that in the intestinal tract. Vitamin D increases the expression of TRPV5, calbindin, NCX and PMCA1b.

Phosphate is reabsorbed by sodium-dependent phosphate carrier proteins (NaPi-IIa and NaPi-IIc) in proximal tubular cells under vitamin D control. In addition, for phosphate reabsorption, a Na/K-ATPase channel located in the basolateral membrane is also needed [1, 2]. The impact of vitamin D on transport channels is not clearly known. While PTH increases the lysosomal degradation of phosphate transport channels, FGF23 causes a decrease in the expression of these channels [1, 2, 82].

4.3 The effect of vitamin D on bone tissue

Calcium, phosphorus and vitamin D are important molecules for bone metabolism and health. Calcium is one of the most abundant minerals in the body and is obtained entirely from dietary sources. In addition to its various biological effects in the body, it is also essential for bone metabolism [83]. More than 99% of the total body calcium is found in the bone tissue as a calcium-phosphate mineral complex, while the remaining <1% is distributed between the intracellular and extracellular compartments [83]. While 40% of calcium outside bone tissue is bound to protein, 9% forms ionic complexes, and the remaining 51% is found as free ions [84, 85]. Ionized calcium balances the calcium pool in the intracellular-extracellular area and plays an important role in bone metabolism. This balance is provided by the cooperation of various hormones (PTH, vitamin D) and the organs they affect (kidney, bone and intestinal system) [83–85]. Where there is vitamin D deficiency (nutritional or genetic) or VDR-inactivating mutations, serum levels of calcium and phosphate, which play an important role in bone development and growth, are reduced and thus rickets/osteomalacia emerge. Rickets is a disease characterized by excessive osteoid tissue accumulation and defective mineralization of the epiphyseal plate, which occurs as a result of insufficient mineralization in the epiphyseal plates of growing bones [1, 2]. Osteomalacia is a disease characterized by a deterioration in the mineralization of the newly formed osteoid and a decrease in bone turnover.

There is a continuous remodeling cycle consisting bone tissue resorption and mineralization. When calcium, phosphorus, and vitamin D are sufficient, this cycle continues in a balanced manner. In the case of negative calcium balance caused by insufficient calcium intake with diet or increased renal calcium loss, vitamin D increases bone resorption in osteoblasts through VDR signaling, resulting in calcium passage from bone to blood, which leads to impaired bone mineralization. Vitamin D increases the expression of RANKL (receptor activator of NF-κB

ligand), which is an osteoclastogenic factor from osteoblasts [86, 89]. RANKL stimulates osteoclastogenesis and increases osteoclast formation by binding to its related receptor, RANK [87]. In conclusion, in the case of negative calcium balance, vitamin D tries to keep the serum calcium level in a certain balance by increasing resorption and decreasing mineralization [1].

In the case of a positive calcium balance, the osteoblastogenic activity of vitamin D is prominent. In this situation where anti-resorbtive effect is in the predominant, bone mineral density increases. The occurrence of this effect has been associated with a decrease in the RANKL/OPG (osteoproteogerin) ratio and an increase in LRP-5 (LDL receptor related protein 5) expression [1]. LRP-5 is controlled by the VDR and is a necessary co-receptor for the anabolic effect of osteoblasts [88]. In addition, vitamin D plays a role in the proliferation of chondrocytes in the growth plate through genomic action.

5. Regulation of vitamin D metabolism

Pro-vitamin-D3, pre-vitamin D3 and then vitamin D3 (cholecalciferol) conversion in the skin is under the control of UV radiation. Serum vitamin D concentration reaches its highest level 24–48 hours after exposure to UV radiation and then shows a gradual decrease. The half-life of serum vitamin D is 36–72 hours. Vitamin D, which is a fat-soluble vitamin, is stored in adipose tissue for later use. The ability of vitamin D to be stored in adipose tissue extends its total half-life in the body up to approximately 2 months.

5.1 Regulation of 25-hydroxylase

There is little information on how this enzyme is regulated because of the few studies performed. What is known is that serum vitamin D level is inversely related to the rate of 25-hydroxylation in the liver, and the synthesis of 25OHD3 from vitamin D (cholecalciferol) is regulated by the 25-hydroxylase enzyme**.** This activity of the enzyme is directly inhibited by 25OHD3. Consequently, serum 25OHD3 levels can be kept at a physiological window ranging from 75 to 220 nmol/L (30–88 ng/mL). However, when an overdose of vitamin D is taken orally, this inhibitory mechanism in 25OHD3 synthesis cannot prevent vitamin D intoxication [2].

5.2 Regulation of renal 1-alpha hydroxylation

Serum active vitamin D levels in healthy adults vary within extremely narrow ranges, so that even in cases of vitamin D intoxication, serum levels may remain normal. 1-alpha hydroxylation activity in the kidney is controlled by PTH, calcium and phosphorus. Hypocalcemia, increased PTH, and hypophosphatemia will stimulate increases in active vitamin D production through renal 1-alpha hydroxylase enzyme activation, while hypercalcemia, FGF-23 secreted from osteoblasts, and active vitamin D itself have an inhibitory effect on active vitamin D synthesis through the renal 1-alpha hydroxylase enzyme. Active vitamin D increases FGF23 synthesis from osteoblasts. FGF23 suppresses the 1-alpha hydroxylase enzyme and increases the activity of 24 hydroxylase enzymes. In addition, hypercalcemia suppressing PTH and hyperphosphatemia by increasing FGF23 levels results in 1-alpha hydroxylase enzyme activity inhbition [1–3]. It is also suggested that calcium and phosphate have a direct regulatory effect on 1-alpha hydroxylase enzyme [89].

Calcitonin is known to reduce serum calcium levels through osteoclast inhibition. In addition, this hormone has been shown to increase the expression of

CYP27B1, the gene encoding the 1-alpha hydroxylase enzyme, in normocalcemic pregnant women due to the increase in calcium need. In this way, active vitamin D synthesis and consequently intestinal calcium absorption is increased [1, 90]. Apart from calcitonin, it has been suggested that prolactin also increases CYP27B1 expression, especially during lactation, and thus contributes to the increased calcium demand of the body [1, 91].

CYP3A4 enzyme in the liver and intestinal system has also been shown to be effective in the inactivation of 25OHD3 and reduction of active vitamin D [92]. Long-term use of drugs such as phenytoin, rifampicin, and carbamazepine may lead to up-regulation of the CYP3A4 enzyme and thus to a decrease in serum 25OHD3 and active vitamin D levels.

5.3 Regulation of 24-alpha hydroxylase

When serum calcium, phosphate and PTH levels are within normal levels, 25OHD3 and 1–25 (OH) 2 D3 are metabolized into biologically inactive forms by activation of 24-alpha hydroxylase enzyme in the kidneys (24–25 dihydroxy vitamin D3 and 1,24, 25 trihydroxy vitamin D3). This enzyme preferably binds to 1–25 (OH) 2 D3, thus limiting the effect of active vitamin D in tissues through inactivation [2]. The low level of 24-hydroxylase enzyme activity leads to high levels of 1–25 (OH) 2D3 and thus hypercalcemia. In addition, it has been suggested that a decrease in this enzyme activity may lead to impairment in intra-membranous bone mineralization [1, 2]. On the other hand, when 1–25 (OH) 2 D3 synthesis decreases, 1-alpha hydroxylase enzyme activity increases and 24-hydroxylase enzyme activity decreases. It is also known that FGF23 increases the activity of 24 hydroxylase enzymes [1, 2].

5.4 Regulation of active vitamin D synthesis in extra-renal tissues

Numerous studies have shown active vitamin D synthesis by 1-alpha hydroxylase enzyme is not only a renal feature [2, 93]. The gene encoding the 1-alpha hydroxylase enzyme and the vitamin D receptor gene can be expressed in many cells or tissues such as skin, placenta, prostate, parathyroid, bone tissue, colon, lung, breast tissue, monocytes and macrophages, as well as renal cells. It has been reported that active vitamin D synthesized in the aforementioned tissues functions mostly as an intracrine or paracrine factor in the tissues where they are located, and does not contribute to the active vitamin D levels in the circulation, except for some special cases [1, 2]. Since PTH and FGF-23 receptors are not found in these tissues, they are not directly involved in controlling active vitamin D synthesis. However, it is propable that PTH increases the effect of vitamin D through posttranscriptional modification [31]. Unlike in other tissues, in activated macrophages, there is also no negative feedback of active vitamin D on 1-alpha hydroxylase enzyme [91]. Moreover, although the 24-hydroxylase enzyme is expressed in these cells, its function is not fully understood. Cytokines such as IL-1, TNF-α, IFN-γ induce the synthesis of active vitamin D in keratinocytes. Unlike macrophages, keratinocytes have a fully functional 24-hydroxylase enzyme activity and is induced by active vitamin D. In this way, active vitamin D limits its own synthesis in the epidermis through alternative catabolism [1, 2, 93].

6. Vitamin D measurement methods

Measurement of serum levels of vitamin D, which plays an important role in calcium and phosphorus metabolism and bone mineralization, is routinely performed

worldwide. For this, it is preferred to measure the 25OHD level, which has a longer half-life (24–36 hours), can be taken exogenously, and can be synthesized endogenously. The half-life of the 1–25 (OH) 2D3 form is short (4–6 hours), and its serum levels are 1000 times lower than 25OHD. For these reasons, the active form is not preferred for routine measurement. In this section, the measurement methods of 25OHD vitamin are discussed.

To date, many methods have been developed for measuring serum vitamin D levels. These methods are basically divided into two groups. One methodology is the use of competitive binding and immunoassays: radioimmunoassay (RIA), enzyme immunoassay (EIA/ELISA), chemiluminescent immunoassay (CLIA), electrochemiluminescence assay (ECLIA), and competitive protein binding assay. The other methodology involves chemical methods. Chemical methods are based on the non-immunological direct detection methods typically after preparative chromatographic separation. Chemical methods include high performance liquid chromatography (HPLC) and LC/MS (liquid chromatography-mass spectrometer).

The first method used in the measurement of vitamin D is the competitive binding method in which VDBP binds. This method was first reported in 1971 and identifies 25OHD2 and 25OHD3 forms equally [94]. Limitations of this method include the incubation period of 10 days and its inability to separate some polar vitamin D metabolites [24,25(OH)2D, 25,26 (OH)2D ve 25,26 (OH)2D-26,23--lactone] [94]. In the late 1970s, the HPLC method was developed that can exclude the effect of polar vitamin D metabolites causing interference to the chromatographic method [95]. The advantages of this method, which uses a UV absorption technique, include the absence of lipid and polar vitamin D metabolite interference, the ability to measure 25OHD2 and 25OHD3 separated at high resolution, and a high specificity and reliability. Its disadvantages include the use of excess sample amounts, equipment cost, a need for preparative chromatography, and interference by other UV-absorbing compounds, and that the method is somewhat complex and not easily practical. It would not be considered a routine diagnostic test, as it is used in only about 2% of laboratories in the world) [94, 95]. With the later development of the RIA method, the value of quantifying vitamin levels improved. The advantages of this method type are that sample amount can be small and not pre-analytical preparative purification process is required. The assay is economical and easily applicable, and results reliable. As to the disadvantages, chemical and radioactive (with the RIA) waste are issues, and there is cross-reactivity with polar vitamin D metabolites as in the earlier competitive binding type assays. The RIA also is 100% specific for 25OHD3 and 75% specific for 25OHD2, so the final calculation requires an adjustment [94, 96]. Nonetheless automated immunoassay methods are widely used in our country and all over the world (approximately 76% of laboratories in the world) [97]. Requiring less sample volume, not requiring sample preparation, easy equipment supply, easy application, fast results, no cross-reactivity with C3-epimer forms, and low user error are among the reasons why this method is used more widely in the world [97, 98]. Despite its widespread use, this method has some significant disadvantages. In this method, 25OHD2 and 25OHD3 cannot be distinguished and both are measured as total of 25OHD. This may lead to misinterpretation in countries that use ergocalciferol in treatment (eg America) [97]. In addition, automated immunoassay results can be affected by pregnancy, whether sampled from intensive care patients, the presence of chronic disease and liver diseases, all of which affect the amount of VDBP synthesized from the liver [99, 100]. In addition, it has been reported that there is a high probability of interferences involving automated immunoassay measurement methods [97, 101].

Due to the low reliability of immunoassay measurements, this method has begun to be replaced by LC–MS/MS, which is considered to be the “gold standard”

method. This method is used in approximately 18% of laboratories around the world, and it is estimated that its prevalence will increase due to its more accurate and precise results [97]. This method provides distinguishing quantitative measurements of both 25OHD2 and 25OHD3 forms in both serum and plasma [102]. Hence, 25OHD2 can be easily monitored in countries where ergocalciferol is widely used. In addition, with this method, C-3 epimer forms of vitamin D, which are present in high levels in serum in the first year, can be separated from other forms, and these metabolites are prevented from causing vitamin D measurement interference [97, 102].

In recent years, instead of measuring the level of vitamin D bound to VDBP, there is a strong belief in the need to measure free vitamin D levels as that is the form that accounts for the principal bioactivity. Routine methods measure the level of 25OHD vitamin bound to VDBP and provide information about the total body pool. In parallel with this, if the total body pool is sufficient, free vitamin D level is estimated to be sufficient. However, the situation is somewhat complex in obese patients, where a negative correlation between the amount of adipose tissue and serum vitamin D levels has been reported. In these cases, it has been reported that serum 25OHD level is lower than those with normal body weight, since large adipose tissue creates a larger pool for vitamin D sequestration [101–105]. In other words, serum 25OHD level in obese patients may not provide information about the body pool of vitamin D. It is thought that it would be more valuable to measure vitamin D levels that are not bound to binding protein in these cases. However, there is a serious standardization problem in the measurement of free 25OHD [103]. Also, Bikle et al. [106] proposed a method by which free 25OHD vitamin can be calculated. However, studies have shown that the results obtained with this method are not reliable [107]. Finally, direct measurement or indirect calculations of free forms of vitamin D are not yet suitable for routine use.

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