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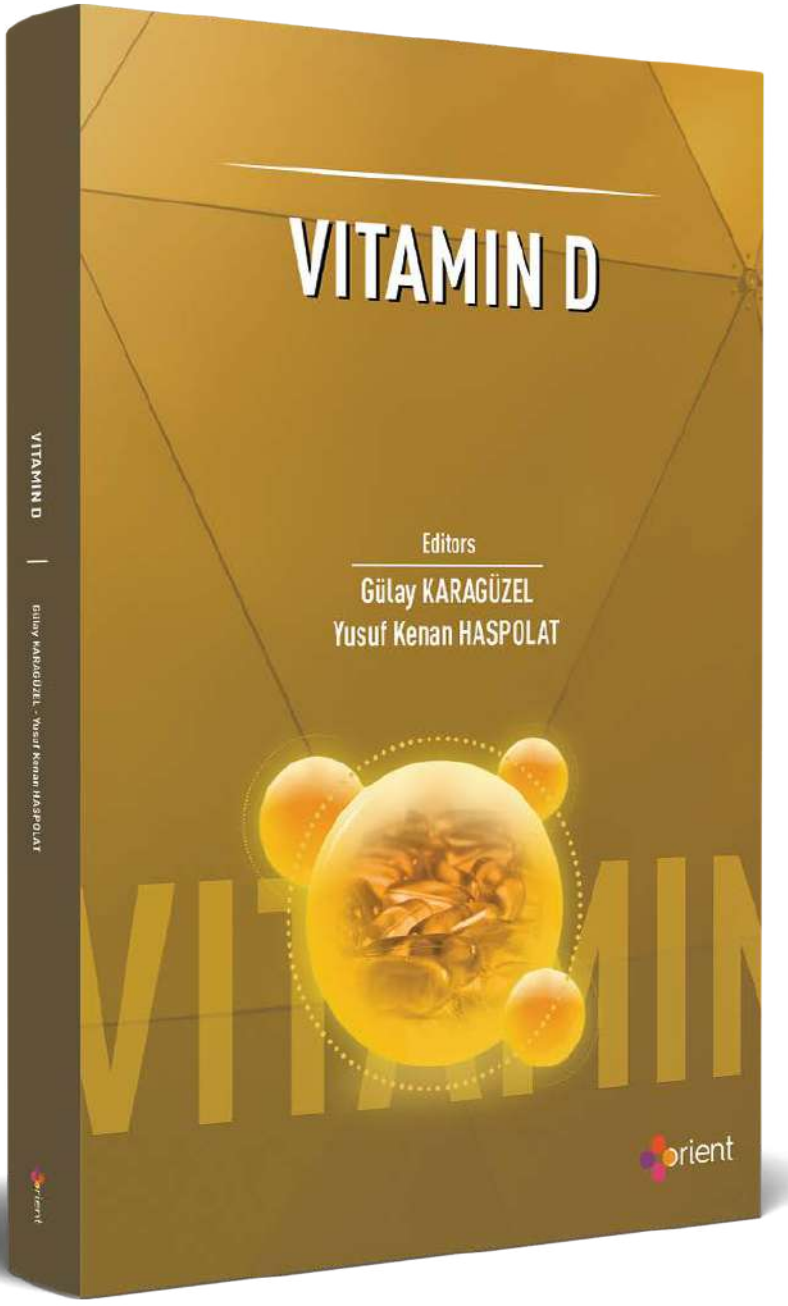
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VITAMIN D

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I dedicate this book to my mother, Zekiye Karagüzel, who has always supported me in all my activities.

Gülay Karagüzel

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CONTENT

VITAMIN D METABOLISM

MİNE KADIOĞLU DUMAN*

Vitamin D is typically a 9, 10-secosteroid hormone with fat-soluble features. Besides its effect on calcium-phosphate equilibrium, this vitamin also involved in the regulation of many organ systems working in the human body (cardiovascular, endocrine, nervous and immune systems etc.). It is found in 2 main forms, one is vitamin D2 (called as ergocalciferol) and the other one is vitamin D3 (called as cholecalciferol). Both of them are converted by UV irradiation. One source is ergosterol that is produced in plants, fungi and phytoplankton naturally or can be produced commercially by irradiating milk and mushrooms via UV (1,2). The other source is 7-dehydrocholesterol (provitamin D3), that can be found in vertebrates.

The vitamin D3 production in the human body derived from 7-dehydrocholesterol occurs via a two-step procedure. This process is performed in the keratinocytes of the epidermis layer of the skin. First UV light (280–320 UVB spectrum) breaks a ring (B ring) then the newly formed thermos-sensitive version isomerized to D3 by a non-catalytic process (1). UVB intensity and pigmentations in the skin can affect the formation rate of D3 (3).

The vitamin D form in fish is vitamin D3, however the vitamin version that is used in fortification procedure is often D2 (ergocalciferol).

The main versions formed in the body of this vitamin have difference chemically in structure and this cause a small difference in pharmacokinetic properties. In the side of chemical difference, one extra methyl group is attached at C24 carbon position and there is a double bond that takes place between the C22-23 carbon positions (Figure 1). As a result, this change cause them to have difference in molecular weights (396.65 g/mol versus 384.64 g/ mol) (2). The different properties in the side chain lower the affinity of D2 for binding to carrier protein. And this causes faster clearance of the vitamin from the circulation. This also affects its catabolism by the 24-hydroxylase (CYP24A1) (4).

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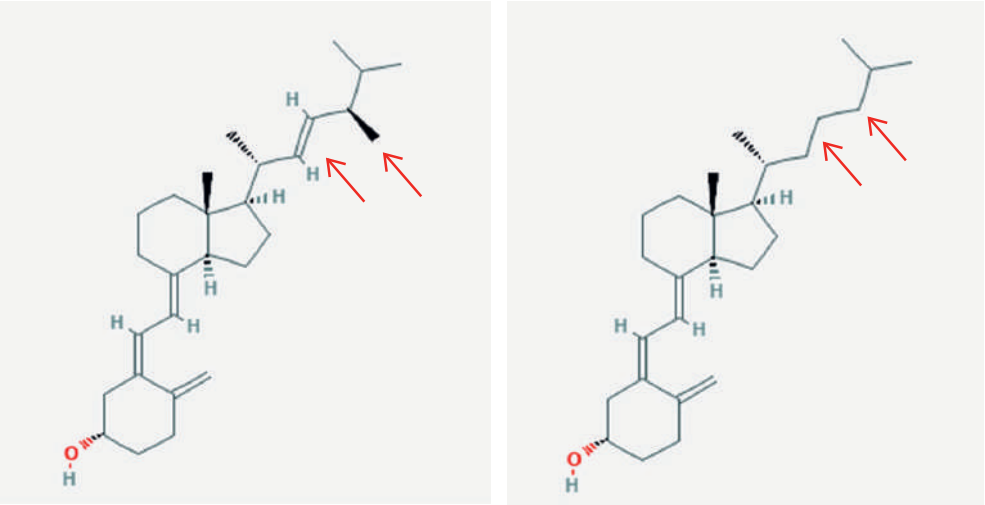

Ergocalciferol (Vitamin D_2) *Cholecalciferol (Vitamin D_3)*

Figure 1. *The two main forms of vitamin D: ergocalciferol and cholecalciferol (the chemical formulas are taken from PubChem https://pubchem.ncbi. nlm.nih.gov)*

The protein that binds vitamin D (VDBP) is used to transport both vitamin D and metabolites from one tissue to another. This is a protein with great amount of isoforms (over 100). It is primarily expressed in the hepatocytes, but it may also be found in other tissues as shown by the animal studies (5). VDBP carries approximately 85% of vitamin D metabolites and the rest is carried by albumin (5). Besides its transportation function of metabolites of vitamin D, VDBP has other actions including to help the clearance of the actin released from cells in response to different injuries in tissues.

Two main process occurred during the metabolism of vitamin D are first 25-hydroxylation and 1α-hydroxylation, and the catabolism step as 24- hydroxylation (Fig 2). All of them are performed by liver cytochrome P450 enzymes (CYPs). The CYP27A1, CYP27B1, and CYP24A1 are found in the mitochondria and CYP2R1 is found in the endoplasmic reticulum (1, 2, 6, 7). Mitochondrial CYPs need two electron transporting proteins named ferredoxin reductase and ferredoxin. In contrast, microsomal CYPs just need a single, NADPH dependent cytochrome P450 reductase (1,7).

25-hydroxylase

First conversion reaction in order to make the vitamin D metabolically active is formed in the liver. Vitamin D2 and D3 are both hydroxylated at position C25 due to the activity of the main enzyme called CYP2R1 to form 25-hydroxyvitamin D (Fig2) (8). However some studies done in knockout animals have shown that other enzymes that are the members of cytochrome P450 family having 25-hydroxylase activity (such as CYP27A1, CYP2C11, CYP2J1, CYP2D11, CYP2J2/3, CYP3A4, and CYP2D25) also contribute this conversion (2,6-10).

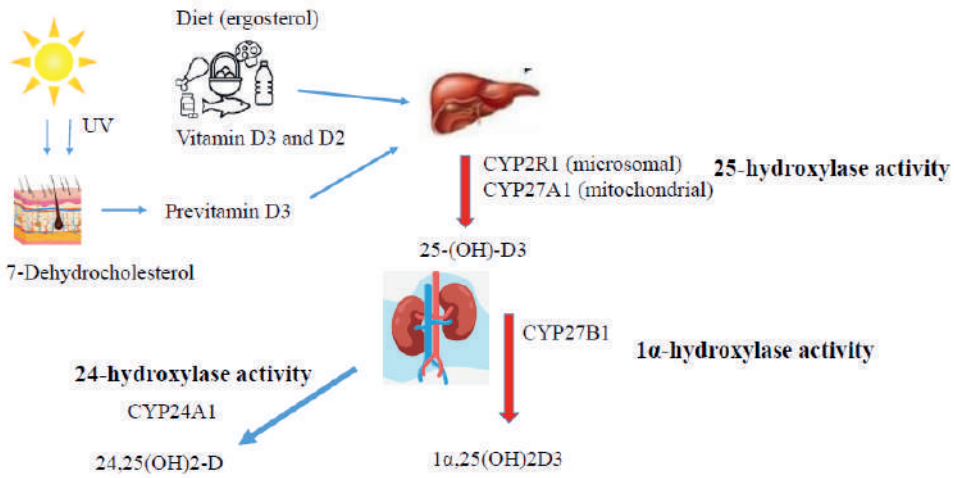

Figure 2. *The metabolism of vitamin D*

CYP2R1, which is consisted of 501 amino-acids, is a liver microsomal cytochrome P450 enzyme (Fig 2) (7). Cloning studies have shown that it is existed both in mouse and human, and it was shown to be mainly expressed in liver and testis (9). In a study done directly on the enzyme, CYP2R1 found to be unable to hydroxylate 25(OH)D, cholesterol and related molecule 7-dehydrocholesterol, showing a high selectivity and specificity for the carbon at 25th position on vitamin D however it is not selective for other sterol substrates (11). This evidence demonstrates that CYP2R1 has the needed enzymatic features for the vitamin D-25-hydroxylase action when appropriate vitamin D precursors are on the scene.

25-hydroxlase activity in liver is found in two parts; in the mitochondria and microsomal fractions. A number of CYPs having 25-hydroxylase activity have been demonstrated in several studies (1,2,6,7). The only mitochondrial 25-hydroxylase is CYP27A1 (Fig 2). This CYP enzyme is widely found in the several parts of the body, besides the liver. However, CYP27A1 does not play a role in the hydroxylation of D2 in the 25th position. Moreover, experiments done in mice displayed that when the gene is deleted, blood levels of 25(OH)D actually increased (12), and mutations playing a role in the inactivation of the gene in humans is shown to cause abnormalities in cholesterol metabolism and bile formation, but does not result in rickets (10,13). The suggested role of CYP27A1 in vivo in vitamin D metabolism is hydroxylation reaction at 25th position for 1α-hydroxylated analogs of vitamin D which are used as prodrugs during the treatment of bone diseases like osteoporosis etc.

Study to explain the relationship between CYP enzymes on hydroxylase activity; CYP27A1 gene is deleted in mice. The expression of CYP2R1 is increased in the CYP27A1 knockout mouse, and this can be an explanation of the increase in blood amounts of 25-OH-D in the.CYP27A1 knockout animal. If CYP2R1 gene is removed in animals, blood levels of 25(OH)D are found to decrease more than 50%, however not to level of

zero (12). When deletion is applied to both CYP27A1 and CYP2R1 genes; the 25(OH) D blood level did not even reduce to zero, this result suggested that a compensation situation occurred by different enzymes having 25-hydroxylase action. In a study on mice done by Cheng et al. microsomal CYP2R1 has been determined in mice liver (9). In that study, unlike CYP27A1, this enzyme hydroxylated both D2 and D3 in the 25th position. Its expression was found primarily in the liver and in the testes of males.

CYP3A4, the most known major drug-metabolizing enzyme mostly that is found in liver and also in intestine, has also 25- hydroxylase activity (14). CYP3A4 enzyme prefers 1α-OH-D as substrate. CYP2J3 that is expressed in hepatic cells of rat has also 25-hydroxylase activity, however CYP2J2, accepted as homologous enzyme in human, has less of this activity, has been expressed mainly in the heart, and seems to play a role in arachidonic acid oxidation (10). The CYP enzyme expressed in the rat liver is CYP2C11. This enzyme shows 25-hydroxylase activity for both D3, D2 and 1-OH-D analogs but its role in the hydroxylation of testosterone is dominant (10,15). It is not known whether it has a human homolog. In general, hydroxylation of vitamin D at 25-C position is not a major point, and 25(OH)D level in blood is more useful in deciding the vitamin D level in nutrition. So, CYP2R1 is accepted as the main 25-hydroxylase enzyme, however some enzymes having 25-hydroxylase activity can affect the amounts of 25(OH)D in circulation.

After the 25-hydroxylation reaction, 25(OH)D is transferred in the circulation to kidney by carrier protein, that it can go on filtration in the glomerular part of kidney and then absorbed back from the proximal part of tubular cells.

1α-hydroxylase

The second most important process in hydroxylation take part in kidney. Only mitochondrial CYP27B1 enzyme plays role in 1α-hydroxylation of 25(OH)D (Fig 2) (16-19). CYP27B1 enzyme has great amount of homology with CYP27A1 and CYP24A1, mitochondrial enzymes playing role in vitamin D metabolism. Kidney is known as the main origin of $1,25(OH)_2D$ in blood, however this enzyme is expressed by some other tissues (20) like the dermal epithelial, cells, breast, lungs, prostate, gastro intestinal system and some secretory glands like pancreatic islets, thyroid, parathyroid gland (PTG), placenta, ovary, testes etc. where it can have autocrine or paracrine actions. Thus, $1,25(OH)_2D$ may be produced in extra-renal tissues, but this extra-renal synthesis does not contribute much to the circulating blood concentration of this vitamin D metabolite (21).

Mutations occurred in the CYP27B1 gene can cause vitamin D–dependency rickets, type 1. The biochemical properties of phenotype of this syndrome is shown by deletion of the Cyp27B1 gene in mouse. The renal CYP27B1 action is essential to provide and maintain the physiologic levels of $1,25(OH)_2D$ in circulation. 1α-hydroxylase activity in kidney is strongly regulated by three hormones: $1,25(OH)_2D$ itself, parathormone (PTH) that is secreted from parathyroid gland and fibroblast growth factor-23 (FGF23). While PTH is stimulating, $1,25(OH)_2D$ and FGF23 limit the activity CYP27B1 enzyme. Elevation in calcium levels restrict CYP27B1 action via preventing the secretion of PTH; increased phosphate levels inhibit CYP27B1 activity via stimulating FGF23 expressed

in bone tissue (1,22). Several factors also play role in regulation of CYP27B1 expression, such as sex and adrenal hormones, growth hormone and prolactin.

Vitamin D catabolism

24-hydroxylase

Catabolism is one of the principle components of vitamin D metabolism. Several side chain hydroxylation reactions during catabolism cause both versions of vitamin D, 25(OH) D and $1,25(OH)_2D$ to be more polar, so they can be excreted easily in both urine and feces. The mitochondrial CYP24A1 enzyme catalyzes the first step. This 24-hydroxylation reaction forms the first step in inactivation process, as a result the biologically inactive calcitriolic acid is occurred (Figure 2).

CYP24A1 is a unique enzyme having 24-hydroxylase activity which is included in vitamin D metabolism. The enzyme in humans has both activities as 24-hydroxylase and 23-hydroxylase (23), but the enzyme in rats is primarily a 24-hydroxylase (24). Mutations in ala amino acid at 326 position to gly at 326 position in the CYP24A1 gene in humans cause the enzyme favor 24-hydroxyation to 23-hydroxylation (25). The product of this enzyme, $1,24,25(OH)_3D$ is shown to have affinity for the vitamin D receptor and has biological activity. 24-hydoxylated vitamin D may have a physiologic role in the growth plate because both $1,25(OH)_2D$ and $24,25(OH)_2D$ seem to be essential for ideal formation of endochondral bone (26). Deletion in CYP24A1 gene in mice, causing elimination of all 24-hydroxylated vitamin D metabolites, has an outcome as defective mineralization in intramembranous bone segments (27). It is shown that the main role of CYP24A1 enzyme is to avoid the increase in levels of $1,25(OH)_2D$ and 25(OH)D to toxic levels.

CYP24A1 has been determined in many tissues (placenta, brain, kidneys, intestines, and bone) expressing the vitamin D receptor (28). It is found both in the proximal and distal tubular parts of Henle in the kidney (29, 30). The CYP24A1 plays a role in the control of amounts of $1,25(OH)_2D$ in blood in order to prevent intoxication (31). CYP3A4 involves also in catabolism of vitamin D.

A number of azole antifungal drugs such as ketoconazole inhibit CYP24A1 enzyme activity.

3-epimerase

It was shown that vitamin D can be metabolized also by C3-epimerase alternatively (1,28). The hydroxy radical at the C-3 carbon position of A ring is isomerized in all natural vitamin D versions by this enzyme. This reaction does not restrict any actions of CYP27B1 or CYP24A1 enzymes. This conversion produces new vitamin D epimers. Epimers have identical structure but difference in stereo chemical configuration. The C3-epi-25(OH)-D which is known as C3-epimer is found to be the most found epimer which was determined in the circulation (31). But this epimer has decreased binding affinity to vitamin D binding protein when compared to 25(OH)D, the C-3 epimerised form of $1,25(OH)_2D$ has also decreased affinity to the vitamin D receptor when compared to $1,25(OH)_2D$, so epimerization seems to reduce the transcriptional activity and so biologic effects.

Clinically, the C-3 epimer of 25(OH)D [or 1,25$(OH)_2$D] cannot be separated from 25(OH)D [or 1,25$(OH)_2$D] by LCMS (liquid chromatography plus mass spectroscopy) when performed alone without using specific techniques of chromatography in order to distinguish the epimers. So, the 25(OH)D measurements via standard laboratory mass spectroscopic procedures cause a false increase in levels. Immunoassays could not detect the C-3 epimer thus the results are not influenced. That problem is partially important in evaluating the 25(OH)D levels in neonates, because amount of the C-3 epimer form of 25(OH)D in blood can be equivalent or more than the non-epimerised form.

The level of C3-epimers (nearly 60 % of the overall vitamin D) that have been measured both in mothers and infants was higher indicating that epimers may have a role during pregnancy and time of early development (21,28,32).

Conjugation changes the solubility of molecules in body that changes their biological activity and elimination. Sulfation reaction is done by the enzyme sulfotransferase (SULT). SULT2A1 is the enzyme primarily converts vitamin D and the main metabolites, and the reaction rate is related with the gene variations. 25(OH)D-3-sulfate, was measured as the most common sulfated form in blood. Other sulfated forms (25(OH)D-sulfate or sulphated forms of vitamins D2 or D3) have been determined in blood but not in urine, that can show these sulfated metabolites may serve as a reservoir of 25(OH)D and may be secreted through bile (28).

The other conjugation procedure for this vitamin with the metabolites is glucuronidation. This reaction is performed by an enzyme called UDP-glucuronosyl-transferases (UGT). Most common glucuronide form of vitamin D found in the circulating blood is 25(OH)D-glucuronide (28).

It is thought that there are more metabolites of vitamin D that have not been yet determined in the circulating blood of humans, so by the development of more reliable laboratory measurement systems, this situation will be understood. Because it has a longer half-life time and higher blood concentration, we can prefer 25(OH)D as the clinical marker (21,33).

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VITAMIN D MEASUREMENT METHODS

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Vitamin D, with its endocrine function, is now widely acknowledged for its crucial roles in maintaining calcium homeostasis and bone mineral metabolism, as well as performing a range of fundamental biological functions such as cell differentiation, growth inhibition, and immune modulation (1). Although vitamin D encompasses a diverse class of secosteroids, vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) are the two most important forms (2), which are metabolized in the same way, and thus, referred to as vitamin D (3). Although synthesized in the skin, vitamin D is also obtained through diet and supplements and absorbed from the intestines. Vitamin D is first hydroxylated from the 25th carbon in the liver and turns into 25-hydroxyvitamin D [25(OH)D](4). While it has been reported that other tissues, such as the skin, intestines, and kidneys, may also contribute to circulating 25(OH)D levels, the extent of their contribution is not well established (3). Subsequently, 25(OH)D is converted to 1,25-dihydroxyvitamin D [1,25(OH)2D], also known as calcitriol, through hydroxylation at the 1st carbon by the enzyme 1-alpha hydroxylase. The serum half-life of calcitriol is approximately 4 hours (4).

Despite its biological inactivity, 25(OH)D is the primary circulating form of vitamin D (2). The 25-hydroxylation process of vitamin D is not well regulated, and as a result, plasma 25(OH)D levels increase proportionally with vitamin D intake, making it a reliable indicator of vitamin D status (3,5). Vitamin D deficiency is a prevalent global health issue (6) and measuring serum/plasma vitamin D levels has become crucial to preventing many diseases associated with deficiency by administering vitamin D supplements.

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Of the over 40 vitamin D metabolites identified to date, 25(OH)D and 1,25(OH)2D have been of significant interest due to their involvement in regulating calcium and phosphate hemostasis (7). 25(OH)D, the primary circulating form of vitamin D, is tightly bound to transport proteins in the circulation and can also be stored in adipose tissue (8). The biologically active form, 1,25(OH)2D, is not ideal for measurement as it has a short half-life (4-6 hours) and circulatory levels 1000 times lower than 25(OH)D. Moreover, in cases of vitamin D deficiency, decreased intestinal calcium absorption leads to decreased ionized calcium levels, causing an increase in parathyroid hormone (PTH) synthesis and release. This increase in PTH secretion results in an elevation in 1,25(OH)2D production in the kidney, renal calcium reabsorption, and calcium mobilization from the bone. Consequently, despite vitamin D deficiency, 1,25(OH)2D levels may appear normal or elevated due to the increase in PTH secretion (9).

Total serum 25(OH)D [25(OH)D2, 25(OH)D3] measurement is widely recognized as a dependable and effective method for predicting vitamin D status, as it reflects both dietary or supplemental intake and the amount synthesized in the skin, making it an ideal marker for monitoring vitamin D supplementation in individuals with vitamin D deficiency (10).

When determining serum/plasma vitamin D levels, it is recommended to measure 25(OH)D instead of 1,25(OH)2D. The following reasons support this recommendation:

- The half-life of 25(OH)D is 2-3 weeks.
- The blood concentration of 25(OH)D is higher than that of other vitamin D metabolites.
- The hydroxylation step in the liver that produces 25(OH)D is poorly regulated, which means that dietary intake of the vitamin and its synthesis in the skin can directly increase 25(OH)D levels in the blood.

Measurement Methods and Historical Development of 25(OH)D

Upon recognizing the association between vitamin D levels and various diseases, efforts were made to quantify vitamin D levels in the blood. However, due to the low concentration of vitamin D metabolites (nano to micromolar levels) in the blood and limitations of the methods available at that time, the implementation of these tests in clinical biochemistry laboratories were delayed (7).

Methods for determining 25(OH)D levels can be classified into two primary categories:

1. Immunoassay methods employing antibodies against the D2 and/or D3 forms of 25(OH)D
2. Chromatographic methods that separate 25(OH)D2 and 25(OH)D3 based on their chemical properties.

In general, immunoassay methods offer several advantages, such as ease of integration into automated systems and shorter turnaround times. On the other hand, chromatographic methods are more complex, require a longer turnaround time, and may necessitate more skilled personnel for operation and interpretation. However, immunoassay methods are typically less expensive and easier to use compared to chromatographic methods (11).

The historical development of vitamin D measurement methods can be summarized as follows:

- Introduction of the first competitive protein binding assay (CBPA) in 1971
- Development of the high-performance liquid chromatography (HPLC) method utilizing the chromatographic method and a UV detector in 1977
- Emergence of the radioimmunoassay (RIA) in 1985 (7,12)
- Implementation of automated immunoassay techniques (enzyme-linked immunosorbent assays (ELISAs), chemiluminescence assays) in 2001 (13)
- Liquid Chromatography Tandem Mass Spectroscopy (LC-MS/MS) methods for 25(OH)D measurements in 2004 (14)

Competitive Protein Binding Assay (CBPA):

Introduced in 1971, the CBPA was developed to assess serum 25(OH)D levels. The method involves extracting 25(OH)D from the sample using an organic solvent, followed by silicic acid chromatography. Subsequently, serum containing vitamin D binding protein (DBP) derived from vitamin D-deficient rats is added, and serum 25(OH)D levels are measured using the protein binding technique. Initially, the method's 10-day incubation period limited its clinical application. Consequently, the method was refined by altering the 25(OH)D extraction stages, reducing the incubation time to one hour. This modification rendered the method suitable for widespread use in clinical laboratories (7). CBPA offers several advantages, such as low cost, small sample volume requirements, and commercially available kits (15). However, the method also has drawbacks, including the inclusion of other polar vitamin D metabolites, such as 24,25(OH)2D, 25,26(OH)2D, and 23-lactone, which can lead to falsely elevated results (16). Additionally, the method exhibits equal sensitivity for measuring both 25(OH)D3 and 25(OH)D2 levels. The subsequent discovery of polyclonal antibodies specific to 25(OH)D, the development of the RIA method, and advancements in HPLC equipment contributed to the adoption of these novel techniques in clinical laboratories, replacing protein binding methods despite their clinically acceptable analytical sensitivity (17).

Immunoassay Methods

Immunoassay methods are widely utilized in both medical and clinical biochemistry laboratories (18). These techniques have been specifically developed for detecting low concentrations of substances and are employed in clinical laboratories to measure various analytes, both urgent and routine. Immunoassay methods are based on the sensitive interaction between antibody and antigen (18,19). Nonspecific binding in the antigen-antibody relationship represents a limitation in these methods' measurement capabilities. As a result, immunoassay techniques have focused on reducing limiting factors by incorporating signal-enhancing methods (19). To create more sensitive and specific immunochemical methods, labeled antigens and antibodies have been integrated into these techniques. Radioactive markers were initially used for this purpose, but non-isotopic markers started

to be used with the emergence of enzyme immunoassays in the 1970s. These methods are named differently based on the marker characteristics.

Markers used in immunoassay methods include:

- Radioimmunoassay (RIA) for radioactive materials
- Fluorescent immunoassay (FIA) for fluorescent materials
- Chemiluminescence immunoassay (CLIA) for chemiluminescent substances
- Electrochemiluminescence immunoassay (ECLIA) for electrochemiluminescent materials
- Enzyme immunoassay (EIA) for enzymes (20)

Automated immunoassay methods typically measure total 25(OH)D (the sum of 25OHD2 and 25OHD3). Compared to alternative techniques, immunoassays require less sample volume, are easier to use, involve fewer user errors, and yield faster results (21). However, 25(OH)D immunoassays inherently face challenges in distinguishing between numerous polar vitamin D metabolites and vitamin D-like seco-steroids. Additionally, due to commercial sensitivities, analytical methodology details, such as method calibration, are not always disclosed (10). Nonetheless, automated immunoassays are preferred in clinical laboratories for their practicality.

Radioimmunoassay (RIA): This sensitive and specific heterogeneous immunoassay utilizes radiolabeled antigens and antibodies. The assay comprises the antibody for the analyte being measured (attached to the tube wall), the antigen labeled with I^{125}, and the analyte to be measured (unlabeled antigen) and antibody. Labeled and unlabeled antigens compete for antibody binding. Following washing and separation processes, the antigen-antibody complex remains. Analyte concentration is calculated from the standard graph by measuring the irradiance of I^{125} with the GAMMA counter. The more the analyte (unlabeled antigen) we want to measure, the less the irradiation amount because the labeled antigen binds less to the antibody (20,22). For measuring 25(OH)D using this method, an extraction step enabling simple and non-chromatographic quantification of total 25(OH)D in the sample is necessary prior to analysis (23).

In 1985, an RIA method incorporating a specific 25(OH)D antibody was developed. This method involved the extraction of 25(OH)D from serum/plasma for 25(OH)D measurement. Based on variations in this step, two distinct RIA methods emerged. First, 25(OH)D and other hydroxylated metabolites were separated from serum/plasma samples using acetonitrile, followed by completion of the measurement using the competitive RIA method containing 25(OH)D-specific antibodies. In the second method, two reagents were employed to precipitate proteins in the serum, allowing separation of 25(OH)D and other hydroxylated metabolites from the serum, and completing the measurement using the RIA method (14). RIA standards and controls applied for calculations in these RIA methods underwent the same procedures as the samples (7). Although calibration curves were generated with 25(OH)D3 in the method, it was suggested that the primary antibody used recognized both forms of vitamin D (vitamin D3 and vitamin D2) equally.

This indicated that the method enabled the measurement of total 25(OH)D by assessing vitamin D2 in addition to vitamin D3 (24).

Variability in extraction steps and antibody diversity employed by manufacturers in RIA-based 25(OH)D measurements contribute to differences in RIA method application steps. Depending on the manufacturer, this may influence the percentage of cross-reactions to vitamin D metabolites, detection limits of the kits for 25(OH)D, and their sensitivity for 25(OH)D3 and 25(OH)D2. These issues are relevant to all immunoassay measurements (17). The limited shelf life of radioactive markers and rendering radioactivity harmless constitute important problems of the RIA method. As described below, in alternative immunoassay methods, these radioactivity-related issues are decreased since chemiluminescent substances (CLIA) or enzymes (EIA) are largely but not entirely employed as markers (14).

The RIA method is generally straightforward to implement, and its results are consistent with HPLC. This method recognizes 25(OH)D2 and 25(OH)D3 equally as in competitive protein binding measurement and includes other polar vitamin D metabolites. Once it was determined that these polar vitamin D metabolites represent a small fraction (6%) of the circulating metabolites, it was considered that their impact could be disregarded for this method. Commercial kits for RIA measurement of vitamin D remain available and in use today (7).

Chemiluminescence Immunoassay (CLIA): In this method, a substance that emits light (chemiluminescence) is used as a marker. Similar to RIA in test principle, the light intensity generated is measured with a luminometer (22).

Electrochemiluminescence Immunoassay (ECLIA): This immunoassay utilizes electrochemiluminescent molecules, such as ruthenium, as markers. The required energy for the electroluminescent property is derived from electrode reactions. Ruthenium (II) tris(bipyridyl) undergoes an electrochemiluminescent reaction (620 nm) with tripropylamine on the electrode surface. ECLIA is a measurement method employing magnetic beads as the solid phase with this marker, and measurements are conducted within the measuring cell. Unbound markers are removed from the cell through washing while the beads are held on the electrode. The marker attached to the bead reacts via electrochemiluminescence, and the light emission is measured by the adjacent photomultiplier tube (20). This method is highly sensitive, demonstrates strong linearity, and can be easily applied to autoanalyzers for numerous analyte measurements (22).

In 2001, a fully automated chemiluminescence test system was developed for measuring vitamin D. In this method, unextracted serum or plasma is directly mixed with human DBP, acridinium ester-labeled anti-DBP, and 25(OH)D3-coated magnetic particles. This method, which is similar to the CPBA method as it uses DBP as a binder, brings the sample analysis environment and the calibrator analysis environment closer together, as the calibrators are in a serum-based matrix. Following this chemiluminescence method, chemiluminescence methods with a similar principle, using antibodies as binders akin to the RIA method, have also been developed (13). The light generated as a result of the chemiluminescence reaction in these methods is measured by the photomultiplier as

relative light units (RLU). The light generated after the reaction is inversely proportional to the 25(OH)D concentration in the sample, calibrator, and standards (14).

Enzyme immunoassay (EIA): This assay utilizes the catalytic properties of enzymes to detect and quantify immunological reactions. Alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and beta-galactosidase are among the most commonly used enzymes in EIA. The technique, similar to RIA, involves an antigen-antibody reaction. The unlabeled antigen serves as the analyte to be measured, while an enzyme is employed as a marker. The products formed by the addition of the enzyme's substrate to the field are used for measurement. Various detection systems are employed in EIA. The products formed after the catalytic effect of enzymes are most frequently detected photometrically (20,22). However, EIA methods utilizing chemiluminescence and fluorogenic substrates are gaining prominence due to their heightened sensitivity (20).

• ***ELISA:*** It is a widely used heterogeneous enzyme immunoassay (EIA) technique. The binding antibody attaches to the solid-phase surface. Calibrator and patient samples containing the target antigen are added and incubated for a period to bind with the solid-phase antibody. After washing away unbound substances, an enzyme-labeled antibody is added, and antibody-antigen-(antibody+enzyme) sandwich complex is formed. Repeat washing is done to remove unbound antibodies and enzyme substrate is added. Enzyme-labeled antibodies convert the substrate to product, and the amount of product is directly proportional to the antigen amount in the sample (20).

After the RIA method, fully automatic chemiluminescence methods and ELISA, an EIA method, have become widely used for 25(OH)D measurement. Currently, two different ELISA methods have been defined. In the ELISA method using biotin-labeled 25(OH)D antibody, there is no need for pretreatment on the samples, the sample, calibrator, and controls pass through the same steps such as washing, incubation, and finally, with the addition of the chromogenic reagent, the color intensity formed on the microplate is measured as absorbance with a microplate reader and calculations are made. In the other ELISA method, antibodies created against serum DBP are used as antibodies, and this method, unlike the other, includes a single extraction step in which samples, calibrators and controls are included before analysis. The other steps of the method are similar to the previous method. It has been reported that 24,25-dihydroxyvitamin D3 and other dihydroxylated vitamin D metabolites may cross-react in both ELISA methods. However, low circulating levels of these metabolites reduce concerns about measurement (7).

Limitations and Interferences in Immunoassay Methods: In immunoassay and protein binding methods, the strong binding of the 25(OH)D molecule to DPB (15) and its lipophilicity render the measurement susceptible to matrix effects. While primarily caused by lipid molecules, any component in the serum, plasma, or sample tubes can contribute to a matrix effect (13), potentially leading to falsely elevated results. Organic solvents that dissociate 25(OH)D from DBP may be incompatible with numerous immunoassay and protein binding methods, and the separation process's sufficiency in these methods remains uncertain, despite the development of novel techniques. This inadequate separation can result in falsely low 25(OH)D measurements. The separation process in immunoassay

and protein binding methods may also induce cross-reactions against 25(OH)D2 and 25(OH)D3 (25).

In immunoassay methods, common sources of interference include cross-reaction, heterophile antibodies, autoantibodies, analyte antibodies (macro complexes), hook-effect at high analyte concentrations, and binding proteins (18). Among human anti-animal antibodies, animal immunoglobulins, particularly HAMA (human anti-mouse antibody), can generate falsely elevated results by mimicking the analyte in sandwich measurements that utilize mouse-derived antibodies in the reagent. Additionally, HAMA's reaction with one of the measurement reagents may produce false-negative outcomes, as it will prevent sandwich formation with the specific analyte (20).

Chromatographic Methods

The high-performance liquid chromatography (HPLC) method, established in 1977, is generally considered the gold standard for comparing other methods in determining circulating 25(OH)D levels (13). Key advantages of the method include the removal of interfering lipids and vitamin D metabolites in the HPLC method and the capacity to separately measure 25(OH)D2 and 25(OH)D3. Nevertheless, this method also possesses some drawbacks such as large sample volume, expensive equipment, labor-intensive processes, and the need for experience and extended time (7,13,21). Subsequently, in the history of vitamin D measurement methods, the LC-MS/MS method was developed after HPLC. Owing to its increased sensitivity, this method has been increasingly utilized in clinical laboratories and is currently deemed the reference measurement procedure for calibrating many commercially available immunoassay kits (26). Chromatography, which underlies these methods, is the process of separating dissolved substances in a sample by exploiting their physicochemical properties against two distinct solvents: one referred to as stationary and the other as mobile phase. As the mobile phase passes through the stationary phase, the dissolved molecules are dispersed between the two phases. Molecules with low affinity for the stationary phase predominantly reside in the mobile phase, thus distancing themselves from the environment. Molecules with high affinity for the stationary phase are collected by separating them from the stationary phase through the alteration of the mobile phase properties. The method is called gas chromatography (GC) if the mobile phase is a gas and liquid chromatography (LC) if it is a liquid. When the stationary phase in LC comprises small diameter particles, it is termed 'HPLC'. Furthermore, the method is also referred to as 'high-pressure liquid chromatography' since the particles in the stationary phase (column) are small, necessitating relatively greater pressure to be applied to the system. HPLC is the most commonly employed form of LC (20).

In general, an LC device consists of the following components:

- Mobile phase source-solvent reservoir
- Pump: Draws the mobile phase from the solvent reservoir and circulates it through the chromatographic system.
- Injector: Allows the sample to mix with the mobile phase before entering the column.
- Column: Serves as the stationary phase for analyte separation.

- Detector: Acts as the flow cell through which the eluate leaving the column passes in the mobile phase. Separated analytes are measured according to the detector feature utilized here (UV photometer, spectrophotometer, electrochemical detectors…).
- Computer and recorder: Control the system operation. It generates chromatographic peaks by recording signals from the detector. Analyte amounts are calculated from the calibration curve based on the height and area of these peaks.
- Waste

High Performance Liquid Chromatography (HPLC): The earlier HPLC method was primarily developed for investigating 25(OH)D3 in serum, making it too complex for routine use. The subsequent method aims to be user-friendly, sensitive, fast, and straightforward in sample preparation. Isocratic elution facilitates separation and quantification of 25(OH)D3 from 25(OH)D2 (24). Initially, silica columns were employed for 25(OH) D measurements by HPLC, but later C18 reversed-phase columns were used, and better reproducibility (stability) was obtained (27).

Sample preparation is a critical step in analysis by HPLC. It involves multiple stages, such as sample purification/concentration, typically performed before chromatographic separation and quantification, and derivatization preparation, which can be done before or after the chromatographic separation stage. These processes enhance the technique's detection capability (20).

Internal standards and 25(OH)D metabolites are rapidly degraded by light, particularly direct natural sunlight. Temperatures exceeding 35 degrees Celsius and prolonged exposure to nitrogen following solvent evaporation may reduce internal standard recovery, leading to inaccurate measurements. Thus, the volumes of extracted calibrators and controls should be identical to the volume of patient samples tested (27).

In this method, false peaks unrelated to 25(OH)D2 may arise, and inexperienced laboratory personnel may interpret these peaks as 25(OH)D2, resulting in falsely high measurements (13). Consequently, the experience of the personnel conducting the analysis is crucial in reaching an accurate conclusion.

Mass Spectrometry: In assessing vitamin D levels, immunoassays can measure total 25(OH)D but frequently encounter interferences such as matrix effects. In contrast, chromatographic methods can measure both 25(OH)D2 and 25(OH)D3 with higher sensitivity. However, immunoassay methods are often preferred in clinical laboratories due to their advantages. Combining chromatographic methods with mass spectrometry makes them the preferred choice for Reference Laboratories. Despite the increased cost, mass spectrometry methods are utilized in numerous laboratories as highly sensitive techniques for measuring 25(OH)D2 and 25(OH)D3 (11).

Mass spectrometers are instruments that measure charged particles based on mass-to-charge ratios. It is an advanced technique employed to determine the chemical structure of all kinds of molecules and compounds (22). By ionizing the target molecule, separating it into ions according to mass-to-charge (m/z) ratios, and generating a graphical mass spectrum, mass spectrometry determines both the structure and quantity of the molecule (20).

Components of Mass Spectrometry:

1. Ion Source: The initial step upon the sample entering the system is ionization. Various techniques are employed to ionize the sample [Electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI)…].
2. Vacuum System: A vacuum is utilized to avoid collisions between ions generated within the system.
3. Mass Analyzer: This component separates ions based on their m/z ratios, enabling them to reach the detector. Various mass analyzers are available (Magnetic Sector Analyzers, "Quadrupole ion trap" Analyzers, Time of Flight Analyzers (TOF)).
4. Detector: Detectors are used to convert the intensity of the reaching ions into an electronic signal. The obtained signal is recorded in the data system in the form of a peak by means of an electronic system.

The molecule's analysis is performed by comparing the mass spectrum, which displays fragmented ion products, with reference spectra. Quantification is made through the retention time of the analyte and the peak heights it has formed (20, 22). MS allows for both quantification and determination of molecular structure.

In mass spectrometry measurements, the analyte's chemical properties remain consistent, but a heavier, stable isotope is utilized as an internal standard (20).

Gas and liquid chromatographs and MS are mapped to each other with interfaces to create devices with strong specificity and sensitivity in measurements (GC/MS; LC/MS; LC/MS/MS). In these systems, only nanogram and picogram levels of the analyte are sufficient for analysis (20).

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method: By integrating LC and MS systems, the diversity of measurement systems is enhanced. Consequently, LC-MS/MS is not a singular, "ready-made" technique, as variability in sample preparation, chromatographic separation, and ionization/fragmentation must be taken into account at each stage. However, the adaptability of LC-MS/MS systems can be advantageous, providing opportunities for customization and standardization of methods (10). As a result, various liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are outlined. These methods necessitate comprehensive sample preparation. Extraction and/or chromatographic separation is a prerequisite for all LC-MS/MS methods, and numerous approaches are employed (5). Consequently, the sensitivity of these systems varies depending on the analytes being measured.

LC-MS/MS is recognized as the gold standard method for 25(OH)D3 measurement (15,28). For instance, LC-MS/MS can differentiate and independently quantify 25(OH) D2 and 25(OH)D3 solely based on the mass-to-charge ratio (m/z). The adoption of the LC-MS/MS technique is increasing due to the automation of the sample preparation stage, the speed of the chromatographic steps, and the sensitivity of the MS method for measuring 25(OH)D. Nevertheless, LC-MS/MS is not an easy method for serum 25(OH) D analysis. Initially, the inter-laboratory CV% values were not satisfactory and one of the

reasons for this variation was that each laboratory prepared its own calibration standards 'in house' for analysis (10). The National Institute of Standards and Technology (NIST), in collaboration with the National Institutes of Health Office of Dietary Supplements (NIH-ODS), has developed a Standard Reference Material (SRM) for determining 25(OH)D, known as SRM 972. This material has certified and reference values for 25(OH) D2, 25(OH)D3, and 3-epi-25(OH)D (29). The use of shared standards has diminished uncertainty between laboratories. Moreover, interlaboratory differences continue to be investigated, despite improved understanding of other factors influencing LC-MS/MS methods, such as the need for method standardization, the tubes utilized for sample collection and preparation, and the interaction of other vitamin D metabolites (10).

LC-MS/MS methods often encounter ion suppression problems (30). This problem is caused by substances eluting with the analyte. These substances enter the ion source simultaneously with the analyte, and become the preferred ion in the measurement, they often cause a reduction in the signal of the measured analyte (28). Ion suppression can cause difficulties with repeatability and precision when examining small amounts of analytes in complex samples such as biological fluids. The accuracy and reliability of the measured analyte may be impacted by matrix components that co-elute. This issue frequently arises when using internal standards that do not share chemical and structural similarities with vitamin D. Recently, deuterated-tetrahydrocannabinol-D3 has been employed as an internal standard to minimize ion suppression while allowing for shorter analysis times. In addition, deuterated vitamin D compounds are now available as internal standards (30). For most HPLC and LC–MS/MS methods, extraction and procedural losses are corrected by using an internal standard (14). In particular, the use of deuterated vitamin D as an internal standard corrects systematic errors related to ion suppression (28).

Tandem mass spectrometry provides good specificity and sensitivity; however, interference can arise from isobaric or isomeric compounds that can affect the measured values. Particularly in the quantification of 25(OH)D3, the C3-epimer of 25(OH)D3, which is present in substantial amounts in some infants, may co-elute with 25(OH)D3 in most HPLC systems, leading toa high measurement of 25(OH)D3 levels. To overcome this shortcoming, a series of LC-MS/MS procedures utilizing chiral HPLC columns, such as Phenomenex Kinetex PFP, have been described to achieve the required selectivity for separating the C3-epimer from 25(OH)D3 and -D2 (31). If the method is not optimized, it may cause results such as high 25(OH)D3 levels in the pediatric population, while some publications indicate that these epimers are also present in adults (15). In summary, HPLC/UV, and LC-MS/MS methods for 25(OH)D may be unable to differentiate 3-epi-25-OHD3 from 25(OH)D3, potentially resulting in overestimated 25(OH)D levels in these measurements (32).

Recently, an LC-MS/MS method that separates 25(OH)D from 3-epi-25(OH)D3 has been developed. In order to make this distinction, in addition to the internal standard used in the normal measurement, the stable isotope standard of the epimer must also be used (28).

While 25(OH)D immunoassays did not exhibit interaction with 3-epi-25(OH)D3, it has been observed that this metabolite cross-reacts in some competitive protein binding (CPBA) methods (32).

The LC-MS/MS method has several limitations in the measuring of vitamin D and its metabolites. Vitamin D's lipophilic nature and strong binding to DBP may result in matrix effects during sample extraction (protein precipitation (PP), liquid–liquid extraction (LLE), solid-phase extraction (SPE), or combination of these techniques). If isomeric and isobaric compounds present in samples such as serum or plasma are not properly separated by liquid chromatography, it can lead to an overestimation of 25(OH)D concentrations. Since the ionization efficiency of vitamin D metabolites under atmospheric pressure chemical ionization (APCI) and Electrospray ionization (ESI) sources, which are frequently used in LC-MS/MS method, is not very good, in LC-MS/MS measurements where these ionization sources are used to measure vitamin D metabolites at low concentrations some limitations may occur (2). In addition, variations are observed in the measurement of 25(OH)D depending on the difference in the ionization sources used in the LC-MS/MS method (16). Another reason for the analytical error is that, as stated above, molecules with the same molecular weight, called isobaric compounds, elute together, causing high measurements. Many isobaric compounds have been demonstrated for 25(OH) D3 in serum. Although many LC/MS methods can easily separate these compounds by providing sufficient selectivity in the chromatographic step, it has been shown that there is difficulty in separating isobaric compounds in the LC method (28).

Although many methods have been defined for the evaluating of vitamin D level, CPBA, RIA and CLIA are among the most preferred methods in clinical practice because they are in kit form, can be easily automated and allow a large number of test studies in a short period of time. Although it has been shown in the literature that these methods are generally equally sensitive to 25(OH)D2 and 25(OH)D3 and have no affinity for 3-epi-25-hydroxyvitamin D3, this is controversial (28). Specificity in immunoassays can be a problem, especially with regard to the measured 25(OH)D2 ratio, while HPLC and LC–MS/MS methods can independently measure two major metabolites [25(OH)D2 and 25(OH)D3] without being affected by this ratio (14). In a study, when HPLC and LC/MS/MS results were compared, they were found to be quite compatible, while 25(OH) D levels were found to be high in cases where the vitamin D3 form was dominant, and low in cases where the vitamin D2 form was dominant, with the CPBA method. It was concluded that this was due to cross-reactions with vitamin D metabolites and serum matrix effect (27).

Table 1: Commercially available 25-hydroxyvitamin D assays.

Manufacturer	Sample type and volume	Extraction	Range of detection (nmol/L)	Sensitivity (nmol/L)	Intraassay CV (%)	Interassay CV (%)	Assay Time*
RIA DiaSorin	Serum or plasma, 50 µL	Acetonitrile		≤6	<8	<12	2 h, 10 min
RIA IDS	Serum or plasma, 50 µL	Two-step reagent extraction	4-400	≤5	5.5	7.9	3 h, 0 min
ELISA IDS	Serum or plasma, 50 µL	None	6-360	≤5	<6	<9	3 h, 0 min
ELISA Biomedica	Serum or plasma, 50 µL	Proprietary extraction reagent	6.3-250	2.4	9	11	5 h, 0 min
CLIA -Nichols Institute Diagnostics	Serum or plasma, 20 µL	Unknown	17.5-300	18	6.6	11.2	75 min
-Abbott Diagnostics**	Serum or plasma, 60 µL	Online Pre-treatment	0-400	7.74	<3.7 (mean conc: 47.50 nmol/L)	Total CV (%): 3.8 (mean conc: 47.50 nmol/L)	36 min
-Beckman Coulter Access2**	Serum or plasma , 30 µL	None	11-418	11	<2.2 (mean conc: 61.50 nmol/L)	<7.2 (mean conc: 61.50 nmol/L)	40 min
-Beckman Coulter UniCel DxI 800**	Serum or Plasma, 30 µL	None	11-525	11	<4.7 (mean conc: 65.00 nmol/L)	<5.7 (mean conc: 65.00 nmol/L)	40 min
- DiaSorin Liaison XL **	Serum or Plasma, 25 µL	None	10-374.4	≤10	2.3	Total CV (%):7.8	24 min
- Roche Diagnostics Elecsys**	Serum or plasma, 9µL	None	7.50-300	7.5	2.5 (mean conc: 48.00 nmol/L)	4.6 (mean conc: 48.00 nmol/L)	27 min
- Siemens ADVIA Centaur XP**	Serum or plasma, 20µL	None	10.5-374.4	10.5	5.3 (mean conc: 43.00 nmol/L)	9.9 (mean conc: 43.00 nmol/L)	18 min
HPLC IDK	Serum, 500 µL	Acetonitrile and C18 cartridge extraction	15-150	4.0	5.2	8.4	20 min
CPB Immunodiagnostik	Serum, plasma or urine, 50 µL	Acetonitrile	8-312	2.5	9.9	14	1 h, 10 min

*Adapted by Zerwekh JE. The measurement of vitamin D: analytical aspects. Ann Clin Biochem 2004;41: 272–281. *Does not include extraction, counting or microplate reading times. ** Kit inserts were used.*

CV, coefficient of variation; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; CLIA, Chemiluminescence immunoassay; CPB, competitive protein binding; Conc, concentration.

When comparing 25(OH)D measurement methods, it should be taken into account that there may be roughly 10-15% variation between methods. Performance criteria of commercial vitamin D measurement kits are given in Table 1.

Measurement of Free 25(OH)D Levels

The majority of total 25(OH)D and 1,25(OH)2D are bound to DBP, with about 10% bound to albumin, while only roughly one-thousandth of circulating vitamin D metabolites exist in free form (33). The predominance of vitamin D metabolites bound to DBP can be attributed to the circulating DBP concentration being 20 times higher than the combined concentration of all vitamin D and its metabolites (34). In addition, the non-genotypic binding coefficient for DBP of both 25(OH)D and 1,25(OH)2D (7×10^8 M^{-1} and 4×10^7 M^{-1}, respectively) is approximately 1,000 times greater than the binding coefficient for albumin (6×10^5 M^{-1} and 5.4×10^4 M^{-1}, respectively) (35). However, due to the higher abundance of albumin in circulation compared to DBP (650 μM and 5 μM), approximately 10% of vitamin D and its metabolites are bound to albumin (36).

The "free hormone hypothesis" posits that the biologically active metabolite is the unbound or free hormone (36). Free 25(OH)D levels can be determined either directly through immunoassay methods or by calculations using protein binding constants of 25(OH)D. A commercial ELISA kit is available for measurement of free 25(OH)D3 levels. The assay has been characterized in terms of precision (4-10% CV, according to concentration), sensitivity (limits of blank = 0.5-1.0 pg/mL and LODs = 1.3-1.8 pg/mL) (37).

Calculation of Free and Bioavailable 25(OH)D3 Levels: The concentration of free hormone for vitamin D is extremely low, and therefore bioavailable vitamin D, which refers to both the free and the loosely bound albumin moiety, is referred to as biologically active vitamin D (36). In the studies, formulas have been devised to calculate bioavailable 25(OH)D3, which is not bound to DBP. These formulas define bioavailable hormone as the fraction that is both free and albumin-bound, or the portion that is not bound to circulating binding proteins such as VDBP (38). The free 25(OH)D3 fraction; The protein binding is calculated using affinity constants.

Kalb = affinity constant between 25(OH)D3 and albumin = 6×10^5 M^{-1}
KDBP = affinity constant between 25(OH)D3 and DBP = 7×10^8 M^{-1}

Calculated Free 25(OH) Vitamin D3

$$= \frac{\text{Total 25(OH)D}}{1+\left(6\times10^{5}\times[\text{Albumin}]\right)+\left(7\times10^{8}\times[\text{DBP}]\right)}$$

Levels of Bioavailable 25(OH)D3= ($6x10^5$ x [Albumin]+1) x Calculated free 25(OH)D3

[Albumin]= (serum albumin g/L)/66.430 g/mol

[DBP]= (serum DBP g/L)/58.000 g/mol

1,25(OH)2D Measurement

1,25(OH)2D is the active metabolite of vitamin D, responsible for regulating calcium and phosphorus balance in the blood. Serum/plasma 1,25(OH)2D levels are generally required for diagnosing congenital and acquired disorders of 25(OH)D metabolism, as well as hypercalcemic syndromes associated with chronic granulomatous disorders and renal diseases (2). Measuring 1,25(OH)2D can be challenging due to its low serum concentrations and the presence of interfering substances (39). Its short circulating half-life of only 4 to 6 hours further complicates the measurement (2).

Currently, RIA, ELISA, HPLC-UV, and LC-MS/MS are employed for 1,25(OH)2D analysis. However, traditional methods may lack adequate sensitivity (40).

The first 1,25(OH)2D measurement was conducted in 1974 using the radioreceptor assay (RRA) method, which required large serum sample volumes, extraction and separation with methanol-chloroform solvent, three consecutive chromatographic purifications, and fresh intestinal vitamin D receptor prepared from rachitic chickens in each measurement (7). Later in 1984, the method was improved by using a vitamin D receptor from bovine thymus, which remained stable for months when frozen instead of fresh tissue (7, 41). In addition, with the improvement of the method and the reduction of the sample volume used, this method has been used as a reference method in many laboratories (7).

In 1978, the RIA method was developed. Requirement of sample preparation and the relative nonspecificity of the antibodies used have limited its clinical applicability. In 1996, the first clinically useful RIA measurement was described, which correlated with RRA results, and both analytical and clinical validation were reported for this test. Although a new RIA method described later was also correlated with RRA, it has been shown to cause interference with vitamin D metabolites such as 26,23-lactone; 1,24,25(OH)3D3; 1,25,26(OH)3D3. In these methods, the interfering metabolites are removed by applying purification steps before their analysis, and the use of HPLC is a very suitable method for this step (7). In addition, it has been reported that radioimmunoassays incorrectly measure the levels of 1,25(OH)2D in vitamin D intoxication (42).

Historically, the 1,25(OH)2D measurement was conducted using the ELISA technique. This method involved separating 1,25(OH)2D from interfering substances through double column extraction, and its results have been shown to correlate with RRA (7). Commercial immunoassay methods for 1,25(OH)2D vary in specificity and may also measure 1-alpha hydroxylated inactive metabolites other than 1,25(OH)2D (43).

The limitations of measuring the 1,25(OH)2D molecule by mass spectrometry are similar to the 25(OH)D measurement. The low ionization efficiency of 1,25(OH)2D, its short half-life of 4-6 hours, and its presence at picomolar levels in the blood make accurate measurement difficult. Additionally, other dihydroxylated vitamin D isomers (e.g., 23,25(OH)2D, 25,26(OH)2D, 24,25(OH)2D) with the same molecular charge can cause false elevations, necessitating chromatographic separation before analysis (2).

Quantification of 1,25(OH)2D by LC-MS/MS is often difficult due to its low concentrations in serum and the presence of interfering substances. The ionization efficiency

is low in ESI and APCI due to the lack of ionizable polar groups. Some researchers have used ammonium or lithium adducts to enhance ionization efficiency. Kissmeyer et al. reported the initial measurement of 1,25(OH)2D in rat and pig serum using ammonium adducts by LC-MS/MS. However, there is no data on human serum in this study (40). The first 1,25(OH)2D measurement in human serum by LC-MS/MS was performed by increasing the low ionization efficiency using a stable lithium adduct. In this measurement, acceptable coefficients of variation were obtained in physiological concentration (42).

Currently, LC-MS/MS is the preferred method for 1,25-dihydroxy vitamin D analysis due to its sensitivity and reproducibility. However, the poor ionization efficiency due to the lack of ionizable polar groups remains a challenge in the use of LC-MS/MS. In addition to the use of lithium and ammonium adducts, derivatizing agents are also used to improve the ionization efficiency (Diels-Alder derivatization, derivatization with 4-phenyl-1,2,4-triazol-3-5-dione (PTAD) reagent, derivatization with Amplifex diene). It has been reported that the sensitivity and selectivity of the analysis of 1,25(OH)2D were increased, and the lower limit of the quantitation and detection limits were decreased by the LC-MS/MS method applied with various derivatization techniques (40). Moreover, the performance of 1,25(OH)2D measurement can be improved through immune affinity extraction with analyte enrichment. It has been shown that isobaric effects and matrix effects originating from human serum are eliminated with this method (2, 42). Consequently, immunoaffinity purification, lithium adducts formation or derivatization techniques have been proposed for measuring low concentrations of 1,25(OH)2D by LC-MS/MS (40), and effective sensitive and specific methods have been developed using combinations of these techniques (42).

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NUTRITIONAL RICKETS AND VITAMIN D DEPENDENT RICKETS

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Rickets is a pediatric bone disease that causes insufficient mineralization in the epiphyseal growth plate. The most common causes of rickets are deficiencies or errors of action in vitamin D, calcium, and phosphate. Rickets is an old disease that was first described in Europe. It became more prevalent in the urban areas of England in the 16th century. For this reason, it was called 'English disease' in old literature. The term "Rhachitis", is thought to come from the English word '*rucket*,' which means to breathe with difficulty. Another possible origin of the term is '*wrikken*,' meaning to twist (1-2).

The first description of bone diseases resembling rickets was reported by Daniel Whistler in the Netherlands in the early 1600s (1-3). By the late 1700s, it has been demonstrated that cod liver oil could prevent Rickets. After about 200 years, in the 1900s, the structure of the vitamin D molecule was first discovered (1).

Rickets predominantly occurs from nutritional defects in calcium and/or vitamin D. Therefore, the etiological cause of rickets depends on the countries' geographical, cultural, and economic variables. While calcium deficiency due to insufficient intake is the most frequent cause in low-income countries, vitamin D deficiency is the most defined causative factor in countries with poor sunlight.

Vitamin D deficiency rickets presents typically in infants, however, rickets due to calcium insufficiency tends to present later. Despite increased awareness and prevention strategies, rickets still remains a global health problem.

This chapter will present nutritional rickets and vitamin D dependent rickets.

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Classification of Rickets and Pathogenesis

Rickets is classified into two major groups; calcipenic and phosphopenic rickets (Table 1). Calcipenic rickets, also named nutritional rickets, predominantly result from vitamin D deficiency. Less frequently, nutritional rickets can arise from inadequate calcium consumption even when serum vitamin D levels are normal. Inborn errors in vitamin D metabolism or target organ resistance to vitamin D may also lead to calcipenic rickets called vitamin D dependent rickets.

Phosphopenic rickets primarily results due to elevated renal excretion of phosphate or less frequently due to deficient intestinal Pi absorption. Urinary loss of phosphate is usually secondary to increased FGF-23 levels or tubular dysfunction.

Vitamin D Synthesis and Metabolism

Vitamin D is structurally similar to steroid hormones. There are two major inactive precursors of vitamin D for humans. Vitamin D3 (cholecalciferol) is provided by dietary (mainly animal) sources or generated endogenously by exposure of skin to UV light. Vitamin D2, (ergocalciferol) is provided by vegetal sources. Ergocalciferol differs structurally from cholecalciferol. The major difference between these components is their half-life and clearance from circulation. Therefore, the biological potency of vitamin D2 is not so much as that of vitamin D3 (4,5). However, their biological effects are similar, and they are metabolized similarly, and commonly called vitamin D (6,7).

Oily fish species such as tuna, salmon, egg yolk, milk, green onions, and watercress are rich in ergosterol or cholecalciferol (8). After oral intake, vitamin D is absorbed from the intestinal system by enterocytes and transported into the liver in chylomicrons. However, oral consumption contributes only a few amounts (10%) of vitamin D status.

Vitamin D is produced mainly in the dermis by the effect of sunlight. Cutaneous synthesis is induced by the effect of sunlight. The type of light required for the production of a sufficient amount of vitamin D from the skin is Ultraviolet B (UV-B) (290-315 nm wavelength). Cholesterol is the main substrate in the two-step process of vitamin D synthesis which happens in cutaneous stratae basale. In the first phase of vitamin D synthesis, provitamin D3 (7-dehydrocholesterol) is transformed to pre-vitamin D3 by the effect of the ultraviolet rays which happens in less than 15 minutes.

In the skin, 7-dehydrocholesterol (7-DHC) is available between the stratum spinosum and stratum basale, in the keratinocytes and fibroblasts. The second phase, *c*onversion from pre-vitamin D to vitamin D (cholecalciferol), occurs very slowly by a thermo-sensitive but noncatalytic isomerization process. After that, vitamin D is transferred to dermal capillaries and binds to DBP (9). The latitude of the country, the seasons, skin pigmentation, severity of air pollution, sun protective creams, and the dressing style affects the synthesis capacity. Regions far away from the equator during winter (34° North and 34° South) can not reach sufficient UV-B intensity (290-314 nm) during winter. Since dietary consumption contributes only a small amount to vitamin D levels, these regions are at high risk for the occurrence of rickets in winter (10-12).

Table 1. Classification of rickets.

Calcipenic Rickets		**Phosphonenic Rickets**	
	Gene		**Gene**
Nutritional Rickets (Calcium or vitamin D deficiency)	-	X-linked hypophosphatemia	*PHEX*
Vitamin D-dependent rickets type 1A (VDDR1A)	*CYP27B1*		
	CYP2R1	Autosomal recessive HR type 1,2,3	*DMP1*
	Autosomal dominant HR		*ENPP1*
			FAM20C
Vitamin D-dependent rickets type 1B (VDDR1B)	*FGF23*		
	Fibrous dysplasia		
	GNAS		
Vitamin D-dependent rickets type 2A	*VDR*	Tumor-induced osteomalacia (TIO)	*NA*
Vitamin D-dependent rickets type 2B	*HNRNPC*	HR and hyperparathyroidism	*KLOTHO*
Vitamin D-dependent rickets type 3	*CYP34A*	Hereditary HR with hypercalciuria	*SLC34A3*
		X-linked recessive HR	*CLCN5*
		Hypophosphatemia and nephrocalcinosis	*SLCA34A1*
		Cutaneous skeletal hypophosphatemia syndrome	*NRAS HRAS KRAS*

HR: Hypophosphatemic rickets

Zenith angle is also a limiting factor of the cutaneous vitamin D3 synthesis. UV-B rays have a short wavelength, thus in the early and late hours, the UVB rays reach the earth at an oblique angle. The time range between 10:00 am and 15:00 pm hours is called solar noon and this is the ideal time that UV-B photons are most effective to induce vitamin D synthesis.

The term 'the minimum erythema dose (MED)' is used to define ultraviolet radiation exposure to the skin. Depending on the skin type, duration of exposure, and zenith angle, it is estimated that 1 MED could trigger to maintain approximately 15.000 IU of vitamin D into circulation in 24 hours. A person with pale skin should exposure to sunlight for at least 4 to 10 minutes to obtain 1 MED at solar noon. However, this time should be 60-80 minutes for a person with dark skin (13). Melanin pigment in the skin competes for and absorbs the UVB lights responsible for the photolysis of 7-DHC. The darker skin type is a risk factor for rickets (8). Protective clothing blocks UVB from reaching 7-DHC, thus diminishing the production of vitamin D (7). Sunscreen use for daily photoprotection can

also decrease vitamin D synthesis only if high protection factors are used. Products with low sun protection factor and high UVA protection are mainly used for daily photoprotection. This kind of usage is unlikely to affect vitamin D production (14,15). In case of prolonged exposure to solar radiation or UV light, pre-vitamin D3 and vitamin D3 are converted into biologically inactive products such as lumisterol and tachysterol. This is a physiological control mechanism that protects against vitamin D intoxication.

After synthesis from the skin, vitamin D is transferred to the liver. Vitamin D circulates by binding mainly (85-88%) to DBP, and to albumin (12%). In the liver, vitamin D is hydroxylated to 25-hydroxyvitamin D [25(OH) D] via the CYP27A1 and CYP2R1 enzymes. The majority of (90%) of the 25-hydroxylation occurs in the liver by these two major enzymes and the remaining 10% in other tissues such as fibroblasts, kidneys, duodenum, and bone. The 25(OH)D, is also called calcidiol and is inactive. It acts as a vitamin D reservoir. Serum 25(OH)D is the major circulating form that is measured for the evaluation of vitamin D status. The 25(OH)D is then transferred to the kidney by binding DBP. In clinical conditions when serum total protein levels are decreased like in chronic liver disease and nephrotic syndrome, a total of 25(OH)D and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] levels also decline however, unbound free forms are not affected. Under normal conditions, 0.4% of calcitriol and 0.03% of 25(OH)D circulate in unbound form (16,17).

In the kidney, 25(OH)D is internalized through endocytosis in proximal tubule cells via transmembrane proteins, called megalin, cubulin, and disabled 2 (17,18). It is further hydroxylated in the mitochondria by 1α -hydroxylase (*CYP27B1*). The end product is 1,25$(OH)_2$D. It is also named calcitriol. In the kidney, 1-α hydroxylation enzyme activity is regulated by itself, and other hormones specific to calcium and bone balance. Parathyroid hormone (PTH), phosphorus, calcium, and fibroblast growth factor 23 (FGF-23) are the modulators of enzyme activity. 1 α–hydroxylase activity is stimulated by PTH, whereas inhibited by FGF23 and 1,25$(OH)_2$D hypercalcemia, and hyperphosphatemia. FGF23 is a phosphatonin that induces phosphaturia. The main source of FGF23 is osteocytes and serum levels of FGF23 are positively correlated by 1.25$(OH)_2$D and serum phosphorus. FGF23 has an important role in phosphate balance and bone healing. Increased FGF23 induces renal phosphate wasting.

After reaching the target tissue, 1,25$(OH)_2$D crosses through the cell membrane, adheres to the vitamin D receptor (VDR), and constructs the 1,25-hydroxyvitamin D-VDR complex. VDR is involved in the steroid receptor family and is described in various types of tissue like the kidney, intestine, bone, parathyroid gland, and keratinocytes of hair follicles (6,19,20). The enzyme capable to catabolize vitamin D and analogs is 24-hydroxylase *(CYP24A1)*. The 24,25-dihydroxy vitamin D, calcitroic acid, is inactive, more polar, water-soluble, and is rapidly excreted by the kidney. However, 1,25-26,23 lactone which is the final product of the 23-hydroxylase pathway, is biologically active (7,21).

Nutritional Rickets

Nutritional rickets is defined as rickets that develop secondary to vitamin D and/or calcium deficiency. The diagnosis of rickets is established on clinical and radiologic features and refers to a disarrangement of chondrocyte differentiation and osteoid mineralization. Clinical presentation may alter depending on the age at onset, the duration of deficiencies, and underlying pathophysiology.

Nutritional rickets is still a common condition worldwide. Since vitamin D synthesis is affected by many factors such as melanin content of the skin, exposure to UV-B, geographical conditions, and dressing style, the prevalence of rickets varies (22). In developed countries, the incidence of nutritional rickets has been reported as 2.9–27 per 100,000 individuals (23). In a recent study from the UK, the incidence of nutritional rickets was found lower than expected (1.39 per 100,000) and the majority of the cases were Black and South Asian children under 5 years, occurred (24). However, in low-income countries like India, and the Middle East, the currency of vitamin D deficiency and insufficiency is 50% to 90% (25-27). A dramatic increase in the frequency of nutritional rickets in young children was reported by Thacher (23). This increase was attributed to the growing number of nonwhite immigrants and undernourishment in this group. Increased use of sunscreen was also accepted as a causative factor (23). An improvement in vitamin D status after the mandatory fortification of dairy products in Finland is also reported (28). One conclusion might be that either decline or an increase in the prevalence of nutritional rickets can be observed in different countries due to the various circumstances.

Nutritional rickets due to calcium deficiency

Rickets can develop in the existence of normal 25(OH)D levels if the calcium intake is insufficient. This type of rickets is reported mainly in countries that have satisfactory sun exposure but low income (29). Calcium and phosphate are the two key mineral components of the bone matrix. Thus, sufficient levels of calcium and phosphate are crucial for ideal bone mass and strength. The Institute of Medicine (IOM), has reported the daily enteral requirements for calcium and vitamin D. The Recommended Dietary Allowance (RDA) for calcium was reported as 700 mg/d for infants between 1-3 years old (Table 2) (30).

A sufficient amount of daily dietary calcium intake is reported as >500 mg/day according to the global consensus guideline. Daily intake between 300 and 500 mg/day is accepted as insufficient, and <300 mg/day is deficient (31). Several studies revealed that nutritional rickets does not develop in the existence of vitamin D insufficiency if the calcium intake is adequate.

Table 2. The recommended dietary allowance for calcium and vitamin D (30).

Age groups	RDA values for calcium (mg/d)	RDA values for vitamin D (IU/d)
0-6 months	200	400
6-12 months	260	400
1-3 years	700	600
4-8 years	1000	600
9-18 years	1300	600
19-51 years	1000	600
51-70 years	1000 males, 1200 females	600
71 +	1200	800
Pregnant and lactating		
14-18 years	1300	600
19-50 years	1000	600

RDA: recommended dietary allowance

It is thought that deficient calcium intake due to undernourishment increases the possibility of nutritional rickets even if the 25(OH)D levels are sufficient (32,33).

Nutritional rickets due to vitamin D deficiency

Vitamin D deficiency and rickets are different terms. Vitamin D deficiency is defined based on circulating serum 25(OH)D levels. Hence the serum levels of $1.25(OH)_2D$ may be normal or compensatory high due to elevated parathyroid hormone, it is not considered in the definition or categorization of vitamin D deficiency. Additionally, the half-life of 25(OH)D is 14-21 days which makes it a more stable marker than $1.25(OH)_2D$.

Serum 25(OH)D levels are commonly detected by chemiluminescent protein-binding assays or radioimmunoassays using highly specific monoclonal antibodies. These assays have been shown to have some variability reaching 30%-50%. HPLC or tandem mass spectroscopy is the ideal technic for the determination of vitamin D metabolites however, they are not universally available and expensive (34-36). There are also heritable and genetic factors that contribute to the serum 25(OH)D concentrations. The pathways included in the metabolism of vitamin D such as sulphonation and glucuronidation have a wide genetic variability and may cause low levels of 25(OH)D levels (37). This interpretation is supported by other work on genetic factors affecting the 25(OH)D levels (38). They also concluded that serum 25(OH)D levels can be low in some subjects as a result of these genetic variable determinants (38).

Distinct limits have been defined for vitamin D deficiency in the literature. A serum level of 25(OH)D ≥ 50 nmol/L (20 ng/mL) is defined as sufficiency. The Global Consensus on nutritional rickets has defined vitamin D deficiency as a level lower than 30 nmol/l (12 ng/mL; 1 ng/mL=2.5 nmol/L). Levels between 30-50 nmol/L (12-20 ng/mL) are considered insufficiency (31). Severe deficiency is defined as a 25(OH)D level of <12.5 nmol/L (5 ng/mL) (39). These cutoffs were based on clinical trials and should be noted that they do not define Rickets. A distinction must be made between vitamin D

deficiency and rickets (40-44). Rickets can develop in the presence of 25(OH)D deficiency accompanied by insufficient daily calcium intake. In the presence of a sufficient calcium intake, the risk of rickets increases only in very low 25(OH)D levels (44).

Risk factors and prevention of vitamin D deficiency

Vitamin D deficiency is frequently observed in infants and toddlers. The possible causes of this situation are rapid growth in this age group, breastfeeding without vitamin D fortification, limited sun exposure, and the presence of vitamin D deficiency in the mother.

During pregnancy, there is a remarkable placental transport of minerals like phosphorus, calcium, and 25(OH)D. Thus, fetal vitamin D levels reflect approximately 75% of maternal concentrations. Therefore, if the mother has a vitamin D deficiency, it is inevitable that the baby will also have it (43,44).

A substantial amount of mineral transfer from mother to baby takes place in the third trimester. Therefore, premature infants have an increased risk of developing rickets due to insufficient transplacental transfer from their mothers. Infants with a birthweight <1000 grams have a higher risk than heavier ones. In these infants, the most common cause of rickets is inadequate calcium and phosphate intake. Vitamin D deficiency is a rarer cause (44).

The major source of vitamin D in an infant is dermal synthesis via UVB or oral intake. Due to the harmful effects of UVB radiation, American Academy of Pediatrisc restricted sun exposure in young infants. The other source, oral intake, is breast milk. Unfortified breastfeeding would maintain only a few amounts of vitamin D which is not enough to reach the suggested serum levels (50 nmol/L). Thus, infants with breastfeeding should be fortified with vitamin D (45-47).

Different dosages were examined as supplementation. Ziegler compared four different dosages and showed that any dose raised 25(OH)D levels (48). However, the study was conducted in a high-income country (48). In contrast to this research, several studies from the low income-countries revealed that 400 IU/day of vitamin D can not prevent vitamin D deficiency in whole healthy-term infants (49-50). They concluded that oral supplementation with 800 IU of vitamin D per day prevented the occurrence of severe vitamin D deficiency. However, the examination periods were short in these studies. As a consensus opinion, 400 IU of vitamin D was suggested as a supplementary dose to achieve serum 25(OH)D concentrations ≥20 ng/mL in infants younger than 1 year old.

Children with gastrointestinal disease accompanied by malabsorption, liver disease, and renal failure are susceptible to vitamin D deficiency. Having dark skin, geographical conditions like high latitude, inadequate ultraviolet B radiation exposure, limited sun exposure due to dressing style or heavy air pollution, breastfeeding without vitamin D fortification, and maternal vitamin D deficiency are among the factors that facilitate the development of rickets. (47).

Pathogenesis

In the existence of vitamin D deficiency, intestinal calcium, and phosphate absorption decrease. Low calcium levels are recognized by the calcium-sensing receptor in the

parathyroid glands and stimulate PTH secretion to increase calcium reabsorption in renal tubules. In contrast, in the presence of insufficient calcium intake despite normal circulating 25(OH)D levels, PTH values can increase, indicating a sensitive biochemical marker of dietary calcium intake. The 25(OH)D level triggering hyperparathyroidism is controversial. Various studies have reported a threshold value between 20-34 nmol/L (51, 52). Increased PTH levels trigger 1-α hydroxylase activity and $1,25(OH)_2D$ synthesis, decrease phosphate transporters from the proximal renal tubules, and cause phosphaturia (53,54). $1,25(OH)_2D$ binds to VDR in osteoblasts. When activated by $1,25(OH)_2D$, the VDR receptor induces the availability of membrane-bound RANKL in osteoblasts. RANKL–RANK interaction triggers the maturation of osteoclasts. Mature osteoclasts induce resorption of the bone, thereby calcium and phosphate shift from bone to circulation (55).

The epiphyseal growth plate is located at the ends of the tubular bones, between the epiphysis and the metaphysis. During the ossification process of cartilaginous tissue, chondrocytes differentiate into successive, well-defined morphological zones within the epiphyseal growth plate. There are four regions are as follows. The resting zone is the hyaline cartilage cell region that does not show morphological changes. The proliferative zone is the region where cartilage cells proliferate rapidly and form long columns. The hypertrophic zone is the area with large cartilage cells. The calcification zone is the zone where new bone tissue is formed. Chondrocytes differentiate, become hypertrophic, and then consequently undergo apoptosis. Apoptosis of chondrocytes is essential for bone turnover and the generation of new healthy bones (56). When the serum phosphate levels are decreased, hypertrophic chondrocytes do not undergo apoptosis and cartilage tissue expands irregularly and deforms, thus, the radiological signs of rickets appear. Osteomalacia is insufficient matrix mineralization of the bone and is seen in addition to rickets (57).

Vitamin deficiency has three different laboratory stages, but often these stages overlap. The clinical condition with hypocalcemia, hypophosphatemia, and low 25(OH)D is classified as stage 1 rickets. Bone turnover is accelerated and alkaline phosphatase and PTH are slightly elevated. Rickets is absent at this stage. Hypocalcemia, hyperphosphatemia, and elevated PTH mimic PTH resistance. In stage 2, PTH increases further, thus plasma calcium can increase slightly, and more obvious hypophosphatemia occurs secondary to hyperparathyroidism. The increase in alkaline phosphatase is more obvious and, rickets starts to develop radiologically. In this stage, $1.25(OH)_2$ D levels are normal to moderately high. In stage 3, hypocalcemia worsens again and clinical signs of hypocalcemia may develop, hypophosphatemia persists. Rickets becomes more serious, the radiological appearances become more dramatic, and serum alkaline phosphatase levels are too high.

Clinical features

The clinical presentation of nutritional rickets varies. The age of the subject, severity, and extent of the deficiency. Rickets can present both with skeletal and extra-skeletal symptoms.

Skeletal symptoms are frequently observed in rapid bone growth areas. The site of the bone involvement depends upon the age of the child. The costochondral junctions and epiphyses of long bones are more frequently affected than the other parts. Weight-bearing limbs are predominantly affected, such as forearm deformities in crawling infants or

bowing of the legs in walking children. Genu varum or genu valgum develops usually in toddlers (58). Osteochondral junctions are also rapid bone growth areas. The rachitic rosaries which are a sign of a disordered hypertrophic growth plate may be observed in an infant who can not walk yet. Other skeletal symptoms include frontal bossing, swollen wrists, craniotabes, delayed tooth eruption, and bone pain.

Decreased growth velocity and worrisome growth might occur as a result of the mineralization defect of the growth plate and progressive leg deformities (59). Fractures were detected in 17% of children, mostly prior to 2 years of age, accompanied by severe radiological features of rickets (60).

Extraskeletal clinical signs seen in children with rickets include muscle weakness, hypotonia, irritability, laryngospasm, decreased muscle tone, tetany, paraesthesia, muscle cramps, and carpopedal spasm. These clinical features may lead to the delayed achievement of motor milestones (61). Life-threatening clinical signs like hypocalcemic seizures and arrhythmia may rarely be the clinical feature in a child with nutritional rickets. Prolonged QTc interval, atrial arrhythmias, and disturbances in ventricular repolarization are reported to be related to vitamin D deficiency (62). The incidence of life-threatening events was reported as 6.1 per 100,000 person-years in a study conducted on children under 5 years old (63). Hypocalcemic seizures were reported mainly in children under two years old with an incidence varied between 12-25% in different studies (64-67). Dilated cardiomyopathy is not rare (%14-21) and is probably underestimated in nutritional rickets since echocardiography is not routinely performed in all centers. It is important to screen for cardiomyopathy to avoid severe complications (67-69).

Radiological features

Radiological changes are seen in the metaphyses. All metaphyses are affected, however, rickets signs are more evident in the rapidly growing bones like knees, distal ulna, or ankles. Therefore, radiographs of the wrist and knee are sufficient to diagnose rickets. In the beginning, a "radiolucent" line is seen between the epiphysis and metaphysis depending on the accumulation of the uncalcified cartilage. Typical radiological changes are the appearance of the provisional calcification zone as frayed or concave. The metaphyses and epiphyseal plate also become wider (47,57) (Figure 1a and 1b). Elevated PTH levels induce subperiosteal bone resorption along the diaphysis and osteopenia. The cortices of the long bones become thin with resorption lines. Pathological fractures and Milkman pseudofractures may be found in severely affected children with long-standing hypocalcemia and hyperparathyroidism. Radiologic findings may be faint during early infancy (57).

A radiological scoring system called rickets severity score (RSS) has been established to evaluate the radiographic rating of rickets and determine the course of treatment. This is a 10-point scoring method established on the severity of metaphyseal fraying, concavity, and percentage of the growth plate involved. The scoring method starts from zero (normal), and progress to ten (severe). Wrists and knees are evaluated (70).

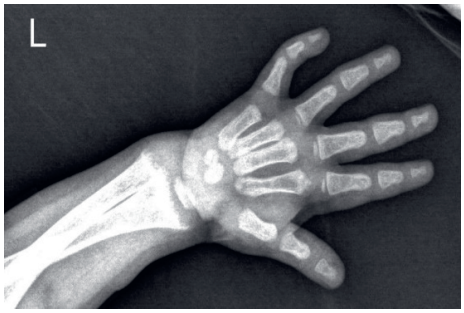
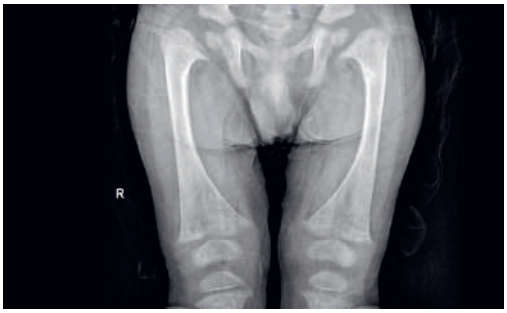


Figure 1a. *Wrist radiograph showing the cupping and fraying of the distal ulnar radial metaphysis*



1b. *radiograph of the distal femur showing fraying and increased physeal widening.*

Laboratory findings

Serum levels of calcium, ionized calcium, albumin, phosphate, alkaline phosphatase, PTH, 25(OH)D, 1.25$(OH)_2$D, and creatinine should be evaluated in children with rickets. Additionally, urinary calcium, urinary phosphate, and urinary creatinine should also be performed. In circulation, calcium is mainly bound to albumin or anions. The biologically active form, the ionized form, is generally 40% of the total serum calcium level. Acidosis diminishes the binding of calcium to albumin. In the presence of hypoproteinemia, total serum calcium levels may be miscalculated. Thus, ionized calcium levels should also be evaluated in the suspicion of hypoalbuminemia or acid-base imbalance (71). Circulating concentrations of 25(OH)D are low in rickets however, 1,25$(OH)_2$D can be low, normal, or increased (72). Serum phosphate concentration varies with age and the highest serum levels are in infants. Therefore, phosphate levels should be interpreted within the age and gender-appropriate reference phosphate values. Low serum levels of phosphate are expected in rickets due to the phosphaturic effect of high PTH levels (73).

There is a variable source of ALP in the human body. Bones, liver, intestines, and proximal renal tubules are the major sources in which ALP is expressed. ALP activity and the type of fraction change with age. Bone-specific ALP is the dominant form (77-89%) in growing children whereas 50% of serum ALP originates from hepatocytes in adults. Since growing children have higher levels than adults, serum levels should be interpreted for age and gender-specific reference ranges. Serum ALP level is used as a reliable indicator of disease activity and is high in rickets. ALP levels become normal during the healing process. High levels exclude metaphyseal diseases like skeletal dysplasias and hypophosphatasia in patients with "ricketic" bone changes (74). Laboratory findings of nutritional and other types of hypocalcemic rickets are given in Table 3.

Treatment

The purpose of nutritional rickets treatment is to improve biochemical parameters, and bone deformities and to relieve symptoms. Calcium, vitamin D, or calcium in addition to vitamin D can be used regarding the etiology.

If the cause of rickets is a calcium deficiency, oral calcium supplements can be used alone for the treatment. The ideal dose and duration of calcium supplementation for the healing of hypocalcemic rickets are controversial. Intestinal calcium absorption is saturable and does not increase even if the amount of elemental calcium taken increases.

Table 3. Laboratory findings of hypocalcemic rickets subtypes.

	Gene	Calcium	Phosphate	PTH	25(OH)D	1.25(OH)$_2$D	ALP
Calcium deficiency	-	↓	↓	↑	N	↑	↑
Vitamin D deficiency	-	N/↓	↓	↑	↓	variable	↑
VDDR1B	*CYP2R1*	N/↓	↓	↑	↓	↓	↑
VDDR1A	*CYP27B1*	↓	↓	↑	N	↓	↑
VDDR2A	*VDR*	↓	↓	↑	N	↑	↑
VDRR2B	-	N/↓	N	↑	N	↑	↑
VDRR3	*CYP3A4*						

PTH: parathormone, VDDR: vitamin D dependent rickets

A comparative study by Thacher found that daily calcium supplementation with 500 mg, 1000 mg, and 2000mg led to bone healing in nutritional rickets due to calcium deficiency. Healing was more rapid with 1000 mg and 2000 mg than 500 mg, however, supplementation dose greater than 1000 mg was not superior in terms of radiographic healing. Therefore, 1000 mg per day of elementary calcium for > 24 weeks is recommended (75,76).

Responses to calcium alone and calcium in addition to vitamin D therapy were compared in some studies. A better and a rapid response was reported to vitamin D plus calcium therapy than to calcium alone (77). The mechanism by which vitamin D improves the cure of rickets in children with calcium-deficiency rickets, even in those without vitamin D deficiency is unclear. Intestinal calcium absorption has been evaluated in these children. Adding vitamin D did not enhance the absorption of calcium and the response to therapy is independent of the 25(OH)D concentrations measured at the beginning of the therapy (78).

Similar results were reported in other studies and a trend to support combination therapy was accepted (79, 80). If the underlying cause of rickets is vitamin D deficiency, vitamin D supplementation is used for treatment. Either ergocalciferol (D2) or cholecalciferol (D3) can be used for the cure of rickets due to vitamin D deficiency. In daily supplementation, there is no superiority of cholecalciferol over ergocalciferol, however, when given as a bolus dose, cholecalciferol is more effective in the improvement of vitamin D status (81). There are various protocols concerning the duration and dose of vitamin D therapy. Vitamin D analogs may be given as a bolus once a time or as low-dose long-term daily therapy.

Low-dose long-term vitamin D therapy: Since nutritional calcium intake deficiency frequently coexists with rickets, administration of calcium with vitamin D analogs is usually recommended even if there is no clinical or laboratory sign of hypocalcemia. The

disadvantage of long-term therapy is non-adherence which results in failure or delay in the healing of rickets. The minimum recommended duration and dose for the rickets treatment is 12 weeks and 2000 IU/day. Age-appropriate treatment doses are given table 4. In this treatment regimen, calcium, and phosphorus recover in 6-10 days, and PTH decreases to normal levels within 30-60 days. High ALP levels may maintain for several months.

High-dose single therapy (stoss): In case of non-adherence to long-term therapy, a single but high dose may be used. In this protocol, 50.000-600.000 IU of vitamin D is given enterally or parenterally in single or divided doses after three months of life. It has been shown that 600.000 IU of vitamin D administration may cause hypercalcemia in infants, however, administration of 150-300.000 IU of vitamin D has been reported to be tolerable with no side effects in many studies. In a comparative study by Tannous, the standard (5000 IU/day, for 90 days) treatment group had significantly greater median 25OHD levels than the stoss (100.000 IU/ week, for 4-6 weeks) group at 12 weeks (82).

A more practical treatment strategy was reported by Malabanan et al (83) for adolescents and adults. Oral vitamin D, 50.000 IU in a single dose, once a week for 8 weeks was reported as safe and sufficient. Maintenance dosing should be added after the bolus dose. If compliance is poor with maintenance dosing, intermittent high-dose vitamin D is needed (85,86).

Some oral vitamin D preparations contain propylene glycol which may be harmful at supraphysiological doses. Therefore, care should be taken if high-dose oral vitamin D is to be given. Clinical response is more rapid with this treatment, biochemical recovery takes a few days, and radiological recovery takes 10-15 days (31,47).

Table 4. Age-appropriate vitamin D doses for treatment

	Treatment Dose		**The maintenance Dose (IU/day)**
Age	Long-term daily dose (IU/day)	Single high-dose (IU) (Stoss dose)	
< 3 month	2000	-	400
3-12 month	2000	50.000	400
12 month-12 years	3000-6000	150.000	600
>12 years	6000	300.000	600

There is a possibility of developing hypocalcemia due to rapid bone remineralization at the beginning of Vitamin D treatment. Therefore, the addition of calcium is recommended in both protocols, regardless of the presence of hypocalcemia. Daily suggested doses of calcium supplementation are between 30 and 75 mg/kg per day. There are several different kinds of calcium compounds with different elemental calcium content (31). The most used types of calcium supplements are, calcium citrate, carbonate, and gluconate which contain 40% 21%, and 9% elemental calcium by weight, respectively. The bioavailability of calcium citrate is greater than calcium carbonate and it doesn't require an acid environment to dissolve, thus calcium citrate may be preferred over calcium carbonate in cases of achlorhydria (87). If the child has hypocalcemic tetany or convulsions, parenteral

calcium (10-20 mg/kg elementary calcium, slow intravenous infusion, generally given as 1-2 mL/kg of 10% calcium gluconate) should be given immediately.

Alfacalcidol or calcitriol are not recommended in the treatment of nutritional rickets. These metabolites can not restore vitamin D levels. In selected cases with severe hypocalcemia, calcitriol should be used in doses of 20 to 100 ng/kg per day to normalize calcium levels. Hence calcitriol has a short half-life, it should be given in 2 to 3 divided doses (88).

Vitamin D Dependent Rickets

Rickets related to disorders of vitamin D constitutes is rare. To date, five types of vitamin D dependent rickets (VDDR) have been described. Mutations in the *CYP27B1* gene cause VDDR1A, which results in defective 1α-hydroxylase activity and failure to synthesize 25(OH)D (89, 90). VDDR1B is caused by mutations in the *CYP2R1* gene, which results in 25-hydroxylase insufficiency. The defect is in the vitamin D receptor in the second type. This type is named VDDR2A. VDDR2B is a very unusual form of rickets that the VDR is normal, however, signal transportation is disordered. VDDR type 3 (VDDR3) is caused by *CYP3A4* mutations leading to increasing vitamin D inactivation (89-91).

Clinical, radiological, and substantially biochemical features of the Vitamin D-dependent rickets (VDDR) group are almost identical to nutritional rickets.

Vitamin D dependent rickets type 1A (VDDR1A)

In 1961, a subject who had clinical signs of rickets but did not have a remission with standard doses of vitamin D therapy was reported. Supraphysiological doses of vitamin D therapy were successful and Prader named the situation as persistent rickets. In 1973, Fraser et al. found that it was developed as a result of 1α hydroxylase enzyme deficiency. After further years, mutations in the gene (CYP27B1) have been identified (92).

In the literature, it was first called 'hereditary pseudo-vitamin D deficiency', then 'vitamin D dependency' due to its response to supraphysiological doses of vitamin D therapy. Recently, 'vitamin D dependent rickets type 1' is used generally. The enzyme "1α hydroxylase" plays a crucial role in the synthesis of vitamin D and is encoded by *CYP27B1.* Although it is frequently found in the kidney, 1α hydroxylase enzyme activity has been also been expressed in keratinocytes, placenta, breast, prostate, colon, macrophage, and osteoblasts (90).

The major determinant of circulating calcitriol level is the renal 1α hydroxylase enzyme. This enzyme is the mitochondrial P450 enzyme that has oxidase functions. Renal 1α hydroxylase activity is adjusted by parathyroid hormone, calcium, and $1.25(OH)_2D$. Vitamin D-dependent rickets type I develops as a result of the inability of calcidiol to convert to calcitriol. Mutations in the 1α hydroxylase gene cause the disorder. The inheritance pattern is autosomal recessive (89, 90).

The 1α hydroxylase gene is on chromosome 12q13.3. It is about 5 Kb and consists of 9 exons. To date, 89 variants in the CYP27B1 gene have been addressed in the Human Gene Mutation Database (HGMD) (http://www. hgmd.cf.ac.uk/ac/index.php). The mutations include "missense" mutation, deletion, duplication, and insertions have been reported as "splice site" mutations.

There is an increased prevalence of VDDR1A in some regions of Québec, Canada. In some regions, the estimated rate of carrier is about 1/27, and the VDDR1A prevalence is approximately 1/3000 (93). A deletion in exon 2, codon 88 (c.958delG) that results in premature termination is called a Charlevoix mutation (90).

Data regarding the relationship between genotype and phenotype is scarce. A study analyzed by Meaux et al. outlined that the partial loss-of-function variation caused a milder phenotype (92). Higher doses of calcitriol were reported for the healing of rickets in patients with truncating variants than in ones with non-truncating variants (94). A more severe presentation accompanied by short stature and higher calcitriol requirement was found in cases with intronic c.195+2T>G mutation, in contrast to milder clinical findings with p.K192E mutations (95). In contrast, several studies reported phenotypical heterogeneity and could not determine the relationship between genotype and phenotype (96-98).

Patients with 25-hydroxylase deficiency display identical clinical findings to vitamin D deficiency. The significant difference is the earlier presentation than nutritional rickets and more serious symptoms which do not improve with nutritional deficiency treatment doses. Failure to thrive, hypotonia, muscle weakness, irritability, and tetany are found in early infancy.

Extensive mineralization loss is particularly evident in the rapidly growing long bones, epiphyses, and costochondral junctions. Clinical signs of rickets occur in 1 month usually between 4-12 months. The first observed findings are rachitic rosary due to costochondral joint hypertrophy, pigeon-chested appearance on the sternum, and Harrison grooves in the costa (89, 96). Craniotabes, enlargement of suture and fontanelle, delayed closure of fontanelle, and prominence on the forehead may develop in patients. Delayed eruption of teeth and enamel hypoplasia due to dental caries can be found. If the diagnosis is not made and treatment is not started, severe deformities occur in the long bone and spinal column. Pathological fractures are common (89).

In laboratory examination, similar to nutritional rickets, hypocalcemia, hypophosphatemia, elevated alkaline phosphatase, and parathyroid hormone levels are detected. Hypocalcemia can be severe and may cause convulsions and tetany. Hypocalcemia triggers secondary hyperparathyroidism and aminoaciduria. Urine calcium levels are decreased and intestinal calcium excretion is increased. Although serum 25(OH)D level is normal, $1,25(OH)_2D$ level is very low. Serum $1,25(OH)_2D$ (calcitriol) levels can not increase with high-dose vitamin D treatment (89,91,97).

The main purpose of treatment is to sustain the serum calcium level in the mid-normal level and to attain a serum level of PTH in the normal range. Physiologic doses of calcitriol are successful in treatment. Calcitriol has a short half-life, so it is usually administered twice daily. The treatment dose is between 0.5-3 μg/day (10-20 ng/kg/day). It can be given initially as 1-2 μg/day, then with a maintenance dose of 0.5-1μg/day (89, 90).

Alpha calcidiol (1α-cholecalciferol), which is converted to $1,25(OH)_2D$ in the liver, is an alternative agent due to its similar effects to calcitriol. It can also overcome the enzymatic block. Alpha calcidiol has a longer half-life, hence it can be given once daily.

High doses of vitamin D may also be used, however, this may cause hypercalcemia. In the course of treatment with vitamin D or calcifediol, serum of 25(OH)D will reach high levels however, the serum concentration of 1,25 $(OH)_2D$ will remain low.

Calcium supplements should also be given at the beginning of treatment to prevent "hungry bone syndrome". Approximately 50 mg/kg of calcium per day is recommended. The serum calcium level begins to rise within 24 hours after starting treatment. Radiological improvement begins in the second or third months of treatment. It has been shown that rickets improved 9-10 months after the treatment (89-91).

During treatment, serum calcium, phosphorus, PTH, alkaline phosphatase, and urinary calcium excretion should be closely monitored every 3–6 months. Due to the risk of nephrocalcinosis or nephrolithiasis, renal ultrasonography should be done once a year (89, 90)

It should be kept in mind that, persistently elevated levels of PTH may cause osteitis fibrosa, a high-turnover bone disease. In contrast, low levels of PTH are a sign of overtreatment and are usually associated with hypercalciuria (89, 90).

Vitamin D dependent rickets type 1B (VDDR1B)

The causative gene of VDDR1B is CYP2R1 which encodes the 25-hydroxylase enzyme. VDDR1B is rare than VDDR1A. It was first reported in two siblings from Nigeria (99). The disease is more prevalent in Nigeria, Saudi Arabia, and Tunisia. This can be explained by the high rates of consanguineous marriages in these countries. Currently, a total of 22 variants in the CYP2R1 gene have been reported in the HGMD (http://www.hgmd.cf.ac.uk/ac/index.php). The variant found in exon 2 of CYP2R1 (c.296 T > C) is the most reported one and is accepted as the founder mutation in the Nigerian population (100,101).

Thacher et al. (100) reported a gene dosage effect of *CYP2R1* on clinical characteristics. While heterozygous mutations present milder clinical manifestations, biallelic mutations cause more severe clinical findings (100-102). The characteristic laboratory findings are similar to VDDR1A. Initial laboratory findings are suggestive of classical vitamin D deficiency, thus it is easy to misdiagnosis. The serum concentration of 1.25 $(OH)_2D$ may be high in some patients. The most plausible explanation for this situation was attributed to the existence of other vitamin D 25-hydroxylases like CYP27A1, and CYP3A4. These enzymes can not generate higher levels of serum 25(OH)D, as a result, the amounts of 25(OH)D produced remain insufficient to prevent rickets during childhood (103). The phenotype may be milder in older ages (89).

The diagnosis of VDDR1B should be considered in the existence of persistent low serum levels despite sufficient vitamin D treatment.

Calcidiol (calcifediol/25-OH vitamin-D) and calcium supplementation are used in the treatment of VDDR1B. High doses of vitamin D2, vitamin D3, or calcitriol (10-20 ng/kg/day) can also be used with calcium supplementation. Mid-normal range of serum calcium and PTH should be targeted with treatment (89, 90).

Vitamin D dependent rickets type 2A (VDDR2A)

VDDR2A is an autosomal recessive disorder that is secondary to biallelic mutations in the *VDR* gene. *VDR* consists of 11 exons and is located in 12q13.11. Currently, there are 81 distinct variants reported in HGMD. Heterozygous carriers generally have no symptoms related to the disease (90).

It was first described in 1978 in a child that had radiological and biochemical evaluation consistent with rickets but with normal 25(OH) D and extremely high $1.25(OH)_2$ D levels and is also called hereditary vitamin D-resistant rickets (89,104).

Vitamin D receptor belongs to the thyroid retinoid receptor group which is a subgroup of the nuclear transcription factor superfamily and consists of 5 domains. The most important and effective domains of the VDR protein are DNA binding and ligand binding domains. DNA-binding domain (DBD) is at the N- terminus of the VDR. Ligand-binding domain (LBD) is in the C-terminal of the VDR. The binding of $1.25(OH)_2D$ to VDR causes a change in the configuration of the VDR which forms a ternary structure with RXRα (90).

Nonsense, insertion/ substitution, insertion/duplication, deletion, frameshift, and combined heterozygous variants have been detected in the *VDR* gene to date. Mutations in the DBD prevent the VDR from binding to DNA, which leads to loss of function, and results in more severe presentation including alopecia. In the VDR-RXR heterodimerization mutations, ligand binding is normal, however, transactivation activity is disordered and all cases have alopecia. Mutations in the LBD usually cause a milder phenotype. Alopecia is rare in this kind of mutation and VDR functions are partially affected (104).

Clinical findings and responses to treatment show heterogeneity. Clinical findings usually appear before the age of two, however late onset (puberty or adult) de novo cases have also been defined. Most of the findings are similar to vitamin D-dependent rickets type I. Bone mineralization is normal at birth, craniotabes, rachitic rosaria, and enlargement of the wrists. The most abundant clinical findings are bone pain, muscle weakness, hypotonia, and growth retardation. Hypocalcemic tetany and rarery convulsions may also occur during the early stages of life. Respiratory distress may accompany lower airway infections. Immune dysfunction and predisposition to infections due to impaired neutrophil chemotaxis have been shown in some cases.

Although spontaneous remission of the rachitic process is rare, the clinical signs of the condition may become faint over time, spontaneous remission has been reported in some cases, particularly after puberty. Biochemical values may return to normal and treatment would be unnecessary in these patients.

Alopecia is one of the specific features that is described in some patients with VDDR2A. It can be variable in appearance and extent, eyebrows and eyelashes and body hair may also be affected. The existence of alopecia is related to the severity of rickets. The association between VDR and the hair follicle is not clear. It has been speculated that the VDR/RXRα heterodimer formation has a substantial role in the generation of epidermal

keratinocytes. The absence of alopecia in vitamin D-dependent rickets type I supports the role of VDR in hair follicle development, rather than vitamin D itself (105)

Low levels of serum calcium and phosphorus, high levels of alkaline phosphatase and PTH, and normal serum levels of 25(OH)D are generally detected in laboratory examinations. One-alpha hydroxylase is activated and 24-hydroxylase is inhibited. These alterations lead to decreased levels of 24,25 $(OH)_2D$ and high levels of 1.25$(OH)_2D$ which are distinctive laboratory findings.

Supraphysiological doses of calcitriol (1-6 μg/kg/day) in addition to elementary calcium (1-3 g/day) are used for the treatment. During the course of therapy, serum calcium, phosphate, ALP, PTH levels, and urine calcium excretion should be monitored regularly during therapy. Prolonged hypocalcemia may cause secondary hyperparathyroidism. More rarely, tertiary hyperthyroidism may occur due to insufficient treatment. Cinacalcet should be used in these subjects with VDDR2A and tertiary hyperparathyroidism.

Response to calcitriol depends on the location of the mutations. If the mutations are at the LBD domain, only binding affinity for 1.25$(OH)_2D$ is reduced. Increasing the concentration of ligand, a supraphysiological dose of calcitriol, may be effective. In contrast, mutations that prevent DNA binding are usually resistant to high-dose calcitriol therapy.

Intensive calcium therapy is used in patients who are resistant to vitamin D or calcitriol. High-dose (1-3 g/day or 0.4-1.4 g/m^2/day elementary calcium) intravenous calcium therapy is used in the treatment. The clinical efficacy of high-dose oral calcium alone or in combination with intravenous calcium therapy has been demonstrated.

Intestinal calcium reabsorption occurs both by VDR-dependent and independent mechanisms. The parenteral route of calcium bypasses the intestinal defect caused by ineffective VDR. Oral calcium (3- 9.0 g/m^2/day) should be continued to provide normocalcemia after the condition stabilizes with high-dose intravenous calcium treatment. High calcium levels in the gut are aimed to maintain passive diffusion. Patients should be hospitalized for intravenous calcium therapy due to serious side effects like cardiac arrhythmia, and tissue necrosis owing to the extravasation of calcium. Additionally, hypercalciuria and nephrocalcinosis should be monitored. Radiological improvement starts after 6 weeks of calcium infusion; complete recovery takes about 3-5 months.

Vitamin D dependent rickets type 2B (VDDR2B)

This is an unexpected and very rare form of vitamin D-dependent rickets. There is an abnormal expression of a hormone response element binding protein that interferes with the binding of the VDR-RXR complex to VDRE (106). The vitamin D receptor is normal.

The disease was first reported in 1995 in a 16-month-year-old infant who was noted to have typical radiological and clinical findings of rickets. She had alopecia and raised serum 1,25$(OH)_2D$ consistent with resistance to Vitamin D. No mutation in the *VDR* gene was defined. Complete healing of rickets with alfacalcidol and large doses of calcium was obtained in the subject (107).

In 1995, Giraldo et al. reported a case series consisting of 64 black children, suffering lower limb deformities such as genu varum, genu valgum, or both with no other abnor-

malities (108). Alopecia was not found. Laboratory investigation revealed normal to low serum calcium and normal to slightly high serum phosphorus, high alkaline phosphatase, and PTH, markedly high 1,25$(OH)_2$D indicating the diagnosis of VDDR II. Subjects had normal VDR cDNA sequences. Authors speculated that VDR dysfunction may result from post-translational events like vitamin D response element (VDRE) (108).

Hence the genetic etiology of the disease is undefined, and the precise number of affected subjects is unknown. Low or normal serum calcium, normal phosphorus, high alkaline phosphatase, increased PTH, and high 1.25$(OH)_2$ D are biochemical findings. Lower limbs are affected more than upper limbs. Irregular metaphyses, genu valgum, and genu varum are detected. Hence the laboratory and biochemical findings are similar to VDDR2A, differential diagnosis cannot be made without genetic testing. Treatment approaches are similar to VDRRA2A (89, 90,107).

Vitamin D dependent rickets type 3 (VDDR3)

The third kind of VDDR, which is a result of a gain-of-function mutation in the gene encoding CYP3A4, was first described in two children by Roizen (89,109). The biochemical and radiological features were consistent with active rickets. The serum levels of 25(OH) D and 1.25$(OH)_2$D were in the lower limit. High-dose calcitriol was required to achieve normal serum vitamin D metabolites and radiological healing (109).

Functional assessment of the variant revealed that the mutant CYP3A4 enzyme had greater activity than the wild type and CYP24A1 enzyme which is the chief inactivator of vitamin D metabolites. They concluded that the disease is due to accelerated vitamin D metabolite inactivation. However, there are only a few reports regarding this type of rickets (89, 109).

Summary

Nutritional rickets is still a common health issue. Therefore, there is a need for identifying the risk group, and prevention policies should be implemented in this group. Since clinical and laboratory findings are very similar, Vitamin D-dependent rickets subtypes can be misdiagnosed. Genetically inherited causes of rickets should be considered in patients at the differential diagnosis who had clinical and radiological signs of rickets but healing could not be achieved with a standard dose of Vitamin D and/or calcium treatment.

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VITAMIN D AND OSTEOPOROSIS

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Osteoporosis is a systemic skeletal disorder and associated weakens bones, making them more susceptible to fractures. The literal meaning of "osteoporosis" is porous bone. Fractures can cause significant morbidity and can reduce quality of life with chronic pain, disability and loss of independence and lead to premature death in patients with osteoporosis (1,2). Optimising bone mass throughout life-time is critically important for preventing fractures at all ages. Therefore, prevention of osteoporosis is as important as early treatment of osteoporosis. The term "osteosaropenia" is used to describe the combination of low bone mass and low muscle mass or strength (3). In general, the pathogenetic factors leading to bone and muscle loss are also similar, e.g. sedentary lifestyle, toxins, etc.(4). The relationship between falls and fractures requires consideration of muscle strength in fracture prevention.

Osteoporosis is more common in women than in men, and as age progresses, this rate increases to the detriment of women with the addition of menopause. Improved bone acquisition in childhood is likely to have benefits in the prevention of osteoporosis and fracture in later life. In this article, the role of vitamin D in the prevention and treatment of osteoporosis will be discussed.

Definition and classification of osteoporosis

Osteoporosis is a disorder characterized by decreased bone mass and microarchitectural deterioration of bone resulting in increased risk of fragility fractures. It is preventable by optimizing peak bone mass, preserving bone mass, and minimizing bone loss especially during old age.

The World Health Organization has defined diagnostic criteria for osteoporosis based on bone density measurements determined by dual-energy x-ray absorptiometry (DXA) in adults. If a patient's bone mineral density (BMD) measures between 1 and 2.5 standard deviations below the mean value in a young reference population, it is classified as osteopenia (low bone mass), osteoporosis is defined if a patient's BMD is 2.5 standard deviations or more below the mean for young normal people (5).

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Osteoporosis in children and adolescents is defined differently from adults. According to the revised pediatric position papers by the International Society of Clinical Densitometry, "osteoporosis" is defined as a size adjusted z-score ≤-2.0 with a clinically significant fracture (2 or more long bone fractures before 10-year old or ≥3 long bone fractures up to 16-year old) or ≥1 vertebral compression fractures occurring without high-energy trauma or local disease, regardless of BMD z-score (6). When bone mineral content (BMC) or BMD z-score is -2.0 or below and no relevant fracture history is present, the term "low BMC/BMD for chronological age" should be used instead of osteopenia or osteoporosis, and z-score of –1.0 to –2.0 is defined as a low range of normality (6,7).

Table 1. Classification of childhood osteoporosis.

Primary
Osteogenesis imperfecta (OI) OI type 1, 2, 3, 4, and other types of OI
Secondary
Immobility induced
Duchenne muscle dystrophy
Poor nutrition and anorexia nervosa Celiac disease, inflammatory bowel disease, gastrectomy, primary biliary cirrhosis, bariatric surgery
Endocrine diseases Glucocorticoid excess, hyperthyroidism, hypogonadism (androgen insensitivity, Turner syndrome, Klinefelter syndrome, hyperprolactinemia, early menopause), anorexia, vitamin D deficiency, hyperparathyroidism, diabetes mellitus
Inflammatory diseases
Rheumatological diseases Ankylosing spondylitis, rheumatoid arthritis
Hematologic diseases Multiple myeloma, leukemia, hemophilia, sickle cell disease, leukemia, lymphoma, thalassemia
Metabolic diseases Homocystinuria, lysinuric protein intolerance
Cardiovascular, renal, pulmonary and miscellaneous disorders Chronic kidney disease, post-transplant bone disease, congestive heart failure, chronic obstructive lung disease, AIDS
Other genetic disorders Ehlers-Danlos syndrome, Marfan syndrome, cystic fibrosis, hemochromatosis, hypophosphatasia
Medications Aromatase inhibitors, heparin, anticonvulsants, methotrexate, cytoxan, gonadotropin-releasing hormone agonists and antagonists, tamoxifen, excess thyroxine, lithium, cyclosporine A, tacrolimus, glucocorticoids, thiazolidinediones, proton-pump inhibitors, selective serotonin reuptake inhibitors, tenofovir

Primary osteoporosis usually occurs due to an underlying genetic defect, with the most common condition being osteogenesis imperfecta (OI). Acute or chronic illnesses and their treatment can also harm bone tissue, leading to secondary osteoporosis. Several diseases are associated with reduced mobility such as cerebral palsy, head and spinal cord injury, and muscular dystrophy (Table 1). Furthermore, in addition to the increase

in circulating inflammatory cytokines in chronic diseases, delayed puberty and low body weight predispose to osteoporosis (8,9).

The diagnostic work-up of children at risk or suspected of osteoporosis should include serum calcium, phosphate, magnesium, creatinine, glucose, total proteins, alkaline phosphatase (ALP), 25-hydroxy vitamin D (25OHD), parathormone (PTH), free thyroxine, thyroid stimulating hormone, and urinary creatinine, sodium, calcium, and phosphate. BMD is usually measured by the DXA method. More detailed tests are planned in special cases. Serum 25OHD is one of the primarily selected laboratory tests when evaluating bone health and bone mineralization. Moreover, one of the general measures to reduce risk of bone loss is ensuring adequate vitamin D and calcium intake (Table 2) (8,9).

Osteoporosis and vitamin D

Vitamin D is an essential nutrient for the bone health. It would be wise to aim to strengthen the bone and muscle at the same time to prevent osteoporosis, and to turn to pharmacological targets that reduce both falls and fractures for the treatment of osteoporosis (2). In this context, a potential nutritional intervention that has attracted interest for years is vitamin D supplementation. Although it is well known that vitamin D deficiency causes pathological findings in the skeleton, it is not clear whether it adversely affects muscle function or contributes to fall risk.

Table 2. Daily requirements of vitamin D and calcium.

Ages	Vitamin D (IU/day)	Calcium (mg/day)
0-12 months	400	260
1-3 years	400-600	700
4-8 years	400-600	1000
9-18 years	400-600	1300
Adults	600-800	1000

The main function of vitamin D is to maintain calcium homeostasis and bone health. Vitamin D deficiency can lead to reduced calcium absorption, secondary hyperparathyroidism, and increased risk of fractures (Figure 1). Vitamin D status in adolescence is particularly important, because the greatest bone gain occurs during puberty, with up to 90% of peak bone mass accumulating at the end of puberty. Therefore, interventions to increase bone acquisition during childhood and adolescence are critical to maximize peak bone mass and thus potentially reduce the detrimental effect of bone loss later in life (10-14). Little is still known about the effect of vitamin D status on the gain of BMD in children and adolescents.

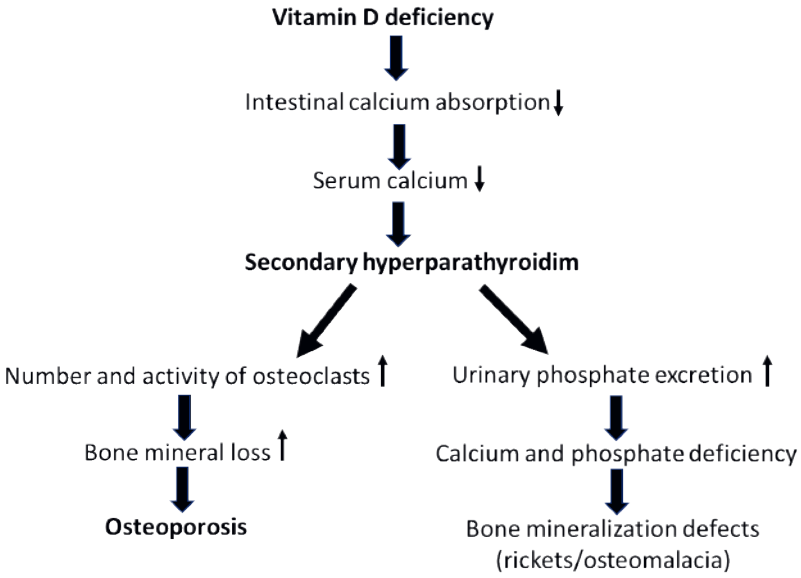

Figure 1: *Relationship between vitamin D deficiency and osteoporosis.*

Bone mass is affected by many factors such as accumulation during growth, gender and race, endocrine and genetic factors, mechanical factors, pharmacological agents and nutritional factors, especially calcium and vitamin D intake (8,12,15). Serum 25OHD has been used as a measure of vitamin D status. Vitamin D deficiency and insufficiency are defined as a serum 25OHD level of less than 15 ng/mL (37.5 nmol/L) and 15-20 ng/mL (37.5-50 nmol/L), respectively (16). Vitamin D insufficiency may not cause symptoms, but contributes to low bone mass. Severe vitamin D deficiency causes rickets and osteomalacia. In addition, although more data are needed, vitamin D deficiency has been associated with proximal muscle weakness, impaired physical performance, and increased risk of falls (17,18).

Pekkinen et al. (19) investigated the influence of both vitamin D levels and physical activity on bone health in children and adolescents aged from 7 to 19 years and the relatioship between vitamin D status and vitamin D intake. They observed that 71% of the study population had serum 25OHD level below 50 nmol/L (20 ng/mL) and reported that serum 25OHD was one of the independent variables and significant determinants of lumbar spine (LS) BMD z-score, total hip BMD, and whole body BMD z-score. The results that insufficient serum 25OHD levels associated with lower BMD z-scores and its effect was greater than physical activity in LS and whole body were the key findings of that study. Another 3-year prospective study investigated the association between changes in BMD or bone mineral apparent density (BMAD, g/cm^3) and serum 25OHD level in healthy girls aged 9-15 years (20). They defined severe and moderate vitamin D deficiency as a serum 25OHD concentration <20 nmol/L (8 ng/mL) and 20-37.5 nmol/L (8-15 ng/mL), respectively. Mean serum 25OHD levels at baseline (in winter), month 12 and month

36 were 34.0±13.2 nmol/, 33.2±11.1 nmol/L, and 40.6±15.8 nmol/L, respectively. There was a significant relationship between the baseline 25OHD level and 3-year ΔBMD and ΔBMAD at the LS and femoral neck among peripubertal girls, that was the main finding of the study. The BMD and BMAD at the LS of the girls with advanced sexual maturation increased over 3 year by 26% and 50% more, respectively. The authors suggested that the dietary vitamin D intake was inadequate to maintain an optimal vitamin D status during winter, thus vitamin D supplementation should be considered to make sure an adequate vitamin D level during the peripubertal years (20).

In recent years, relatively higher prevalence of vitamin D deficiency has been reported in the pediatric population and it is of great importance to evaluate the effect of vitamin D supplementation on bone health in this critical period for bone mineralization (16,21). However, there are very few studies systematically assessed dose-responce effects in children and adolescents and most of the randomized controlled trials (RCTs) of vitamin D supplementation to optimize bone health have been performed in females (21-25). Winzenberg et al. (13) reported a meta-analysis of six RCTs of vitamin D supplementation in children for improving BMD and suggested that was unlikely that vitamin D supplements were beneficial in children and adolescents with normal vitamin D levels, however vitamin D supplementation in vitamin D deficient (<35 nmol/L = 14 ng/mL) children and adolescents could result in clinically useful improvements, particularly in LS-BMD and total body bone mineral content, but they stated that required confirmation in further studies targeting vitamin D deficient children and adolescents.

In black preadolescent children aged 8.5±1.4 years in South Africa, the LS-BMD was significantly higher in vitamin D insufficient group (n= 35; 25OHD 21-29 ng/mL) than the deficiency group (n= 4; 25OHD ≤20 ng/mL), but not significantly higher than the sufficient group's (25OHD ≥30 ng/mL, n= 20) (26). However the small sample size of the study could had affected the significance of associations between 25OHD and BMD measurements. In a recent prospective randomized study, the effects of vitamin D supplementation on BMD in healthy children aged 5-7 years during 3 winter months was evaluated and determined that BMD remained higher in dark than fair-skinned children throughout the study (27). When children were randomized to receive either a vitamin D supplement or a placebo, changes in BMD did not differ between the vitamin D and placebo groups, but all children who received vitamin D had improved their vitamin D status. Song et al. (28) investigated the effect of vitamin D status on BMD z-score in 1063 adolescents aged 12 to 18 years who were divided into three groups according to serum 25OHD levels. They defined vitamin D deficiency as 25OHD <12 ng/mL, 25OHD 12-20 ng/mL insufficiency, and 25OHD >20 ng/mL sufficiency in the adolescents they included in the study. They determined that the frequency of vitamin D deficiency as 20%, insufficiency as 59%, and sufficiency as 21%. They reported that the subjects with vitamin D sufficieny and insufficieny had higher BMD z-scores compared to vitamin D deficient subjects. Therefore, they emphasized that vitamin D deficiency should be prevented in order to achieve optimal BMD in the growing age. Borg et al. (29) investigated the hypothesis in an animal model that vitamin D deficiency at an early age results in decreased bone mass and strength and an abolition of response to mechanical loading during both

childhood and adulthood. They observed that vitamin D deficient mouse offspring showed lower BMD and early life vitamin D deficiency resulted in a decreased anabolic response of bone to mechanical loading in growing bone, and that effect on cortical bone persisted into adulthood.

Many studies have shown that correction of severe vitamin D deficiency in older adults reduces the risk of hip fracture (2,30). Vellingiri et al. (31) reported that impaired bone mineralization accompanied by low serum 25OHD levels was of significant importance in the etiology of femoral neck fractures, and normal serum 25OHD levels with normal serum PTH levels could reduce the incidence of femoral neck fractures. In a randomized, placebo controlled trial (Women's Health Initiative [WHI] study) planned to investigate whether calcium and vitamin D supplementation (participants received 1000 mg of elemental calcium and 400 IU of vitamin D3 or placebo) decreased the risk of hip fracture in healthy postmenopausal women was showed that calcium with vitamin D supplementation diminished bone decrement at the hip, but the reduction in the incidence of hip fracture was not statistically significant and there were no significant reductions in the incidence of vertebral fractures or total fractures (32). The authors suggested that the effect of calcium with vitamin D supplementation on reduced fracture might require higher doses of vitamin D than were used and the most of studies supporting an advantage from calcium with vitamin D supplements evaluated vitamin D at doses of 600 IU or higher (10,13,14,33-35). However, Bolland et al. (33) evaluated 42 RCTs that assessed supplemental vitamin D and fractures outcome, they reported that vitamin D had a very limited effect on BMD and the results did not vary by dose. In a recent review, it was stated that vitamin D supplementation has no effect on BMD and does not decrease the risk of fracture in individuals with adequate vitamin D levels (36). Nevertheless, up to 60% of patients with hip fractures had one or more biomarkers consistent with a decreased calcium, such as secondary hyperparathyroidism, low 25OHD levels, or fallen urine calcium evacuation (37). The fact that the participants in the WHI stud were healthy postmenopausal women, with an average daily calcium intake exceeding 1000 mg at baseline, close to current national recommendations, and were already calcium-replate women might have influenced the results (32).

In some previous studies, a positive association betweeen serum 25OHD levels and BMD of forearm and whole body in prepubertal children was reported (26,38). Serum 25OHD levels have been reported to be an independent predictor of lumbar spine and whole body BMD in Finnish children and adolescents aged 7-19 years (19). Lehtonen-Veromaa et al. (20) examined the relationship between serum 25OHD and bone deposition at the LS and femoral neck in healthy 171 peripubertal Finnish girls (age range 7-15 years) taking a multivitamin containing 10 micrograms (400 IU) vitamin D2 in the first 2 years of the study and 20 micrograms (800 IU) in the third year of the study during winter, and calcium supplementation (500 mg/day) was given to those who consumed <1000 mg/day calcium. They reported that there was a significant association between the baseline 25OHD level and 3-year bone gain at the LS and femoral neck amongst peripubertal girls. The LS-BMD of the girls with advanced sexual maturation increased over 3-year by 26%. After 3 years, there was a 4% difference in BMD in the LS in terms

of BMD accumulation from baseline between those with severe vitamin D deficiency and those with adequate vitamin D. None of the subjects with a baseline serum 25OHD level >50 nmol/L (20 ng/mL) lost BMD at the LS. Their findings support the importance of maintaining vitamin D adequency during the growth period of the skeleton.

Several special cases in terms of vitamin D and osteoporosis

Decreased BMD and increased frequency of fractures in **colestatic liver diseases** have been demonstrated previously (39,40). Although the etiopathology of hepatic osteodystrophy is not fully understood, it results from calcium and vitamin D malabsorption (41). Chongsrisawat et al. (42) reported that cholestatic patients with osteoporosis presented decreased vitamin D and calcium levels. In a recent study, the frequecy of vitamin D deficiency was found higher in patients with cholestasis (n= 50, aged 1-18 years, <30 ng/ml in 26% of patients and <20 ng/ml in 30% of patients) than controls and was reported that the z-score of total body BMD and serum calcium were correlated positively, but not found a significant correlation between BMD z-score and serum 25OHD level (39). The authors suggested that low BMD was correlated to the serum calcium rather than the 25OHD level.

Glucocorticoids that are used in many chronic inflammatory diseases have a variety of effects on calcium and bone metabolism. There is conflicting data regarding glucocorticoids effects on BMD in children (43-45). However, it is known that glucocorticoids increase osteoclastic activity and reduce osteoblastic function, thus enhancing bone loss with reduced bone accretion. In other words, glucocorticoids increase RANKL and decrease OPG production, resulting in increased bone resorption. The patients with prolonged glucocorticoid exposure have a risk for osteoporosis. It is suggested that normalization of vitamin D may ameliorate the effects of glucocorticoids on bone (46). Korean guideline for the prevention and treatment of glucocorticoid-induced osteoporosis in adults recommend that the patients administered ≥2.5 mg/day of prednisolone or an equivalent drug for ≥3 months should use adequate calcium (1000-1200 mg) and vitamin D (800 IU) and have adequate 25OHD concentrations (≥20 ng/mL) maintained throughout treatment, the use of supplements should be considered when dietary intake of calcium and vitamin D is insufficient (47). They stated that the minimum adequate dosage should be determined, because high doses of calcium and vitamin D supplementation may increase the risk of gastrointestinal side effects and renal stones.

In epileptic patients, one of the most important side effects of **antiepileptic drugs** is on bone metabolism, including decreased vitamin D levels, changes in bone turnover markers, and high parathyroid hormone levels. Miziak et al. (48) emphasized that in addition to the side effects of antiepileptic drugs, decreased calcium absorption due to low serum 25OHD levels may lead to low BMD and contribute to an increased risk of fracture in epileptic patients. Therefore, periodic monitoring of serum 25OHD level and vitamin D supplementation are recommended in children receiving long-term antiepileptic therapy (48-50). However, newer antiepileptic drugs with minimal or no enzyme-inducing effects have been reported to show a better safety profile on bone metabolism (51). On the other hand, a recent study showed that despite calcium and vitamin D supplementation, 67% of

children with epilepsy and intellectual disability had a low BMD, and 42% had a history of at least one fracture (52).

Minimum desirable 25OHD level in children is 20 ng/mL and levels <12-15 ng/mL are classified as deficient (16,53). Due to limited dietary sources of vitamin D and inadequate consumption of fortified foods, supplemental vitamin D is required for those conditions. Recomended daily intake of vitamin D is a minimum 600 IU daily to preventation and treatment of bone fragility, but higher doses may be required in patients with obesity or malabsorption disorders (54,55).

Conclusions

Studies have shown that low calcium intake, vitamin D deficiency, and inadequate physical activity are major contributors to osteoporosis in children and adolescents with recurrent fractures, and their correction should be addressed before considering other medications. In order to prevent osteoporosis and maintain normal bone mineralization, daily supplementation of vitamin D is considered reasonable which allowing maintenance of a serum 25OHD level of approximately 30 ng/mL in children and adolescents. Sufficient vitamin D and calcium levels should be maintained through dietary intake and/or supplements during the peripubertal years, in accordance with current guidelines.

Fundamentally, maintenance of vitamin D sufficiency in children at increased risk for bone fragility is an important modifiable risk factor. Although there are publications reporting different results, most evidences support that low serum 25OHD levels associate with impaired bone quality and bone health parameters, and vitamin D supplementation can increase BMD in children and adolescents. Therefore, in childhood and adolescence which are important for skeletal growth, it would be a rational approach to avoid the use of osteotoxic drugs and to contribute to strengthening the bone with an optimal diet containing adequate vitamin D and calcium.

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NATURAL AND ANALOG VITAMIN D METABOLITES; WHICH VITAMIN D METABOLITES SHOULD BE GIVEN TO WHICH PATIENT?

NURULLAH ÇELİK*

Introduction

Dietary sources of vitamin D [as cholecalciferol (Vitamin D_3) or ergocalciferol (Vitamin D_2)] which is essential for maintaining serum calcium homeostasis and bone health, are fairly low. So, more than 80% of the vitamin D in circulation is synthesized from 7-dehyrocholesterol by exposure to Ultraviolet B in the skin(1). Calcitriol, the active form of vitamin D $(1\text{-}25\text{-}OH)_2\text{-}D_3$, is produced by 25-hydroxylation in the liver, and then 1-hydroxylation in the kidneys from vitamin D_3 or D_2 (2). Active vitamin D provides calcium and phosphorus hemostasis by affecting renal, intestinal, and bone metabolism on its intracellular receptor. In clinical practice, different natural and analogs of vitamin D metabolites have been used for the treatment of many diseases, related to calcium and phosphorus metabolism. On the other hand, vitamin D receptors are present in many normal and cancerous tissues. It has been demonstrated that stimulating these receptors influences cellular proliferation and differentiation and that active vitamin D has also immunomodulatory and anti-inflammatory effects (3). Because of the extra-skeletal effects, active vitamin D has been investigated for clinical and experimental studies in the treatment of diseases other than bone metabolism, such as psoriasis, malignancy, multiple sclerosis, inflammatory diseases, etc. (3–6). Due to the side effects of calcitriol, especially at supraphysiological doses, such as hypercalcemia and hypercalciuria, analogs with a lower calcemic effect but capable of affecting vitamin D receptors have been developed (Table 1).

Which vitamin D preparation (natural or analogs) should be used for which patient is an important question for clinicians. The type of the disease, the patient's kidney and liver function tests, and the drugs half-life should be considered for the decision. In this review, vitamin D natural and analog metabolites will be summarized, and the reasons for preference in various diseases will be discussed in light of the literature.

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Natural Vitamin D Metabolites

a. Cholecalciferol (Vitamin D_3): More than 80% of circulating vitamin D is cholecalciferol synthesized from 7-dehyrocholesterol by exposure to Ultraviolet B in the skin (7). However, it can be taken in a small amount from the diet. Many preparations used for vitamin deficiency contain cholecalciferol. It is the most commonly used molecule in the treatment of vitamin D deficiency in patients without liver and kidney problems (8). In addition to its well-known role in bone mineral metabolism, cholecalciferol supplementation has been suggested to be associated with better outcomes for a number of diseases, including cancer, rheumatoid arthritis (RA), polycystic ovarian syndrome (POS), multiple sclerosis, type 1 and type 2 diabetes, cardiovascular disease, and hypertension(9).

The most important side effect of the cholecalsiferol is hypercalcemia. When using cholecalciferol, monitoring of alkaline phosphatase, BUN/creatinine ratio, calcium, magnesium and phosphate levels is recommended. Excessive vitamin D consumption can cause vitamin D toxicity, which is characterized by fatigue, weight loss, constipation, dehydration, irritability, and disorientation. These symptoms, which are mostly related to hypercalcemia, may persist for more than two months.

Table 1. Natural and Analog Vitamin D Metabolites

1. **Natural Vitamin D Metabolites**
   a. Ergocalciferol (D_2)
   b. Cholecalciferol (D_3)
   c. Calcifediol (25-OH-D_3)
   d. Calcitriol (1,25dihydroxyvitaminD3 1-25-$(OH)_2$-D_3)
2. **Analog Vitamin D Metabolites**
   a. Paricalcitol (19 nor 1,25dihydroxyvitamin-D_2)
   b. Falecalcitriol
   c. Eldecalcitol
   d. Seocalcitol
   e. Elocalcitol
   f. Falecalcitriol
   g. Maxacalcitol
   h. Tacalcitol
   i. Calcipotriol
   j. Alfacalcidol
   k. Seocalcitol
   l. Lexicalcitol
   m. CD578
   n. Inecalcitol
   o. TX527
   p. 2MD
   q. WY1112
   r. PRI-2205

b. Ergocalciferol (Vitamin D_2): Ergocalciferol, a natural plant-derived vitamin D is also known as vitamin D_2. Ergocalciferol structurally varies from cholecalciferol because of the C22 and C23 double bonds, as well as a methyl group at C2 (Figure 1). In the bloodstream, vitamin D_2 and its metabolites are typically undetectable (10). It is produced by plants and yeasts. It undergoes 25-hydroxylations in the liver and 1-alpha hydroxylation in the kidneys, just as cholecalciferol, before becoming the active form calcitriol. It can be

used in the treatment of vitamin D deficiency. They were previously known to be used in the same doses and interchangeably with vitamin D_3. However, it is suggested that higher doses (3:1) should be used because the 25 hydroxylation step of ergocalciferol is slower than vitamin D_3 (11,12). The most important side effect of ergocalciferol is hypercalcemia. Although a small number of animal studies have shown that vitamin D2 supplementation causes aortic valve stenosis, there are also studies to the contrary (13,14).

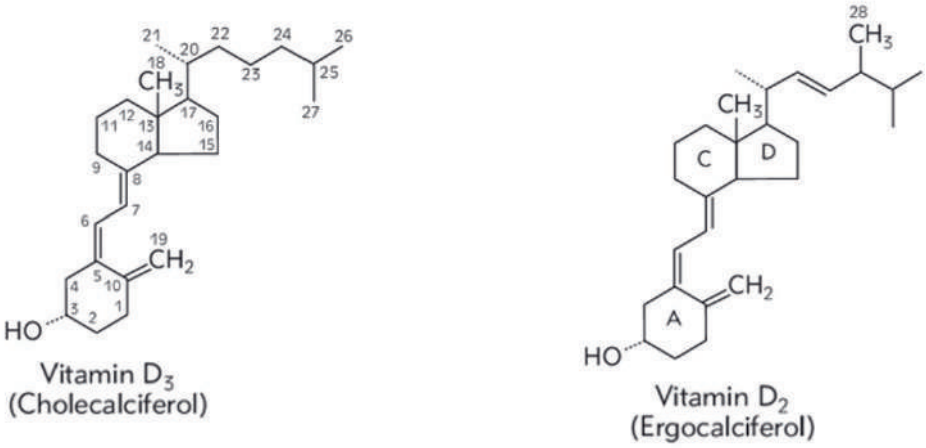

Figure 1. *Structural form of cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2)*

c. Calcifediol (25-OH-D_3): Calcifediol is another natural vitamin D metabolite formed by the hydroxylation of ergocalciferol and cholecalciferol in the liver. Compared to the active form of vitamin D, calcifediol has a longer half-life but lower efficacy due to its high affinity for vitamin D-binding proteins. The most detectable form of vitamin D in the circulation is calcifediol (10). It is an ideal treatment option for the treatment of vitamin D deficiency in patients with liver failure(15). However, it should not be used in cases with chronic renal failure, since 1-alpha hydroxylation cannot occur. In animal studies, it has been shown that patients with chronic renal failure have lower calcifediol levels due to the downregulation of liver CYP450 isoforms, including 25-hydroxylase enzymes (16).

d. Calcitriol (1-25 $(OH)_2$-D_3): Calcitriol, the active form of vitamin D (Figure 2) is used in end-stage renal failure related to seconder hyperparathyroidism, vitamin D-dependent rickets, hypoparathyroidism and pseudo-hypoparathyroidism (17). It is a potent parathyroid hormone (PTH) inhibitor, especially in chronic renal failure (CRF). Seconder hyperparathyroidism, characterized by abnormal mineral levels and bone disorder, is one of the most important mortality and morbidity in patients with uremia. On the other hand, it has been shown that a low level of calcitriol may be related to mortality in patients with CRF with stage three or four (18). So, significantly and progressively elevated PTH levels in CRF should be treated with calcitriol or the other active vitamin D analogs, such as paricalcitol, doxercalciferol, and alfacalcidol (15,19).The most important side effects are hypercalcemia, and hypercalcemia related conditions such as nefrocalsinosis, vascular calcification, renal impairment.

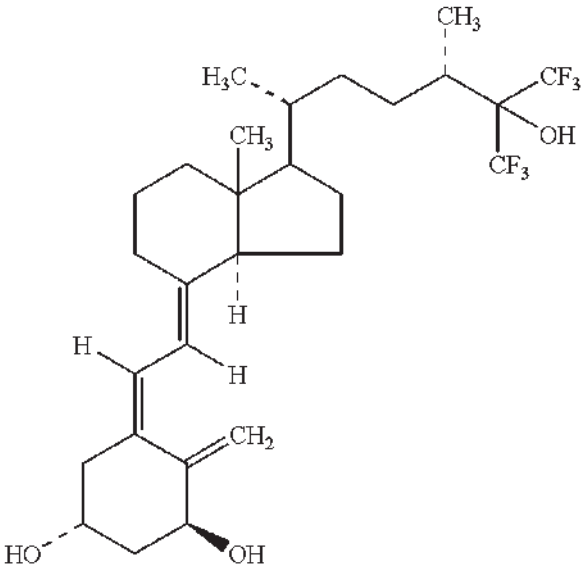

Figure 2. *Structural form of calcitriol [1-25 $(OH)_2$-D_3].*

Vitamin D Analogs

a. Alfacalcidol (1-alpha-OH-D_3): 1-alpha hydroxylated cholecalciferol needs 25 hydroxylation in the liver to become an active form. Alfacalcidiol and calcitriol are the same clinical effect. With treatment of alfacalcidol, bone matrix production and bone growth factors rise, osteomalacia and the rate of trabecular fractures decreases (20). After being chemically synthesized for the first time in 1971, it has been used in various diseases such as osteoporosis, seconder hyperparathyroidism (21–23). Many studies have shown that alfacalcidiol is as effective as calcitriol in the treatment of primary hyperparathyroidism (24,25). Saha et al. showed that the normocalcemic effect of patients treated with alfacalcidiol was similar to that of calcitriol (26). The most important side effect is hypercalcemia. Additionally, it has been shown that using alfacalcidol is related to increased vascular calcification in uremic patients. This effect is thought to be due to the effect of increasing calcium and phosphorus levels. As a matter of fact, this effect is partially reduced after parathyroidectomy (15).

b. Paricalcitol (19-nor-dihydroxyergocalciferol): It is an active form of ergocalciferol (1-25 $(OH)_2$-Ergocalciferol), so not need activation. Due to the lower hypercalcemic impact, it is utilized in patients with CRF and high PTH levels (27). The weak hypocalcemic effect of paricalcitol is thought to be associated with lower intestinal calcium and phosphate absorption when compared calcitriol (28,29). In cases with secondary hyperparathyroidism, not only intravenous but also percutaneous local injection of paracalcitol has been successfully used in selective cases (30). Paricalcitol, on the other hand, has several other possible effects, including proteinuria reduction, anti-inflammatory effects, and immunological and cardiovascular system support. (29,31,32). It has also been shown

that Paricalcitol protects the kidneys and liver damage by reducing oxidative stress and inflammation in mice (33).

c. Eldecalcitol: Eldecalcitol is a new calcitriol analog used in the treatment of osteoporosis. It is formed by a hydroxyproproxy at the 2 beta position to 1-25-OH-D (34). Mean half-life is fifty hour that it is very long when compared the calcitriol (35). Fristly approved in Japon in 2011(36). Eldecalcitol therapy has been demonstrated in studies to enhance bone mineral density and decrease the number of vertebral fractures (37). In a meta analysis trials including eight study and 2368 patients, it has been shown that eldecalcitol was superior to alfacalcidol in improving vertebral fracture risk and BMD (38). Eldecalcitol has also been investigated for its potential antitumor effect (39). Yupu et al (40) showed that it potently inhibits cellular proliferation in vitro and in vivo in oral cancers.

d. 22-Oxacalcitriol (Maxacalcitriol, 22oxa1,25dihydroxyvitaminD_3): Oxacalcitriol, an analog of calcitriol, specifically effect on the parathyroid gland. So, it has a lower calcemic effect compared to calcitriol and the other analogs. Therefore, it can be used in the treatment of secondary hyperparathyroidism (15). Maxacalcitriol can be transferred into cells at a higher rate than other vitamin agonists due to its high affinity for vitamin D receptors in human epidermal keratinocytes. On the other hand, it inhibits lymphocyte proliferation and production of interleukin 6 and 8 (41). Due to these properties, it is also used in inflammatory skin diseases, such as psoriasis, especially in Japan. In a recent phase 3 study, it has been shown that it can be used safely and effectively in the treatment of palmoplantar pustulosis(42).

e. Calcipotriol: MC903, also known as calcipotriol and a 1-25 $(OH)_2$-D_3 analog, is used in the topical treatment of psoriasis, a common inflammatory skin disease (43). Calcipotriol is among the first-choice treatments in the treatment of psoriasis due to its positive effects on keratinocyte proliferation and differentiation and suppression of proinflammatory T cell cytokine production (44). Since skin irradiation is frequently observed during treatment, it is often used in combination with betamethasone-17,21-dipropionate (43). This combination is available in the market as ointment, gel, and foam, and it is claimed that especially the foam formulation is more effective (45).

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VITAMIN D AND AUTOIMMUNITY

EMİNE AYÇA CİMBEK*

Introduction

Vitamin D is largely provided in the skin and may also be supplied from diet and dietary supplements. Cholecalciferol is derived from 7-dehydrocholesterol after ultraviolet B radiation exposure. The CYP2R1 allele directs conversion to 25-hydroxyvitamin D, 25(OH)D by 25-hydroxylase in the liver. The 25OHD is a reliable measure of vitamin D status that measures the supply from dietary sources and skin production. CYP27B1 allele encodes the transformation of 25OHD to the active form, calcitriol, by 1α-hydroxylase, which occurs mainly in the kidney. The $1,25(OH)_2D_3$ shows an effect binding to the vitamin D receptor (VDR) (1). Thus, vitamin D acts in calcium metabolism via the conversion of 25OHD (circulating) into $1,25(OH)_2D_3$ (active). $1,25(OH)_2D_3$ is produced after hepatic 25-hydroxylation by cytochrome CYP2R1 and 1α-hydroxylation by CYP27B1 in peripheral tissues. After transitioning into the cell, $1,25(OH)_2D_3$ forms a complex with VDR, which modifies gene expression in the nucleus. Vitamin D, in both forms, is broken down by the 24-hydroxylation enzyme (CYP24A1) (2). 1α-hydroxylase is also found in immune cells, and CYP27B1 is expressed in myeloid cells. These findings support the observations of high $1,25(OH)_2D_3$ levels in granulomatous diseases, which may be accompanied by hypercalcemia (3).

Sun exposure, diet, pigmentation, aging, obesity, and gender have been shown to affect vitamin D status (1). In addition, living at higher latitudes leads to vitamin D deficiency due to poor solar ultraviolet B irradiance and insufficient sun exposure (4). Therefore, screening for vitamin D deficiency in the broad population is not a recommendation. Instead, groups with a risk for vitamin D deficiency, such as patients with osteoporosis or renal diseases, should be evaluated (5).

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Besides being an essential regulator of calcium and bone metabolism, vitamin D has been the topic of increasing interest because of its other effects, particularly in regulating the immune system and autoimmunity. Vitamin D controls the growth and differentiation of immune cells. Vitamin D signaling is a crucial innate and adaptive immune system mechanism (6–8). Active vitamin D regulates various genes in immune responses and inflammation and controls cell proliferation, growth, apoptosis, oxidative stress, and DNA repair (9).

Vitamin D, with its immunomodulatory characteristics, is a potential supplementation therapy for immune-related diseases. However, different outcomes are observed in studies due to inter-individual differences with complex gene expression in immune cells. Presently, clinical use is limited, and there is no consensus for vitamin D treatment in autoimmune diseases (10).

The VDR and enzymes related to vitamin D homeostasis are expressed throughout the immune system enhancing vitamin D as a multi-facet moderator of the innate and adaptive immune system (11). With the advances in the field of genomic approaches, various VDR-regulated genes modulating the immune system functions have been identified (12). In addition, vitamin D signaling, an essential factor of different immune system traits, regulates inflammatory responses related to autoimmunity (10).

Studies analyzing gene expression regulated by vitamin D in the immune system have shown that vitamin D signaling improves immune responses, including the expression of CD14 (11). Vitamin D signaling also makes dendritic cells less inflammatory, suppressing T cell-associated inflammation and induction of suppressive T-regulatory (Treg) cells (2,13). In addition, vitamin D moderates the intracellular toll-like receptors (TLRs), and the downregulated expression of TLR9 leads to decreased IL-6 secretion, inhibiting T helper-1 (Th1) responses (14). Furthermore, vitamin D promotes antigen-presenting cells (APC) to turn out to be more tolerogenic, and the expression of major histocompatibility complex (MHC) class II is reduced (15).

Vitamin D enhances a more tolerogenic phenotype by inducing Th2 lymphocytes and downregulating proinflammatory Th9 and Th17 cells. It also prevents the synthesis of proinflammatory Th1 cytokines (i.e., IL-2, tumor necrosis factor) with the induction of anti-inflammatory Th2 cytokines (i.e., IL-3, IL-10) directly or indirectly (16). In addition, vitamin D induces Treg cells, which leads to self-tolerance and the prevention of autoimmune disorders. Vitamin D may directly contribute to B cell proliferation, suppressing antibodies and auto-antibodies production and suppressing B cell variation into antibody-secreting plasma cells. (11). While decreased CD4/CD8 ratio correlates with reduced levels of vitamin D (17), its administration leads to an improved CD4/CD8 ratio, meaning immune suppression (11).

Vitamin D is related to various autoimmune diseases, and multiple trials have studied vitamin D supplementation in autoimmune diseases with variable outcomes (3). Vitamin D can affect the manifestation and development of many immune disorders, such as alopecia areata, psoriasis, systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes (T1D). In addition, low vitamin D levels are correlated with an augmented risk of immune-related disorders such as psoriasis and multiple sclerosis (11).

Autoimmune and Immune-related Diseases and Vitamin D

Type 1 Diabetes

There is growing literature on the connections between vitamin D and autoimmunity, particularly T1D. However, proof for the correlation between vitamin D deficiency and the risk of islet autoimmunity and T1D remains mixed. The mechanisms participating in T1D include the immune-mediated damage of the pancreas with the generation of autoantibodies and autoreactive T lymphocytes (18). Animal experiments demonstrated that vitamin D supplementation in several autoimmune diseases, such as encephalomyelitis prevented and improved the disease course (19). Early intervention in T1D might help reserve the remaining beta cell function (8). In animal experiments, vitamin D administration resulted in Treg stimulation and Th1 inhibition (11). Vitamin D deficiency during early life was related to the occurrence and acceleration of disease onset in the NOD mouse. Moreover, the favorable effect of vitamin D in inhibiting insulitis and diabetes in NOD mice is more prominent if directed before the immune attack on the pancreas (10). Vitamin D can also be defensive by provoking insulin secretion via direct binding (11).

Several factors alter vitamin D levels, including dietary intake, genetic factors, and exposure to sunlight (20). It should be considered that rather than leading to the disease, poor vitamin D levels may also result from the disease itself (21). An association between a minor reduction in vitamin D levels and islet autoantibody early in the disease course to T1D has been shown in population studies. However, it should be taken into account that this association is not evidence of interconnection (22).

Type 1 diabetes (T1D) is more common in countries at higher latitudes with lesser sunlight exposure and a higher incidence of vitamin D deficiency (23). An inverse connection between vitamin D levels and developing T1D was shown in a meta-analysis of 16 observational studies, including over ten thousand participants (24). A quantitative correlation between serum vitamin D levels of 100–150 nmol/L (40-60 ng/mL) and a significantly lower hazard of T1D was established. The association was present irrespective of age, duration of disease, or ethnicity. The seasonal onset of T1D, associated with low circulating vitamin D levels, could suggest that poor vitamin D status may accelerate disease onset (10). The TEDDY study showed a relationship between low serum vitamin D levels through childhood and islet autoimmunity (25). However, some studies, such as the DAISY study, did not confirm these results (26). Several authors supported that vitamin D supplementation during early life is related to T1D prevention (27). A reduced incidence of T1D with early vitamin D supplementation was reported (10). One nationwide trial concluded that long-term supplementation of vitamin D- added omega-3 fatty acids or not- could decrease the risk of autoimmune disease (28). Hahn et al. (28) showed that in their study comprising older adults, vitamin D (2000 IU/day) and omega-3 fatty acids (1 g/day) for five years reduced the incidence of autoimmune diseases. The effect increased with time. However, omega-3 fatty acids alone did not affect the occurrence of the disease. They highlighted the importance of their findings as these supplements are well tolerated and easily administered.

A lower probability of T1D was demonstrated in children with an advanced vitamin D intake. A significant reduction in T1D risk was found in studies including children supplemented with daily vitamin D during the early years of life. Additionally, some studies indicated that vitamin D supplementation reduced the daily insulin dosage and increased C-peptide levels (11). Miettinen et al. (20) showed that in children with islet autoimmunity, compared to controls, vitamin D levels were lower before the detection of the first seroconversion of the cases. Additionally, in children with T1D, vitamin D levels were lower 18 months priorly and at 12 months. They concluded that early postnatal vitamin D might help prevent T1D.

Based on the hypothesis of gestational programming, several factors throughout the maturation path of the immune system might be related to various irreversible consequences on immunity and health in the long term. Studies analyzing the association between vitamin D levels or prenatal intake and T1D in offspring showed inconsistent results (29). While some authors found a linkage between maternal low vitamin D levels and the risk of T1D in the offspring, others did not demonstrate such an association. In addition, several other authors could not depict an association between T1D and serum vitamin D levels during pregnancy or neonatal vitamin D levels (30,31). In one of these studies, Jacobsen et al. (31) evaluated vitamin D levels utilizing neonatal dried blood spots, unlike the other studies relying on maternal vitamin D supplementation/intake questioning through pregnancy. They performed the analyses using reliable, highly sensitive liquid-chromatography–tandem mass spectrometry in a large-scale and population-based sample.

If greater vitamin D levels through pregnancy and higher supplementation doses might prevent T1D in the child could not be interpreted from the findings of current studies. Moreover, the vitamin D levels at birth primarily reflect fetal vitamin D exposure during the end of pregnancy. Thorsen et al. (30) found similar results using serial measurements throughout the pregnancy and cord blood samples in the largest cohorts of pregnant women in their study, which is the first to analyze umbilical blood vitamin D levels concerning childhood T1D. On the other hand, Granfors et al.(32) did not show a relation between vitamin D supplement usage during pregnancy and childhood T1D.

Norris et al. (25) demonstrated that higher childhood vitamin D levels were related to lower islet autoimmunity risk. Moreover, this association was especially shown in the group carrying a particular variant in the VDR gene (rs7975232), suggesting that the two factors might have a combined part in the risk of emerging islet autoantibodies. It can be speculated that the underlying mechanism includes factors related to vitamin D action. Vitamin D and VDR may contribute to T1D development in children having an increased genetic risk (25). Genetic factors have been demonstrated in T1D, but in genome-wide association analyses, only one SNP was statistically significant (rs10877012, CYP27B1 gene) (20). It was depicted that vitamin D deficiency might contribute to T1D in individuals carrying specific VDR gene genotypes (33). A combined effect of multiple risk alleles (DHCR7, CYP2R1, GC, and CYP24A1 genes) was demonstrated in another study (34).

VDRs are present in most immune system cells. Immune cells also involve enzymes related to vitamin D metabolism that locally convert vitamin D to the active form. In

addition, vitamin D may also affect gut microbiota which is associated with T1D and is one of the significant target localizations of vitamin D (35). Moreover, VDR is also expressed in the intestines. Furthermore, vitamin D interferes with gut integrity, sustaining the balance between the host and gut microbiota. This way, intestinal bacterial translocation is inhibited, and homeostasis is achieved, which has a role in developing various auto-inflammatory disorders (11). These findings inform the intricacy of the potential relationship between vitamin D and T1D.

It should be added that a lower serum vitamin D level may be a consequence of ongoing inflammation during autoimmune disorders, thus showing immune system disturbances. Altogether, vitamin D maintains adequate immunological defenses and has a role in autoimmunity in various scenarios (21). Notably, individuals with classical vitamin D deficiency do not usually develop T1D, even if not vitamin D supplemented for prolonged periods to cause osteoporosis or rickets (22). However, there is no agreement regarding the vitamin D levels needed for immune homeostasis (7).

Autoimmune thyroiditis

The relationship between autoimmune thyroid diseases and vitamin D is controversial. The topic has previously been studied in limited research. In a recent meta-analysis, it was shown that autoimmune thyroid disease was more frequent in individuals with vitamin D deficiency, suggesting that vitamin D status might also predict the disease process. The authors concluded that the cost-effectiveness of the supplementation therapy and the required dose of treatment needs to be evaluated comprehensively (36).

Feng et al. (37) showed that vitamin D levels were lesser in individuals with autoimmune thyroid diseases than in controls. They also depicted that vitamin D levels were negatively linked to thyroid autoantibodies. Furthermore, vitamin D levels were negatively associated with serum IL-21 levels in children with Hashimoto or Graves' disease irrespective of the treatment status (37). Similarly, Hanna et al. (38) showed that FokI AA genotype was more frequent in patients with Hashimoto compared to cases with other causes of hypothyroidism. Moreover, FokI AA genotype was related to better vitamin D levels. They suggested that this observation could be due to a vitamin D receptor dysfunction, mainly present in this genotype. They added that patients with Hashimato thyroiditis and appropriate vitamin D levels should be analyzed for the genetic variant FoKI AA rs2228570.

Rheumatic diseases

Data regarding the association of vitamin D with rheumatic diseases in children is scarce. Many children with pediatric rheumatic diseases may demonstrate vitamin D deficiency or insufficiency, reflecting disease processes. In addition, glucocorticoid treatment may disturb vitamin D metabolism, inhibiting optimal bone mass achievement. Therefore, several authors have explained the requirement for vitamin D supplementation as an adjunct treatment in rheumatic diseases (39). They analyzed the connection between light exposure and rheumatoid arthritis and established a subsided risk of rheumatoid arthritis and ultraviolet B exposure until youth period.

Similar results have been demonstrated also for juvenile idiopathic arthritis (39). Juvenile idiopathic arthritis is a chronic autoimmune disorder with varying severity of an autoimmune and destructive course and may cause disability. A relatively high occurrence of vitamin D deficiency is observed in juvenile idiopathic arthritis. However, in a meta-analysis on this topic, the connection between vitamin D deficiency and the disorder could not be established, mainly because of the unavailability of an evident classification of vitamin D deficiency in children in the studies (40). However, patients with juvenile idiopathic arthritis had lower vitamin D levels compared to controls.

Stagi et al. (41) have shown that patients with juvenile systemic lupus erythematosus have lower vitamin D levels than controls. Robinson et al. (42) demonstrated findings in line with the others. In their study, a significant proportion of the study population had vitamin D deficiency and insufficiency, which was related to increased inflammation in children with lupus. However, disease activity was not associated with vitamin D levels at baseline. They further showed that vitamin D status contributes to atorvastatin's effect on carotid intima media thickening over time in patients with lupus despite adjusting covariates, highlighting its role in cardiovascular risk. In addition, it was shown that low vitamin D levels in systemic lupus erythematosus are correlated with disease activation and pathophysiology. Therefore, several authors suggested that vitamin D supplementation should be involved the treatment of the disease to reduce the inflammatory process (11).

Polymorphism of genes regulating vitamin D metabolism may be linked to the occurrence and severity of rheumatic diseases. It was found that FokI f variant VDR polymorphism was related to the outcome and necessity for biological treatment in juvenile idiopathic arthritis (39).

Inflammatory bowel diseases

A sufficient vitamin D status is significant for gut homeostasis through various mechanisms including calcium metabolism, maintenance of the barrier task and regulation of the intestinal microbiota. The importance of vitamin D action in the gut is demonstrated by the observations of several disorders with vitamin D deficiency including rickets and inflammatory bowel diseases (35). Inflammatory bowel diseases are chronic diseases of the gastrointestinal system characterized by a need of long-term treatment. The pathogenesis is related to a defective immune system and chronic intestinal inflammation. Inflammatory bowel disease is frequent in localizations with a higher latitude and lesser sun exposure. In addition, vitamin D absorption in these disorders is disturbed, and vitamin D level is a predictor of disease activity in these patients. Sub-optimal dietary intake, intestinal losses and cooccurring corticosteroid treatment also contribute to the insufficiency. Thus, vitamin D deficiency is frequently seen in individuals with inflammatory bowel diseases (11).

Several studies supported the immune-modulator role of vitamin D in these disorders. In a randomized, placebo-controlled, double-blinded research by El Amrousy et al. (43) oral vitamin D supplementation of 2000 IU/day for 6 months was evaluated in children and adolescents with inflammatory bowel diseases and it was found that the intervention decreased the disease activity, and improved the quality of life. Inflammatory markers, including erythrocyte sedimentation rate, were reduced in the treatment arm. In addition,

vitamin D was inversely related to hospitalization and emergency visits. No major adverse effects were detected related to vitamin D administration. It was the first study proving that while vitamin D inhibits inflammatory cytokines, it improves anti-inflammatory reaction in children with inflammatory bowel diseases.

A recent systematic review demonstrated that studies with various treatment regimens were scarce in managing vitamin D deficiency in children with inflammatory bowel disease (44). Veit et al. (45) did not show a difference in vitamin D levels among children and adolescents with disease and controls. However, individuals with increased erythrocyte sedimentation rate had lower vitamin D levels than controls.

Dermatologic diseases

The potential implications of vitamin D in the management of patients with autoimmune skin diseases have been of interest. Vitamin D deficiency in patients with alopecia, a T-cell mediated autoimmune skin disease with hair loss, effects disease severity and duration (46). Vitamin D deficiency is frequent in patients with alopecia. Currently, no successful treatment for alopecia is accessible. Alopecia is also commonly seen in patients with vitamin D dependent rickets type 2. In a study by Papadimitriou et al. immunomodulating therapeutic effect of active vitamin D was observed in children with alopecia areata (47). Kim et al. analyzed the factors associated with vitamin D insufficiency in children and adolescents with alopecia (48). Over half of the study population including 96 patients living in a geographical area with four seasons had insufficient vitamin D levels. They found that non-summer season and race were associated with insufficient vitamin D levels but the monthly ultraviolet index was negatively correlated.

Vitiligo is a pigmentary autoimmune disorder. In a study conducted in children and adolescents, vitamin D levels were not different between the patient and control group. However, combination therapy of oral vitamin D supplementation and a topical agent was more successful in treatment than only topical treatment. The authors suggested for a potential role of vitamin D supplementation in children with vitiligo and vitamin D deficiency (49). The study was the first to analyze vitamin D levels in this patient group along with the detection of the size of the lesion with a reliable method. Moreover, any adverse event was not observed. Alike results have been demonstrated in adult studies (50). However, one should remember that skin pathologies may affect vitamin D synthesis (7).

Table 1. Autoimmune disorders associated with vitamin D.

Rheumatoid arthritis
Sjögren's syndrome
Systemic lupus erythematosus
Inflammatory bowel diseases
Multiple sclerosis
Type 1 diabetes
Guillain-Barre syndrome
Chronic inflammatory demyelinating polyneuropathy
Psoriasis
Vitiligo
Autoimmune thyroid disease
Myasthenia gravis
Vasculitis
Antiphospholipid syndrome
Immunoglobulin A nephropathy
Autoimmune hepatitis
Immune thrombocytopenic purpura
Autoimmune hemolytic anemia

Miscellaneous

Comparable observations were obtained for multiple sclerosis, antiphospholipid syndrome, immune thrombocytopenic purpura, autoimmune hemolytic anemia, Sjögren syndrome and various diseases (Table 1). In line with the finding observed for T1D, multiple sclerosis is more commonly seen in countries with a higher latitude and insufficient sun exposure (7,11). Vitamin D deficiency has been commonly observed in autoimmune hepatitis and it was found to be related with disease progression. Several authors suggested that vitamin D analogues may be an additional supply to the current treatment strategies of autoimmune hepatitis (1).

Legitimo et al. (51) evaluated vitamin D levels and the immune functions, mainly T cell groups and dendritic cells, in 22q11.2DS patients suffering from increased risk of infections and autoimmune disorders. They showed a direct connection between vitamin D levels, recent thymic emigrant, and dendritic cell numbers. They concluded that deficiency of the plasmacytoid dendritic cell subset might be one of the factors resulting in the increased prevalence of autoimmune diseases in these patients. They suggested a probable function of vitamin D supplementation in immune disorders in 22q11.2DS patients.

VDR agonists have immunomodulatory and anti-inflammatory effects. Suppression of inflammation by VDR agents led to a change in the immune response regarding Th1-Th2 dominance. In addition, antagonism of the inflammatory circle among immune and resident cells was detected, along with cytokine release impairment. Targeted locally absorbed drug

therapy is under investigation for vitamin D deficiency, mainly for autoimmune disorders. β-glucuronides of 25(OH)D and $1,25(OH)_2D_3$ are of interest and have been shown to have more favorable effects compared to the native form (7). Vitamin D analogues with super-agonistic effects and low calcemic consequences are on the way (1).

Conclusion

In summary, vitamin D, crucial in calcium homeostasis and an immunomodulator, affects the innate and adaptive immune systems. The wide distribution of the enzymes related to vitamin D (CYP24A1, CYP27B1) and VDR in the immune system, with the various genes linked to vitamin D, provides the base for the divergent effects of vitamin D in the immune system. As serum vitamin D level is related to numerous immune disorders, including autoimmune diseases, vitamin D supplementation may be significant in the prevention and treatment. The possible connection between vitamin D and type T1D relates to the diverse effects of vitamin D on the immune system. As vitamin D regulates inflammatory immune responses related to autoimmunity, it may be beneficial as an immunomodulatory agent.

Vitamin D intake during the early years of life may protect the development of T1D and other autoimmune disorders. However, there is substantial heterogeneity among studies, with trials presenting mixed results. These contradictory results may be related to analysis designs or a failure to consider the inherent genetic variation regarding the vitamin D pathway. Genetic adjustment of the effect of vitamin D may explain the inconsistent findings concerning the alleged securing role of vitamin D. A child having susceptibility genes related to autoimmune disorders may be more defenseless to insufficient levels of vitamin D when compared to their counterparts.

Healthcare organizations should develop programs to lessen the risk of vitamin D deficiency, and awareness regarding vitamin D in the general population, particularly at-risk individuals, should be improved. In addition, during follow-up of individuals with autoimmune diseases, possible vitamin D deficiency needs to be evaluated and treated by appropriate supplementation. Lastly, the role of vitamin D in limiting autoimmune disorders, including T1D development, needs to be further explored.

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VITAMIN D AND INFECTIONS

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Vitamin D is obtained from the diet, supplements, or seasonal exposure of skin. Vitamin D3 (cholecalciferol) is obtained naturally from ultraviolet B (UVB) sunlight by conversion to 7-dehydrocholesterol, a steroid precursor in the skin. Vitamin D3 and orally taken vitamin D are transported to the liver and converted to 25(OH)D by the enzyme CYP2R1, also known as cytochrome P450 2R1 (1). 25(OH) D is then hydroxylated by the CYP27B1 enzyme in the kidney, converted to its active form, 1,25-dihydroxycholecalciferol [$1,25(OH)_2D$]. The active metabolite, $1,25(OH)_2D$ (also known as calcitriol), is the most potent steroid hormone in the human body and its role in calcium homeostasis and skeletal health is well known. Outside of these systems, vitamin D potentially regulates many other cellular functions. $1,25(OH)_2D$, is activated by signaling via the vitamin D receptor (VDR), a member of the nuclear receptor family of ligand-regulated transcription factors (2). Vitamin D receptors is broadly expressed in many tissues of the body such as intestine, kidney, parathyroid gland, bone, brain, immune system and many sites of the genome. Vitamin D receptor activation regulates the expression of more than 1,000 genes directly and indirectly in the human genome (3). $1,25(OH)_2D$ potentially regulates many other biological roles in numerous systems in the human body via VDRs, including the immune system, cardiovascular system, musculoskeletal system, endocrine system, neuropsychiatric system, and immunomodulatory system (4,5). In this part of the book, the association between Vitamin D and infections will be discussed.

The causal association among low vitamin D levels and infections remains unclear. Studies in recent years have shown that vitamin D also plays prominent function, regulating the functioning of the immune system as well as providing protection against various infections. Evidence for the function of vitamin D has emerged from important discoveries that CYP27B1 expression in immune cells is controlled by a number of immune cascades (6). The VDR and vitamin D metabolic enzymes are present throughout the immune system, and, importantly, CYP27B1 production in immune cells is induced by pathogen detection is regulated by a complex cytokine network, which is independent of calcium homeostatic signals. Thus, $1,25(OH)_2D_3$ can be produced under conditions of pathogen threat, and plays a role against infections. In almost all immune cells; VDR is

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expressed on CD4 + and CD8 + T cells, B cells, monocyte and antigen presenting cells such as macrophages and dendritic cells (DCs). VDR acts as modulator of both innate and adaptive arms of immune system (7).

The innate immune responses are the first line of defense against invading pathogens. Vitamin D activated the innate immunity, particularly monocytes, macrophages and DCs in response to infections. The relationship between monocytes and vitamin D is mediated by the enzyme CYP27B1. Mechanisms of vitamin D-innate immune signaling include its production of cytokines, antimicrobial proteins, and pattern recognition receptors. Innate immunity activated by VDR is mediated by CYP27B1 through stimulation of TLR1/2 pathogen-recognizing receptors (PRR) and activation of TLR-4 ligand lipopolysaccharides (8). Pathogens are identified by pathogen-associated molecular patterns that bind to these PRRs. Studies have shown that there is amplification of CYP27B1 activity with the recognition of pathogens in this way. CYP27B1 amplification leads to an increase in the production of $1,25(OH)_2D$, which binds to the endogenous VDR and activates gene expression in monocytes (9). Vitamin D has a function in innate immunity by taking part in the transforming monocytes into macrophages, then increasing the phagocytic capacity of macrophages (10,11). And also, with TLR2 and TLR4 inhibition, a natural immune response occurs by fighting pathogens. Vitamin D enhances innate immunity by increasing the production of antimicrobial peptides (AMPs) such as ß-defensins, cathelicidins, human β-defensin 4 which have the ability to kill viruses, bacteria and fungi (12). AMPs are innate immune elements with small cationic molecules that show broad spectrum activity against pathogens, especially on skin and mucosal surfaces (13, 14). LL37, which is the active form of an antimicrobial peptide named cathelicidin, recruits neutrophils, monocytes and T cells to infectious sites and promotes apoptosis of infected cells. In addition to these effects, cathelicidin-LL 37 has the functions of supporting the antimicrobial activity of neutrophils by increasing the production of reactive oxygen radicals. Thus, cathelicidin-LL37 shows activity against both gram-negative and gram-positive bacteria, many viruses and fungi, which are also expressed in macrophages (13,15). Moreover, vitamin D inhibits maturation of DCs. Inhibition of DCs also contributes to the formation of anti-inflammatory response by increasing anti-inflammatory cytokine (IL-10) and T-cell inhibitory molecule (PD-1) and downregulation of antigen presenting molecules (MHC-class II), costimulatory molecules, and pro-inflammatory cytokines (16). Vitamin D reduces Th1 and Th17 differentiation and shifts to more tolerogenic Th2 response, enhances Treg cell differentiation. All these leads to hypo-responsiveness and anti-inflammatory responses. T cell proliferation is down-regulated when DCs are stimulated with $1,25(OH)_2D_3$ (9,16).

Studies have suggested that vitamin D plays a role not only in innate immunity, but also in adaptive immunity. In particular, it suppresses the transformation of B cells into plasma cells and T cell proliferation, modulates immunoglubulin production. Vitamin D decreases the generation of IL12, an important cytokine for T cell proliferation. Decrease of IL12 suppresses the Th1-mediated response by suppressing the production of proinflammatory cytokines, IL2 and IFN-γ which are pivotal for T cell proliferation (17, 18). $1,25(OH)_2D$ restricted Th1/Th17 differentiation enhancing Th2 differentiation and stimulate the formation of IL-10-secreting Treg cells. The effects of vitamin D on B

cells are to inhibit memory cell formation and to favor B cell apoptosis (16). These results suggest that, vitamin D modulates the innate and adaptive immune system and shows positive effects against infections through many mechanisms including cell, receptor and signaling pathways. Therefore, vitamin D deficiency may well have an effect on the emergence of infections.

Vitamin D and Respiratory Tract Infections

Many studies have been conducted to reveal the relationship between vitamin D deficiency and respiratory tract infections (RTIs). When viral infection develops, inactive vitamin D is transformed to $1,25(OH)_2D_3$ in respiratory tract epithelial cells. Vitamin D in its active form increases the production of AMPs such as cathelicidin and shows activity against infections. Studies have reported that the antiviral response of vitamin D is regulated by cathelicidin. In addition to its direct effect on cathelicidin, vitamin D constitutes an antiviral response against respiratory tract viruses by regulating cytokine production through the innate and acquired immune system (19). Its supplementation may also play a protective role in preventing acute RTIs. The spike of RTIs during the winter months may be related to lower vitamin D level as a result of the decrease of solar UVB presence during this season in countries. In addition, it has been stated that $1,25(OH)_2D_3$ protects against microorganisms by affecting the barrier integrity in respiratory system (20). In a study conducted on 18.883 volunteers 12 years of age and older, it was determined that serum vitamin D level and upper respiratory tract infections (URTIs) were inversely related (21). A meta-analysis report of 11 placebo-controlled studies including 5660 patients suggested that the use of vitamin D may be effective in preventing respiratory diseases (22). Studies have shown that low vitamin D levels are associated with acute otitis media (AOM), pharyngotonsillitis, rhinosinusitis. In a randomised, single-blind study vitamin D level was statistically significantly lower in the patient with AOM than in the control group (23). It was thought that vitamin D limits biofilm formation and it could prevent recurrent tonsillopharyngitis by taking advantage of this effect. Studies have indicated that vitamin D levels were lower in these patient groups (24,25). There are not enough studies that reveal the direct relationship between rhinosinusitis and vitamin D. In a meta-analysis in which 5 clinical studies were evaluated, the effect of vitamin D and placebo supplementation on URTIs was evaluated. Upper respiratory tract infections were found to be statistically significantly less in patients receiving supplementation (26). Further studies are needed to demonstrate the effectiveness of vitamin D supplementation against RTIs.

One of the important causes of upper respiratory tract agents is rhinoviruses. In a study investigating the antiviral effect of vitamin D on rhinovirus replication, no direct antiviral activity was detected. Proinflammatory chemokines CXCL8 and CXCL10 are increased when interacting with vitamin D. These chemokines recruit macrophages, neutrophils and T cells to the infection site, and as a result, inflammatory cells and cytokines show antiviral activity against rhinovirus (27).

Lower respiratory tract infections are still among the top five causes of death in childhood. The association among vitamin D and pneumonia first started to attract attention as early as 1975, with more frequent pneumonia in pediatric population with rickets. In a

case-control study (500 cases, 500 controls) reported from Ethiopia, the risk of pneumonia was 13 times higher in the rickets group (28). In two studies reported from Israel and Bangladesh, it was stated that vitamin D level was lower in the pediatric patient group with lower respiratory tract infection (LRTIs) when compared to the control group. In different studies, vitamin D was lower in children with bronchiolitis than in healthy children (29,30). In an another study supporting these studies, wheezy bronchiolitis is observed more frequently in children with rickets than in the control group. respiratory syncytial virus (RSV) is the most common cause of bronchiolitis. In vitro studies have shown that vitamin D reduces the inflammatory response in respiratory epithelial cells in RSV infection. This suggests that vitamin D may be protective against severe RSV infections. In a meta-analysis, it was determined that the genetic polymorphism in the VDR is associated with the course of RSV as severe bronchiolitis (31-33). The rate of hospitalization due to RSV-related LRTIs was found to be high in GC1 haplotype carriers (34). It has been suggested in studies that it can suppress the severity of the disease that will lead to morbidity and mortality by reducing the inflammation caused by RSV. Antimicrobial peptides LL37 and β-defensin 2, whose production is stimulated by vitamin D, block the entry of RSV into the cell, thus preventing epithelial cell necrosis (9). However, contrary to these studies, no relationship could be established between acute lower respiratory tract effects and vitamin D in other studies (35).

In the study designed to determine the vitamin D level in the cord blood and the risk of developing pneumonia in the first two years of life, cord blood was collected from 206 newborns. Cord blood vitamin D levels of newborns who developed acute LRTI in the first two years of life were found to be lower than those who did not have pneumonia (36). In another study, vitamin D levels of mothers of newborns with LRTIs were investigated. Maternal vitamin D levels in the patient group were found to be lower than in the control group (37). In another study evaluating 922 cord blood vitamin D levels, an inverse association was found between cord blood vitamin D level and risk of respiratory infection (38). As a result of these studies, it was thought that vitamin D supplemented to mothers during pregnancy could prevent ALRI. With this thought, in the study in which children who were supplemented with 14,000 international unit (IU) vitamin D per week were followed up for 3 years, vitamin D did not have a protective function against respiratory tract infections (39). Although it has been shown that vitamin D deficiency is associated with respiratory tract infections, the protective effect of vitamin D supplementation from these diseases has not been definitively proven.

Vitamin D and Influenza

Many studies have been conducted to determine the relationship between influenza and vitamin D levels. Influenza peak occurs in winter and also vitamin D level will decrease depending on the reduce in solar UVB doses in winter. Therefore, it is difficult to establish the existence of a direct relationship between vitamin D level and influenza. LL37 antimicrobial peptide binds directly to the influenza virus, causing membrane damage and destroying the virus. Similarly, CAMP reduced influenza A replication in infected mice and reduced disease severity (2). A double-blind, placebo-controlled trial from Japan com-

paring vitamin D supplementation (1200 IU/d) with placebo in schoolchildren suggested that vitamin D supplementation during the winter months may reduce the incidence of influenza A (40). In a multicenter, randomized, open, controlled clinical trial involving 400 infants, high-dose vitamin D (1200 IU) has been found to be effective in preventing seasonal influenza (41). Influenza-infected mice fed a high-dose vitamin D diet showed a reduction in inflammatory cytokines such as interleukin and interferon gamma, viral replication and improved clinical response compared to mice not supplemented with vitamin D (2). It is thought to be effective in rapid recovery in symptoms, rapid reduction in viral load and recovery. In a randomized controlled trial during the 2009 H1N1 pandemic, vitamin D supplementation was not protective for Influenza A (42). In most studies other than these, no correlation was found between vitamin D levels and seasonal influenza. In a randomized controlled study examining 1300 children in Canada, the relationship between vitamin D level and respiratory viruses was investigated. Vitamin D supplementation reduced the incidence of flu but moderately reduced non-flu respiratory viral infection. It shouldn't be forgotten that vaccination is the most effective method of preventing influenza.

Vitamin D and COVID-19

While a wide variety of studies have been conducted on the relationship between infections and vitamin D, with the onset of the COVID-19 pandemic, the investigation of the relationship between vitamin D status and COVID-19 infection and its consequences has aroused great interest. In the light of data obtained from previous studies, it was thought that vitamin D levels may affect SARS CoV-2 infection and COVID-19 prognosis. There have been studies revealing the association among lower vitamin D serum level and COVID-19 disease and severity. The effects of vitamin D during COVID-19 infection occur via different immune pathways. SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) on alveolar cells and reverses the ACE2/ACE ratio, then angiotensin 2 increases, leading to a severe course of COVID-19 (43). Vitamin D increases ACE2 expression and inhibits renin, both the increase of ACE 2 and the inhibition of renin; decrease the level of angiotensin 2, which causes the severe course of COVID-19 (44,45). COVID-19 leads to cytokine storm by increasing pro-inflammatory cytokine production. This cytokine storm causes tissue damage in the lung. Vitamin D prevents the formation of cytokine storm by reducing proinflammatory cytokine production (46, 47). And also, vitamin D plays an active role in SARS-CoV-2 elimination in the immune system by increasing autophagy in macrophages (15). Vitamin D level was found to be lower in COVID-19 patients in intensive care unit compared to other COVID-19 patients. Respiratory complications were more common in COVID-19 patients in South Asia and Switzerland due to low vitamin D values (48). A large cohort study (348,598 participants,499 with COVID-19) reported no association between Vit D level and COVID-19 risk or mortality after excluding other risk factors (49,50). Similarly, in a retrospective cohort study (780 patients) reported from Indonesia, mortality was found to be higher in patients with low vitamin D levels (51). In a study with the participation of 489 people, the risk of COVID-19 test positivity was higher in those with vitamin D deficient (25[OH]D level <20 ng/mL) than

those without (52). Not only vitamin D deficiency but also comorbid diseases pose a risk for COVID-19, such as obesity, diabetes mellitus.

There is no conclusive evidence that vitamin D supplementation reduces disease risk and severity on COVID-19 outcomes. In a randomized study comparing a single dose of 200,000 international units of vitamin D versus placebo in 240 moderately ill patients hospitalized due to COVID-19, no difference was found in the length of hospital stay, mortality, admission to intensive care unit or mechanical ventilation requirement (53). In a pilot, randomized clinical study, higher dose vitamin D supplementation was shown to reduce viral load in intensive care patients. In addition, in another case series, it was reported that high-dose vitamin D contributed to clinical improvement, reduced oxygen requirement and shortened hospital stay (54). In a cross-sectional observational study involving 986 COVID-19 patients from 3 different hospitals, 151 patients with vitamin D deficiency received ≥280000 high-dose booster therapy over a 7-week period. It has been reported that booster therapy has a reducing effect on COVID-19 mortality (55). Knowing that overdose has harmful effects, vitamin D supplementation can be given to those with vitamin D insufficiency. Moreover, futher studies are needed to understand the immune mechanisms and therapeutic effect of vitamin D in the course of COVID-19.

Vitamin D and Human Immunodeficiency Virus

Epidemiological studies have revealed that 70-85% of human immunodeficiency virus (HIV)-infected people have lower vitamin D level. This situation is not only related to HIV, but may also be due to different reasons. Comorbid diseases, malabsorption, malnutrition, infectious complications, decreased exposure to sunlight lead to vitamin D deficiency in patients with HIV. Low vitamin D level has been associated with poor prognosis in HIV-infected individuals (9). Besides these the natural course of HIV infection, chronic inflammation, drugs used for HIV therapy, are among the causes of vitamin D deficiency in patients with HIV.

High vitamin D upregulates anti-inflammatory IL-10 and increases anti-HIV-1 defensins in the mucosa of seronegative individuals exposed to HIV-1 (56). In studies, increased VDR expression was associated with increased expression of anti-HIV molecules (57). Vitamin D blocks the entry of HIV into monocytes by reducing surface CD 4 expression and monocyte proliferation (58,59). Furthermore, Vit D inhibits HIV-1 replication by stimulating autophagy in HIV-infected macrophages (60). All these studies revealed that high-dose vitamin D may have a role in HIV resistance. Some studies suggest that antiretroviral therapy and vitamin D supplementation contribute to prognosis. Some studies suggest that antiretroviral therapy and vitamin D supplementation improve prognosis and obstruct disease progression (9). However, contrary to these, some studies have reported that vitamin D increases HIV replication in monocytes (61-63).

Vitamin D deficiency can cause complications such as organ dysfunction, progression to acquired immunodeficiency syndrome and death in HIV-infected individuals as a result of increased inflammation and activated monocytes. Chronic inflammation can also lead to vitamin D deficiency. However, high levels of vitamin D, known to suppress inflammation, were associated with higher levels of proinflammatory cytokines, contrary to expectations

in HIV patients on cART therapy (64). In addition, HIV increases the expression of CYP27B1 and CYP24A1 in monocytes and macrophages, leading to a decrease in vitamin D and a reduction in the expression of VDR and antiviral peptides (65,66).

In conclusion, low vitamin D level is effective in the development of HIV infection by affecting the innate and adaptive immune system. Lower vitamin D may increase the risk of comorbid diseases and mortality by triggering inflammation. It has been demonstrated in some studies that vitamin D supplementation can be used as prophylaxis to correct these negative results. Further studies are needed to clarify the relationship between HIV and vitamin D.

Vitamin D ve Bacterial Infections

Vitamin D modulates the cytokine profile and homeostatic parameters in response to bacterial endotoxins (LPS). It has functions of generating macrophage response against *Mycobacterium tuberculosis* (MTB), bronchial cell response against *Pseudomonas aeruginosa* and cathelicidin-mediated urinary bladder response against *Escherichia coli* (67). It has been shown that vitamin D has an antibacterial effect against urinary tract infection by increasing the expression of cathelicidin in the uropathogenic *E. coli* CFT073-infected bladder (68). Lower vitamin D level has been related with bacterial vaginosis Against E. coli and P. aeruginosa, $1,25(OH)_2D_3$ has been indicated to stimulate antimicrobial protein release owing to VDREs located in the promoter regions of the cathelicidin and ß-defensin-2 genes. It has been reported that cAMP is a potential anti-sepsis agent by stimulating the expression of cAMP by showing a synergistic effect with LPS, which are microbial ligands in neutrophils (69). Autophagy was a catabolic pathway that contributed to antimicrobial defense. Cathelicidin is essential for the induction of autophagy by vitamin D in bacterial infection. And also, activation of VDR signaling has been reported to affect autophagy.

Vitamin D and Tuberculosis

To date, many studies have been conducted to reveal the relationship between vitamin D and tuberculosis. But there was no clear relationship between vitamin D level and tuberculosis disease, progression of latent infection to the active disease or response to treatment. Since most of the studies are retrospective, it cannot be determined whether low vitamin D levels cause tuberculosis infection or a result of tuberculosis infection. Meta-analysis studies have shown a relationship between vitamin D deficiency and tuberculosis disease (70-72). Unlike these studies, no statistically significant risk for tuberculosis infection was found in some studies (73,74). In a meta-analysis included 5 studies involving 2 case–control studies and 3 cohort studies, serum vitamin D level was not found to be associated with latent tuberculosis and high vitamin D level was not found to be protective from latent tuberculosis (75).

Before the introduction of anti-tuberculosis therapy, sun exposure was known to be beneficial. Vitamin D can destroy tuberculosis bacillus-infected macrophages by stimulating the production of nitrogen and oxygen radicals (76). As mentioned before, the increase of proinflammatory cytokines and AMPs via the VDR breaks down the *M. tuberculosis* bacterial wall and the bacteria are destroyed by perforation of the cytoplasmic membrane

(77). Studies have suggested that VDR activation induces autophagy in TB-infected macrophages and destroys bacillus (78,79). It has also been suggested that vitamin D creates an anti-inflammatory response by increasing the activity of Treg lymphocytes, limiting Th1 activity and regulating the expression of metalloproteinase encoding genes (80).

The relationship between VDR polymorphism and tuberculosis disease was investigated. Polymorphisms in the form of homozygous dominant (TT, BB, FF), heterozygous (Tt, Bb, Ff) or homozygous recessive (tt, bb, ff) form have been associated with tuberculosis. Studies have shown that FokI-ff polymorphism is associated with pulmonary tuberculosis, TaqI-tt polymorphism reduces the likelihood of active disease, while non-tt gene polymorphism predisposes to tuberculosis (81-84). Those with Tt or FF polymorphism had faster bacillus negativity in sputum than those with non-TT or FF polymorphism. Inverse association with BsmI-bb genotype and little correlation with TaqI and ApaI polymorphism were detected in other studies on this subject (85).

These findings raise the question of whether vitamin D contributes to prophylaxis and treatment. It is also thought that vitamin D supplementation may reduce the risk of latent tuberculosis progression to tuberculosis disease. In a randomized controlled trial of 8,851 children with latent tuberculosis, 14,000 units of vitamin D supplementation per week was compared with placebo. There was no significant reduction in progression to tuberculosis disease was detected (39). Several meta-analyses supporting each other concluded that vitamin D deficiency predisposes to active tuberculosis disease. (39,86). In an animal experiment in mice, when vitamin D and pyrazinamide were used together, it was observed that the reproduction of tuberculosis bacillus was interrupted (87). In addition, it does not contribute to the negativization *M. tuberculosis* bacillus in sputum compared with placebo (88,89). Existing studies have many limitations such as the small number of samples, data inaccuracies, and the presence of multiple factors affecting the sample. Therefore, further clinical trials should be conducted to elucidate these questions.

Vitamin D and Brucellosis

Another bacterial infection whose relationship with vitamin D has been investigated is brucellosis. In a study reported from Iran, vitamin D levels were not different from the control group (90). In a study, including 86 brucellosis and 86 control cases, vitamin D and soluble VDR levels were found to be lower in patients with brucellosis than healthy groups (91). Although there are not as many studies as tuberculosis, it suggests that vitamin D plays a role in the pathogenesis of brucellosis like tuberculosis.

Conclusion

Increasing publications reveal that vitamin D is active in the immune system throughout life and is physiologically important in protection from infections. As a result of the interaction between vitamin D and innate immune system signaling mechanisms; cytokine, antimicrobial peptide and pattern recognition receptors are produced. On the other hand, vitamin D has a suppressive effect on the adaptive immune response. The protective role of vitamin D against infections has been revealed by several clinical trials. Yet, more clinical research, including randomized and controlled clinical trials and large-scale cohort investigations,are essential to further understand the therapeutic mechanisms of vitamin D and infections.

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VITAMIN D AND MALIGNANCIES

GOKÇE PINAR REİS*

Vitamin D

Most of the vitamin D needed by humans is produced by adequate exposure to the sun (1-3). Endogenous synthesis of vitamin D begins with the active conversion of the precursor 7-dehydrocholesterol to previtamin D3 as a result of exposure of the epidermis to ultraviolet (UV) radiation (especially UVB) in sunlight. Previtamin D3 is transferred to the liver by blood and hydroxylated to 25-dehydroxyvitamin D. This form comes to the kidney through the blood and is hydroxylated to $1,25(OH)_2D$ (calcitriol), known as the active vitamin D form. It was reported that exposure to excessive sunlight does not cause vitamin D intoxicity (4-8). Vitamin D intake through diet is limited. The highest amount of vitamin D is found in oily fish (salmon, sardines, mackerel) and egg yolks. Dietary vitamin D forms are absorbed in the small intestine, are combined with chylomicrons and participate in the venous circulation through the lymphatic system. Vitamin D2 and vitamin D3, which are made endogenously or taken through diet, are stored in the body's fat cells and released into the circulation in case of need (9). Although the exact excretion of vitamin D and its metabolites is not known, it is thought to be primarily excreted in feces and bile salts, and a small amount is excreted in the urine. Vitamin D taken in the diet and synthesized in the skin is inactive. Vitamin D and its metabolites circulate in the circulatory system bound to the vitamin D binding protein. These proteins show high affinity for 25(OH) D, 1α, $25(OH)_2D$ and $24,25(OH)_2D$ in circulation. Vitamin D and its metabolites, which are transported to the liver by binding to the vitamin D binding protein, are hydroxylated by the 25 hydroxylase enzyme and converted to 25-hydroxyvitamin D, a 25(OH)D form (9). In contrast with 25-hydroxylation, only CYP27B1 enzyme has 1α-hydroxylase activity. Mutations in this gene were shown to underlie pseudovitamin D deficiency due to insufficient production of $1,25(OH)_2D$. There is a highly conserved homology between YP27B1 and other mitochondrial enzymes participating in vitamin D metabolism (10). Although the main source of CYP27A1 and CYP24A1 is the kidney, enzyme is also expressed in some other tissues, and the regulations of renal and extrarenal CYP27B1 are different. Examples of these tissues are epithelial cells of skin, breast, lungs, intestine, prostrate, parathyroid gland, pancreatic islets, endocrine glands including thyroid, testicles, ovaries and placenta, osteoblasts, dendritic cells, and T and B lymphocytes, chondrocytes and various tumors (10-12).

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Vitamin D receptor

The vitamin D receptor (VDR) expression was demonstrated in various tissues such as fat, liver, lung, muscle, ovary, prostate, brain, colon, pancreas, stomach, thyroid and skin (13,14). Upon entering the cell, $1,25(OH)_2D$ binds to the vitamin D receptor in nucleus (15). In addition to being present in many tissues, $1,25(OH)_2D$ receptor is also present in benign hyperplastic and malignant epithelial and fibroblastic tissues. $1,25(OH)_2D$ directly or indirectly regulates about two hundred genes including those responsible for insulin and renin production, cytokine release, proliferation and growth of cardiomyocytes and vascular smooth muscle cells (14).

Vitamin D and malignancies

The anticarcinogenic role of vitamin D has been well established. The experimental and epidemiological studies revealed that vitamin D has a protective role against the development of colorectal, breast, ovarian, prostate cancers and others.

The anticancer effects of active vitamin D have been investigated for many years. In vitro cell culture and in vivo animal studies showed that active vitamin promotes cell differentiation, inhibits cancer cell proliferation, and has antiangiogenic and anti-inflammatory properties. In laboratory studies, active vitamin D was shown to inhibit the cancer cell growth by binding to VDR and regulating various genes responsible for cell proliferation (6-8). Active vitamin D in keratinocytes was found to improve the repair of DNA damage caused by ultraviolet and reduce apoptosis.

Vitamin D receptor and 1-a hydroxylase enzyme expression were demonstrated in colorectal tissue (4). It was suggested that active vitamin D can affect colon cancer progression and development by increasing intracellular calcium flow and directly affecting calcium balance (16). It has been shown that individuals with low vitamin D value have results such as larger tumor size and higher lymph node metastasis, and they metastasize significantly. (17,18). It was stated in the previous studies that low vitamin D level in cancer patients is a risk factor that can be remedied with necessary supplements or ultraviolet rays.

Anti-proliferative effects: The main mechanism of this effect of vitamin D takes place by its interrupting the progression of the cell cycle (19). The mechanism of the cell cycle, which takes place in four stages, is carried out by cyclin-dependent kinases, involved in specific tasks such as the transcription of the cell, and their inhibitors (20). Vitamin D provides its effects by suppressing the activity of kinases or inducing kinase inhibitors (2).

Anti-inflammatory effects: Vitamin D mediates its effects by acting on the metabolism of prostaglandins (21), which are known to be involved in cancer development and progression. These effects were reported to be through down-regulation of NAD+ dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) gene and up-regulation of cyclooxygenase-2 (COX-2) expression (2), thereby decreasing prostaglandin levels.

Proapoptotic affects: Activation of the apoptosis pathway in tumorous cells is the main mechanism for the destruction of tumorous cells with chemotherapeutic drugs (22,23). Vitamin D induces apoptosis in many cancer cells by suppressing the expression of anti-apoptosis proteins or by mediating the expression of apoptosis-inducing proteins.

Vitamin D in some specific cancers

Prostate Cancer

Today, with the prolongation of the average human lifespan, the incidence of prostate cancer has increased. In some studies, prostate cancer was shown to be more common in winter. This was associated with less sun exposure in winter and insufficient vitamin D synthesis. In addition, single nucleotide polymorphisms of vitamin D receptors were thought to be linked to the prognosis and risk factors of prostate cancer (16).

Breast Cancer

Vitamin D is a steroid hormone that affects the functions of numerous organs in the human body, such as the heart, skeletal system, lungs, intestines, and mammary glands. Its effect on mammary gland development is mediated by the VDR. Recent epidemiological research has reported associations between vitamin D deficiency and various critical key adverse health outcomes, particularly cardiovascular and cancer-associated morbidity and mortality (3). Vitamin D has also been shown to exhibit VDR-mediated anti-proliferative, pro-apoptotic, and pro-differentiating activities in various malignant cells (4,5) More than 90% of the vitamin D in the body is produced endogenously in the skin. This is subsequently subjected to two hydroxylation reactions to yield the active hormonal form 1,25(OH)2D. The half-life of 1,25(OH)2D is approximately 6 h, while that of 25(OH) D is nearly 1000 times higher compared to the active form (6). A number of longitudinal studies regarding serum 25(OH)D and multiple cancer risks have reported that while 25(OH)D concentrations exhibit an inverse correlation with the incidence of colorectal cancer (8–12), no association has been determined with the incidence of either prostate cancer or breast cancer (BC) (10,13–15).

The question of whether vitamin D inhibits BC development has attracted growing interest in recent years. Lower serum 25(OH)D levels have been determined in patients with BC compared to healthy controls, and have also been linked to worse prognostic outcomes. The known anticarcinogenic capacity of vitamin D derives from its active, hormonal form, 1,25(OH)2D. Experimental research has shown that 1,25(OH)2D can also be synthesized in a local manner from 25(OH)D present in other tissues, such as mammary, intestinal, and pulmonary tissues. VDR is present in nearly all human tissues and organs and activated by 1,25(OH)D. Due to its responsibility for the transcription of large numbers of genes associated with cell proliferation, differentiation, metastasis, and apoptosis, it is also the subject of significant interest in the context of numerous cancers, including BC. Circulatory levels of the biologically active metabolite 1,25(OH)2D are strictly regulated. Due to the close relationship between circulating 25(OH) and 1,25(OH)2D in breast tissue, and the fact that circulating 1,25(OH)2D is subjected to homeostatic control, studies have suggested that circulating 25(OH)D is may be relevant to the development of BC (24).

Novel options are currently being sought for the treatment of BC, with its very high incidence among women. Cathelicidin, which exhibits an apoptotic effect against cancer cells, is less secreted in estrogen receptor negative and HER2-receptor positive breast tumors, which are among the highest risk subtypes (24). Active vitamin D exhibits an anticancer effect in BC by increasing levels of cathelicidin.

Thyroid Cancer

The incidence of thyroid cancer has been increasing in recent years. There are studies reporting that vitamin D prevents the proliferation and growth of tumor cells without damaging normal cells, thereby positively affecting the prognosis. High levels of vitamin D in some metastatic thyroid cancers have made vitamin D use controversial in terms of its safety in thyroid cancer (25).

Melanoma

It is very difficult to investigate the effect of vitamin D in melanoma because the skin is protected from sunlight in patients with melanoma. The increase in the incidence of melanoma in countries where the sun exposure is less common has been associated with this condition (24).

Pancreatic Cancer

Pancreatic cancer is one of the cancer types with the highest mortality. Most patients are diagnosed at an advanced stage. Ergocalciferol plays an active role in the proliferation of cells, programmed cell death and tumor formation. Calcidiol helps in stopping the development of tumors in pancreatic cancer. In order to prevent high calcium levels in the blood, it is recommended to use cholecalciferol as the precursor of the hormone together with artificial forms of vitamin D during the cancer treatment. With this treatment, high levels of calcium in the blood were prevented (26,27)

In a study with a group of men who smoked, it was observed that although vitamin D levels were high, the frequency of pancreatic cancer increased. Although smoking was indicated to be the culprit, the association of vitamin D levels with the pancreatic cancer incidence has not been fully elucidated (27).

Gastrointestinal tract cancers

Calcitriol is thought to slow tumor growth, especially in squamous cell tumors originating from the oral mucosa. This was hypothesized to be through the toxic effect to malignant cells and stimulation of programmed cell deaths. For this reason, regular control and supplementation of vitamin D levels is recommended. Since vitamin D is very low in foods, it should be supplemented and more sunlight exposure should be ensured (23).

In countries with more sunshine, the incidence of oral cancers is lower. This applies not only to oral cancer, but also to laryngeal and esophageal cancers. Low vitamin D receptor gene expression in people diagnosed with squamous cell carcinoma in the mouth adversely affects the prognosis of the disease (28).

Colorectal Cancer

Colorectal cancer (CRC) is not only the third most common form of cancer, but also the second leading cause of cancer-related deaths worldwide. A close association exists between the etiology and prognosis of CRC and dietary and lifestyle factors, not least vitamin D (2,29). In addition to its key role in bone health, research indicates that vitamin D performs numerous anticancer functions, including reducing inflammation, inhibiting cellular proliferation and angiogenesis, and promoting cellular differentiation and apoptosis (3-5). Both normal and neoplastic colon cells are capable of manufacturing the biologically active form of vitamin D from the major circulating form, 25(OH)D, and researchers have therefore suggested vitamin D may be directly involved in regulating the growth of both colon cell types. However, epidemiological evidence concerning the association between vitamin D status and CRC risks and prognosis remains inconclusive.

Advanced age, intestinal diseases due to inflammation, malnutrition, diabetes, and genetics are the principal factors implicated in CRC (24,28).

According to a study conducted in the USA in the 1980s, individuals with CRC tended to live in areas receiving less sunlight. Based on this finding, vitamin D was considered to exhibit a preventive effect on CRC (29,30). It does this by increasing apoptosis in tumor cells without damaging healthy cells.

In contrast to normal colonic epithelial cells, which possess low vitamin D receptor levels, polyps and well-differentiated tumor cells have much higher vitamin D receptor levels. VDR and 1,25(OH)2D lower the proliferative effect in CRC cells while increasing differentiation and changing the transcription of genes that inhibit carcinogenesis (30).

Lung cancer

Lung cancer is the most frequent malignancy observed in the general population and the leading global cause of cancer-related mortality. However, wide variations, depending on the geographical region involved, occur in the onset and incidence of the disease, and also in associated mortality rates (3). The exact etiopathogenesis of lung cancer is currently uncertain, although studies have implicated various environmental, genetic, and epigenetic factors (4–9). Smoking, an environmental factor, is particularly implicated in the development of lung cancer (10,11). Other predisposing factors include air pollution, infections, environmental carcinogens, and nutritional deficiencies. Genetic risk factors have also been linked to susceptibility to lung cancer, with extensive genome-wide association studies (GWAS) having identified numerous genetic risk loci linked to an increased risk of lung cancer susceptibility (14,15).

Research has shown that vitamin D is capable of regulating various different signaling pathways in tumor cells in vitro. These include mitogen activated protein kinase (MAPK) signaling in breast cancer and epidermal growth factor receptor (EGFR) signaling in BC and colon cancer (30,31). Studies have also detected mutations in various genes encoding molecules with roles in the modulation of the proliferation and differentiation of lung tumor cells (31). Vitamin D and VDR have also been implicated in the modulation of various of these oncogene signaling pathways. For example, EGFR gene is particularly

commonly mutated in NSCLC, resulting in upregulated transcriptional levels of the relevant protein in tumor cells. EGFR signaling has been linked to a range of cancerous behaviors, such as angiogenesis, proliferation, migration, invasion, and tumor cell survival (38). VDR is capable of suppressing the transcriptional function of EGFR in BC cells. Vitamin D therapy has also been observed to promote that suppressive activity. Vitamin D treatment applied to the EGFR mutant lung cancer cells has also been shown to result in dose-dependent inhibition of clonogenic growth. It has been shown to be capable of inhibiting the mitogenic signaling of EGFR in ovarian tumor cells, resulting in the induction of cell cycle arrest (31). Despite the limited nature of data concerning the effect of vitamin D on the EGFR signaling pathway in lung tumor cells, such studies suggest that vitamin D /VDR therapy may also be beneficial in lung cancer through modulation of EGFR signaling.

Renal cell cancer

In a study on the association between renal cell carcinoma and VDR, it was found that while VDR and metabolic pathway enzymes are expressed in normal kidney tissue, they are lost in malignant transformation (29).

Childhood age cancers

Every year, approximately 400,000 new cancer diagnoses are made in children between the ages of 0-18 throughout the world. Significant advances have been made in the treatment of cancer after the recent developments. However, cancer treatment is still a major problem in developing countries (30,31).

Nephroblastoma (Wilms' Tumor)

Healthy kidneys were reported to have a VDR expression pattern similar to that of Wilms' tumor. Tubular epithelial cells of intact kidneys and nephroblastoma have typical expression of VDR (31). Thus, VDR presence could be evident in early differentiation stage, and thus nephroblasts also share this characteristic. It was reported that good nephroblastoma prognosis was associated with pattern of VDR in mature renal tissue and the signal pathway associated with the maturation.

Hepatoblastoma

Some studies found that only some cell types in the liver (sinusoidal endothelial, stellate and Kupffer cells) have considerable VDR expression and other cells do not (32). However, other studies found that healthy liver tissue has undetectable VDR level.

Neuroblastoma, Ganglioneuroma

In study on neuronal development, cell differentiation was achieved by overexpression of VDR. The antiproliferative effect of VDR in neuroblastoma cells was described. Based on the findings of this study, it was concluded that vitamin D and its analogues can be used in the treatment of neuroblastoma (33).

Role of vitamin D in prevention and treatment of cancers

Vitamin D deficiency is a widely accepted health problem in the world. VDR whose importance has been better understood recently is thought to be associated with cancer. Previous studies indicated an association between high serum vitamin D levels and lower risk of developing cancer. Vitamin D may help in cancer prevention by regulating cell growth, preventing infection, boosting the immune system, and supporting DNA repair system (34-37) (Figure 1).

The vitamin D has a high-affinity nuclear receptor and carries properties found in steroid hormone receptors. By binding to the $1,25(OH)_2D$ VDR, it activates the retinoid X receptor (RXR). This activation process facilitates the transfer of RXR-VDR-ligand complex from cytoplasm to nucleus (4, 37). Vitamin D receptor exerts its antitumor effect through its active metabolite, $1,25(OH)_2D$. This activation of VDR is associated with the regulation of numerous genes taking part in cellular functions and cellular processes. Therefore, low vitamin D levels were found to be associated with human cancers (38,39).

$1,25(OH)_2D$ exerts its major effect by inhibiting G1/S of cell cycle phases. Its inhibitory effect on tumor invasion and metastasis, on the other hand, is by inhibiting serine proteinase, metalloproteinase and angiogenesis.

Although cell studies showed 1000 times higher binding of 1,25-(OH)2D3 to VDR than that of 25-(OH)Vit D_2, population-based studies suggested that the serum metabolite associated with cancer risk is 25(OH)Vit D. The reason for this is that $1,25(OH)_2D$ is synthesized locally via the CYP27b1 enzyme outside the kidney. This enzyme is found at a low rate in the kidney but it is abundant in skin, colon, brain, placenta, prostate epithelial cell, pancreas and adrenal medulla, lymph nodes and MCF-7 breast cancer cells (40-42).

While normal cells have growth inhibiting response on 25(OH) vitamin D treatment, this response is not evident in cancer cells with reduced CYP27b1 activity. Expression of CYP27b1 is not found in human metastatic colon tumor cells (43). There are two conditions that determine the dose relationship of vitamin D with its cancer-protective role. First, the cancer-protective effect of high vitamin D values depends on the CYP27b1 values in the tumor cell which is possibly to develop. If the CYP27b1 value is low, higher doses of Vit D are needed to have a protective effect. Second, high Vit D values are needed in case of increased CYP24 activity and/or decreased VDR values or signaling (44). While Vit D receptor density increases in hyperplastic polyps and early stage tumor cells, it decreases in late stage tumors (45).

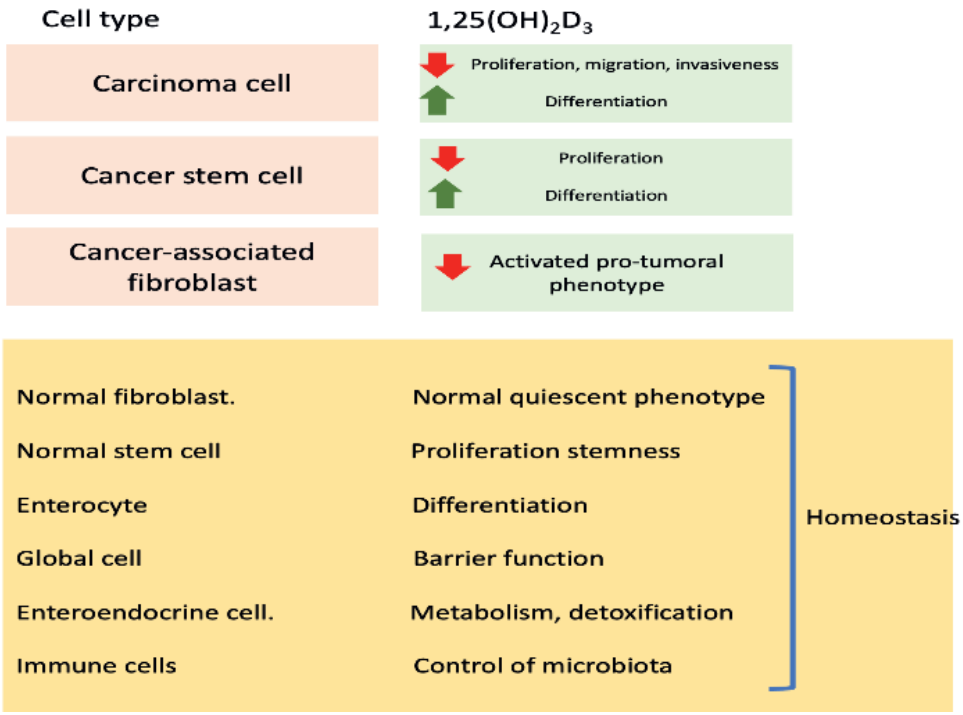

Figure 1: *Vitamin D may help in cancer prevention by regulating cell growth, preventing infection, boosting the immune system, and supporting DNA repair system*

Vitamin D regulates many different proto-oncogenes and tumor suppressing genes. The most prominent of them, transforming growth factor beta (TGF-β), is located in the target pathway of the growth factor and inhibits epithelial cell proliferation. Vitamin D augments the negative regulation of interferon-gamma (IFN-γ) and interleukin 12 (IL-12). Vit D3 derivatives increase the expression of P21 and P27 in many cancer cells. The $1,25(OH)_2D$ prevents cancer formation by inhibiting proto-oncogenes c-Fos and c-Myc. Besides, Vit D decreases BCL-2 expression, which is responsible for programmed cell death, and increases the expression of BAX gene. $1,25(OH)_2D$ induces apoptosis in breast, colon, and glioma cells (46).

A daily intake of 700-800 IU Vit D is required for a remarkable reduction in the risk of colon cancer. The protective effect may occur with higher Vit D levels along with exposure to sunlight. In prospective studies, an inverse relationship was found between circulating 25(OH) Vit D level and rectal cancer, colorectal cancer and adenoma (47-49). In a study conducted by the World Health Organization, an inverse relationship was observed between the Vit D plasma level and the risk of CRC. Although the relationship between CRC and Vit D is not certain, at least 30 ng/mL Vit D level is required for a healthy state.

Conclusion

Cancer is one of the most investigated diseases in recent years. The reason for this is that the treatment has not been fully elucidated in parallel with the increasing number of patients. There are many new emerging treatments. Treatment can be strengthened by giving vitamin D supplementation to cancer patients. Further studies are needed to reach clearer conclusions about the relationship between vitamin D and cancer.

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VITAMIN D IN MENTAL HEALTH

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Vitamin D is the main regulator of calcium and bone homeostasis. Vitamin D shows its biological effects through the vitamin D receptor (VDR) (1). This vitamin has effects on skeletal muscle (2), cardiovascular function (3), immune cells (4) as well as neurons and glial cells. Due to its effect on glial cells and neurons, it can influenced various functions in the central nervous system such as growth, development and cognition (5). This means that vitamin D has an effect on mental health. For this reason, there have been studies investigating the relationship of vitamin D with other diseases, as well as studies examining its relationship with mental health.

While the relationship between vitamin D and mental health was investigated in experimental studies, the benefit of vitamin D supplementation was also investigated in clinical studies. A systematic review by Guzek et al (6) analyzing the effects of vitamin D supplementation on mental health in healthy adults did not confirm the positive effect of vitamin D supplementation on mental health, either. Vitamin D supplementation has been supported by some studies that mainly involve depression rather than other mental health issues (6). Although findings from observational studies in children and adolescents appear to suggest a beneficial role for vitamin D in a variety of mental health conditions, randomized controlled trials are urgently needed to confirm these observations (7). In this section, a summary was created by examining the articles aiming to determine the relationship between vitamin D and mental health. Studies in both adults and children were included.

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Vitamin D and Central Nervous System

Low vitamin D status is associated with a number of adverse neuropsychiatric outcomes such as depression, schizophrenia, Alzheimer's disease, and autism (8,9). VDR and vitamin D metabolizing enzymes are expressed in the human brain, particularly in large (possibly dopaminergic) neurons in the hypothalamus and substantia nigra (10). Vitamin D potentially regulates calcium transitions and neuronal development in the brain; It also provides protection against reactive oxygen species (11). Vitamin D is involved in the expression of nerve growth factor (NGF), neurotrophin 3 (NT-3), NT-4, and glial cell line-derived neurotrophic factor (GDNF) (12), but not directly in brain-derived expression neurotrophic factor (BDNF) (13). This suggests that vitamin D deficiency makes the brain more vulnerable to secondary side effects (14). 1,25(OH)2D3 can also modulate differentiation and maturation of dopaminergic neurons and affect brain serotonin concentrations, e.g. via induction of brain tryptophan hydroxylase (TPH2) mRNA, which encodes the rate-limiting enzyme in serotonin synthesis (15,16). In addition, 1,25(OH)2D3 suppresses leptin mRNA levels in mouse adipocytes and induces leptin mRNA in human glioblastoma cells, thus supporting the role of vitamin D in action control and eating behavior (14).

Vitamin D deficiency has also been shown to reduce peripheral nerve fiber density, and vitamin D has been recognized as a potential therapeutic in spinal cord repair. In addition to study showing that vitamin D is neuroprotective through the production of neurotrophins, there are studies showing the regulatory effects of vitamin D on calcium in the brain and its neuroprotective effects against reactive oxygen species (ROS) and reducing stress as well as inflammation. Vitamin D increases antioxidants such as glutathione and cytochrome C to mediate antioxidant effects in neurons and brain in cell cultures. (17). According to experimental studies, vitamin D deficiency in the adult brain, together with extracellular calcium, increases ROS, causing disturbances in the secretion of gamma-aminobutyric acid (GABA) and glutamate. More importantly, reintroducing vitamin D into the diet normalizes all deficiencies (18). Another study found that when cholecalciferol was added to hypoxia-exposed cultured primary neurons, cholecalciferol was anti-apoptotic and preserved calcium signaling, although this effect was reversed at high doses. Upregulation of hypoxia-induced factor (HIF)-1a and/or BDNF of vitamin D has also been considered as possible protective mechanisms (17). In another result found in experimental studies, in a lead-induced neurotoxicity and oxidative stress model, reductions in the antioxidant molecules GSH, superoxide dismutase (SOD) and catalase and increased ROS production were observed in the rat cortex. Cholecalciferol likely reverses these changes via the Nrf2 and/or NF-kB mechanism (19)

A neurological disorder commonly associated with ROS-mediated brain injury is Parkinson's disease (PD). PD-related motor deficits are believed to result from selectively dying dopamine neurons in the substantia nigra and associated decreased dopamine release in the dorsal striatum. This has been modeled in animals by intracranial administration 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, the primary modulator of NADPH oxidase activity, and restored some motor functions (20). Experimental studies in PD showed that vitamin D prevents characteristic losses in dopamine synthetic enzymes and transporters, protects motor function and reduces lipid peroxidation. It has been shown that administration of calcitriol improves motor deficits, reduces dopamine neuron toxicity, and decreases ROS production (17).

ROS production is also linked to other degenerative processes such as Alzheimer's disease. Animal models for Alzheimer's disease often use genetic models that overexpress Taurine or amyloid (Aβ) proteins, which are closely linked to disease pathology. In a model with vitamin D deficiency, SOD1 downregulated glutathione peroxidase 4, contributing to increased expression of IL-1β, IL-6, and TNFα, and increased Aβ production and Tau phosphorylation exacerbated ROS production (21).

Glucocorticoid release is the classical endocrine response to stress and prolonged exposure induces neuronal shrinkage followed by cell death. The effects of 1,25(OH)2D3 and glucocorticoids in the body can be considered antagonistic. Similarly, in the brain, 1,25(OH)2D3 antagonizes the effects of dexamethasone (a corticosterone agonist) on hippocampal neuron differentiation and glucocorticoid receptor function (17). In rats, chronic mild stress causes increased corticosteroids, inflammatory markers, and decreased anti-oxidant enzymes SOD and glutathione peroxidase and catalase in the hippocampus and prefrontal cortex. Concomitant cholecalciferol treatment reverses these stress-mediated effects (22).

Chronic unpredictable stress creates behavioral despair and increases inactivity. Cholecalciferol treatment reduces this stress-induced immobility. In this case, it is thought that vitamin D acts by increasing the synthesis of serotonin. Other studies that chronically administer corticosterone replicate cholecalciferol's reversal of these stress-induced behaviors and suggest that changes in glucocorticoid signaling in the brain or reductions in stress-induced ROS production in the brain are protective mechanisms. Cholecalciferol supplementation also reduces serum corticosterone/ACTH levels and increases BDNF and NT-3/NT-4 levels in the hippocampus (23).

Hypervitaminosis D is rare in humans and is usually caused by excessive vitamin D supplementation or diseases such as sarcoidosis that produce excess 1,25(OH)2D3 due to active macrophages. Although not focusing on neurological conditions, an earlier review concluded: "There is accumulating evidence that both high and low serum calcidiol concentrations are associated with an increased risk of chronic disease". Hypervitaminosis D always results in hypercalcemia, which can be toxic to brain function. Animal studies have shown that high cholecalciferol intake for four consecutive days reduces brain wave activity by 25,000 IU/kg. In the accelerated aging mouse phenotype, it showed a progressive increase in serum 25OHD3, which is associated with decreased cognitive function and increased capillary permeability (24).

Due to all these effects, many studies have been conducted on embryonic or neonatal vitamin D deficiency, schizophrenia, autism, and more recently attention deficit hyperac-

tivity disorder (ADHD). There are also numerous studies suggesting that adult vitamin D deficiency may be associated with certain neurodegenerative conditions. In this section, the effects of vitamin D on schizophrenia, autism spectrum diseases (ASD), ADHD, cognitive disorders and depression will be explained.

Vitamin D and Cognitive Function

Cognition is understood as aspects related to knowledge, learning and understanding, as well as the ability to develop these functions. It consists of a set of skills and cognitive domains that act in synergy for the greater development and maintenance of intellectual, communicative and social improvements. Cognitive function is the result of several complex systems involving many local neural circuits (25). Vitamin D may exert its effect on neurocognition through a number of mechanisms, including induction of neuroprotection, modulation of oxidative stress, regulation of calcium homeostasis, and inhibition of inflammatory processes (26).

The behavior of rats and mice exposed to vitamin D deficiency has been observed in various experimental conditions. In rats, developmental vitamin D deficiency resulted in impaired latent inhibition, reflecting the animals' attentional function, but pre-stimulus inhibition and working memory were normal. However, these animals showed hyperlocomotion and increased exploratory activity, which may improve some types of cognitive function or be a confounding factor in various behavioral tests used to assess cognitive function. What this means is that developmental vitamin D deficiency can affect various components of cognition differently. Animal models of vitamin D deficiency have shown that low serum calcidiol induces behavioral changes, suggesting that calcitriol is involved in a number of important neuronal and glial processes. Interestingly, Vitamin D deficiency did not cause cognitive impairment in rodents as expected, but vitamin D supplementation improved some cognitive processes (27).

In a birth cohort study conducted in Australia, the presence of maternal Vitamin D deficiency was associated with neurocognitive difficulties in 10-year-old children (28), but in a similar study in Denmark, the school achievement of children of Vitamin D deficient mothers was not statistically different from other children. However, in the same study, a significant relationship was observed between maternal vitamin D status and offspring depression (29). A recently published meta-analysis and systematic review found only a borderline positive association between 25(OH)D levels and cognitive development, but a strong inverse association between 25(OH)D levels in mothers and risk of ADHD and autism in offspring. Therefore, they concluded that higher prenatal 25(OH)D concentrations may reduce the risk of developing ADHD and autism-related features (30).

Two large-scale prospective studies involving the elderly suggested that low vitamin D levels are a predisposing factor for cognitive decline (27). Licher et al. (31) found that subjects with vitamin D < 25 nmol/L (defined as deficiency) had an increased risk of developing dementia compared to subjects with 50 nmol/L (adequacy) without reaching statistical significance. However, lower vitamin D concentrations have been associated with a higher risk of dementia and Alzheimer's disease (AD). AD is a progressive neurodegenerative syndrome, characterized by deterioration of cognitive function and behavioral

alterations (31). A large body of evidence confirms the association between low serum vitamin D levels and cognitive impairment, especially in older people. The biological mechanism underlying this link includes the role of vitamin D in processing and clearance of β amyloid, the accumulation of which is known to cause and trigger AD. However, studies addressing the relationship between cognitive function decline and vitamin D deficiency may present critical limitations. First, a set of cognitive tests that cover general cognitive function and a few specific domain processes can be difficult to administer; therefore, studies evaluating cognitive function using a single-domain outcome measure should be taken with suspicion. Second, most studies have a cross-sectional design that sharply limits their strength due to the concern of reverse causality and the presence of confounding factors. Third, interventional studies are few and very heterogeneous, so findings should be interpreted with caution (26).

While there are studies showing the relationship between vitamin D serum level and AD, there are studies reporting that vitamin D supplementation has no effect on the development of AD. It can be argued that vitamin D supplementation has failed to prevent cognitive decline in the healthy elderly population, and there is insufficient evidence that vitamin D can improve cognition in elderly people with AD (26).

Vitamin D and Depression

Insufficient serum 25(OH)D levels are common among humans and can cause mood disorders. A growing body of literature also supports the link between vitamin D and the pathophysiology of depression (9,32,33). The proof of this was in three ways; first, the presence of lower serum vitamin D levels in depressed individuals compared to controls (9,34,35), secondly, the presence of vitamin D receptors in various parts of the brain's limbic system, cerebellum, and cortex that control emotions and behaviors (36,37); and third, the important modulatory role of vitamin D in regulating the immune inflammatory pathways found to be related to the pathophysiology of depression (10). VDR and specific cytochrome P450 enzymes responsible for converting vitamin D to its active form have been found in brain regions and brain cells associated with the pathophysiology of depression (38). On the other hand, vitamin D appears to be a mediator in the sleep-wake cycle, as vitamin D deficiency is associated with poor sleep quality and shorter sleep duration. Melatonin is produced through the metabolism of serotonin, a hormone that regulates the circadian rhythm. The vitamin D receptor was found in postmortem human brain samples in areas related to sleep patterns and in brain regions with the highest activity of the enzyme 1-α-hydroxylase, responsible for synthesizing 1,25-dihydroxycholecalciferol. In conclusion, it is reasonable to consider that chronic vitamin D deficiency and sleep-wake cycle dysfunction caused by isolation and social isolation may play an important role as modulators of magnified depressive symptomatology and in the pathogenesis of major depressive disorder (39).

Clinical evidence also reveals that low serum 25(OH)D levels are closely associated with depressive symptoms. Vitamin D deficiency or insufficiency is associated with depression-like symptoms such as low mood, muscle weakness, and feelings of fatigue. Individuals with stress, anxiety or depression seem to have lower serum 25(OH)D levels

(42,43). Seasonal affective disorder can also be explained by low serum 25(OH)D levels due to less exposure to sunlight during winter, especially in northern latitudes (41). It remains unclear whether vitamin D supplementation will reduce depression in depressed patients. Mikola et al. (44) found that vitamin D had a positive effect on depressive symptoms in adults in the general and clinical population in a meta-analysis of 7 randomized controlled trials in adults with major depressive disorder. Meta-analyses pointed to an unresolved conclusion about the effectiveness of vitamin D in reducing the severity of depression in depressed patients. In addition, some factors that may reduce this effectiveness, such as age, concomitant physical diseases and vitamin D administration methods, were not investigated in studies (42).

Heterogeneous data suggested that vitamin D supplements have moderate efficacy in reducing depressive symptoms. Adults with depression may respond better to these supplements than depressed children and adolescents. Vitamin D supplements administered as bolus doses (intermittent high doses by mouth or a single high intramuscular dose) have been shown to be more effective than those administered orally daily. Patients with more severe depression may respond better than those with less severe depression. However, the certainty of vitamin D efficacy is very low because the available data appear to be extremely heterogeneous, raising concerns about publication bias (42).

Although not yet fully understood, vitamin D supplements may alleviate depressive symptoms through a variety of mechanisms. First, vitamin D can modulate neurotrophic agents. Serum 1,25(OH)2D3, which can cross the blood-brain barrier and bind to vitamin D receptors distributed in brain regions, can increase the expression of brain-derived neurotrophic factor required for neurogenesis (42). Second, vitamin D can directly affect depression by affecting neurotransmitters. Vitamin D is involved in dopaminergic neuron development and may increase serum dopamine levels. Third, vitamin D can prevent abnormal activation of the immune system related to the pathophysiology of depression, by leading to a reduction in inflammatory cytokines such as interleukin 1, interleukin 6 and interleukin 8. While these lines of molecular evidence seem to support our clinical findings of vitamin D efficacy, there are some missing links between these two extremes. In some studies, no relationship was found between vitamin D activity and serum 25(OH) D levels at baseline and after supplementation. However, it suggests that the relationship between vitamin D and depression is complex and cannot be fully explained by the available evidence (47).

In conclusion, limited evidence suggests that vitamin D supplements are considered beneficial, have moderate efficacy for depressive symptoms, and may be a complementary option for depressed patients. Adults with severe depression may respond well to bolus doses of vitamin D supplementation. Patients with more severe depression tended to respond better than those with less severe depression. Although vitamin D supplements for depression are said to be safe for depressed patients, there are no clear studies investigating side effects in patients receiving vitamin D. Depressive adults may respond better to vitamin D supplements than depressive children and adolescents. The precision of this activity is very low and future studies are needed in this area (42).

Vitamin D and Schizophrenia

Schizophrenia is classified as a neurodevelopmental condition based on epidemiological and neuropathological evidence. Many studies have reported that schizophrenia is more common in people born in the spring and winter, living in high latitudes, and living in metropolitan areas as children. Additionally, discrete findings showing an increased risk of schizophrenia in offspring of dark-skinned immigrants who settle in cold regions may be attributed to vitamin D deficiency during early life and pregnancy, as dark skin requires greater sunlight exposure to produce appropriate amounts (48).

A recent comprehensive analysis found that 70% of people with schizophrenia are vitamin D deficient. Vitamin D levels and cognitive function in the population have been associated with psychotic illness. However, it was determined that there was no causal relationship between neurocognitive performance and vitamin D levels. These findings suggest that vitamin D supplementation does not affect cognitive domains related to schizophrenia (49). According to the findings, vitamin D deficiency was linked to slower processing speed and verbal memory in people with psychotic illness. As mentioned earlier, processing speed in particular has been found to be a unique predictor of cognitive abnormalities in schizophrenia. However, no significant correlation was found between low vitamin D levels and verbal memory (50). It also revealed that low vitamin D levels were closely linked to impairment in all cognitive domains examined, including verbal fluency, attention, processing speed, and working memory. Some studies have revealed a strong correlation between vitamin D levels and neuropsychological function (48,51).

Vitamin D supplementation has been proven to marginally improve cognitive status but not affect other outcome measures. Researchers looked at the link between vitamin D levels and brain regions important for memory. Vitamin D level was strongly associated with hippocampus volume after adjusting for age and education. A comparative study found that vitamin D supplementation reduced the hippocampus volume of people with psychosis (48,52). Meta-analyses and systematic reviews, at the top of the evidence-based medicine hierarchy, have found no link between cognitive performance and vitamin D levels in people with schizophrenia. In conclusion, convincing evidence for the effect of vitamin D on the cognition of patients with schizophrenia is still pending (48).

Vitamin D and Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by two main areas: social interaction disorders and limited, repetitive patterns of behavior, interest, or activity. The prevalence of ASD has been increasing in the last 20 years. It is currently believed that ASD is driven by genes and may be triggered by environmental risk factors (53,54). Nutrients are important environmental factors (55). Compared with other childhood and adolescent mental health disorders, the most evidence has accumulated for an association between vitamin D deficiency and ASD in cross-sectional and longitudinal studies and uncontrolled studies. A negative association between serum 25(OH)D concentrations and ASD has been detected in 85% of the cross-sectional studies (12 out of 14). It has also been found to be negatively correlated

with ASD severity and 25(OH)D levels. All uncontrolled studies identified have found a positive effect of vitamin D supplementation on ASD symptoms (14).

Vitamin D treatment reduces the signs and symptoms of ASD. According to one study, children with a history of autism had significantly lower vitamin D levels than healthy children, and there was a strong link between autism severity and low blood vitamin D levels (56). Low blood vitamin D levels in children with ASD can result from a variety of causes, including inadequate vitamin D consumption, poor vitamin D absorption, and geographic and lifestyle variables, possibly due to inadequate sun exposure (48). In a study in China, serum 25(OH)D levels of children with ASD were lower than healthy children. Serum 25(OH)D levels were primarily associated with baseline symptoms in children with ASD, and individuals with relatively lower serum 25(OH)D levels exhibited worse autistic symptomatology (57).

Vitamin D deficiency can cause oxidative stress and mitochondrial malfunction in the brain of ASD patients (55). Glutamate and gamma-aminobutyric acid are two neurotransmitters that play an important role in synaptic transmission (GABA) in the brain. Glutamic acid decarboxylase 67 (GAD 67) and glutamic acid decarboxylase 65 (GAD 65) convert glutamate to GABA. Several studies have found that the brains of autistic children contain dysregulated glutamatergic and GABAergic neurotransmission. ASD brains have higher glutamate levels in the amygdala and hippocampus than controls. In autopsy studies, the levels of GAD 67 and GAD 65 in the brains of ASD patients were 48-60% lower than in the control group. Dentate cerebellar nuclei and Purkinje cells from the brains of ASD patients showed significant decreases in GAD 65 and 67 (58). Vitamin D has been shown to increase GABA production in many brain regions and upregulate GAD 65 and 67 in mice (59). Serotonin is a neurotransmitter involved in neurogenesis and neuronal differentiation during brain development. According to various studies, ASD brains have a dysfunctional serotonin system. According to studies using positron emission tomography, the brains of autistic people contain low amounts of serotonin transporters (60). Compared with controls, postmortem ASD brain studies reveal decreased density of serotonin receptors, particularly 5-HT2A and 5-HT1A. Unlike the brain, it is found in extraordinarily high amounts in the blood. Hyperserotonin has been described in 32% of autism spectrum disorder patients (54). Vitamin D can activate tryptophan hydroxylase 2 (TPH2), which acts as a serotonin synthesizer in the brain (61).

There is a clear link between maternal infection and ASD in offspring. ASD brains were discovered to be subject to inflammation, as evidenced by the activation of inflammatory cells in astrocytes and microglia in post-mortem studies (54). Various studies have proven that vitamin D induces an anti-inflammatory response in various tissues, including the brain. Vitamin D suppresses the proliferation of active B lymphocytes in autoimmune diseases by targeting the activation of the mitogen protein kinase phosphatase, which is important in the regulation of immune responses. Vitamin D suppresses the proliferation of active B lymphocytes in autoimmune diseases by targeting the activation of the mitogen protein kinase phosphatase, which is important in the regulation of immune responses (62). Vitamin D inhibits the growth of active B cells in autoimmune disorders, so vitamin D deficiency can affect the normal function of the immune system (54).

However, the causation remains unclear: On the one hand, vitamin D deficiency may be explained by behavioral symptoms of children with ASD, such as unusual preferences for certain types of food or dislike and insufficient exposure to sunlight. On the other hand, vitamin D deficiency may be a risk factor for impaired brain development due to its dubious effect on neuronal growth factors, neural transmitters, immunological factors, and myelination. In conclusion, the biological effect of vitamin D on the etiology of ASD is still an unanswered question (14).

Vitamin D and Attention Deficit Hyperactivity Disorder

Impulsivity, hyperactivity, and inattention are common symptoms of ADHD, a neurodevelopmental condition. ADHD can be divided into three subtypes: inattentive, hyperactive, impulsive, and compound. For a diagnosis of ADHD to be made, at least six symptoms must be present for at least six months. ADHD is generally thought of as a childhood condition that presents after the age of seven, with symptoms that interfere with functioning in two or more settings, such as home and school. ADHD, on the other hand, can persist into adulthood, with inattention being more common than hyperactivity (48).

Vitamin D adequacy plays a role in prefrontal cortex and hippocampal synchronization. Vitamin D-deficient animals had a significant reduction in the volume of brain regions and poor spatial learning ability (62). One of the critical neuropsychological aspects of children with ADHD is impairment in spatial learning, which is sometimes linked to what is known as attention-like "working memory" (48).

Decreased dopamine activity is one of the features of ADHD in children and adults (63). There is evidence that ADHD may occur as a result of decreased serotonin levels in the brain caused by vitamin D deficiency (60). Vitamin D promotes serotonin synthesis by maintaining the expression of tryptophan hydroxylase 2 (TPH2). Low serotonin levels are also a feature of ASD and schizophrenia, as explained earlier. Neuropsychiatric diseases such as ASD and ADHD are also triggered by a number of other processes, such as oxidative stress (20,21,22).

Two of the drugs used in the treatment of ADHD, methylphenidate hydrochloride and amphetamine, act by increasing the activity of the dopamine signaling pathway. The effect of methylphenidate in the treatment of ADHD is enhanced by vitamin D supplementation (63). One of the functions of dopamine is to control the tonic excitatory impulse, which regulates the frequency of brain rhythms. Individuals with ADHD are known to exhibit too many slow theta brain waves, resulting in relaxation along with a decrease in the faster alpha/beta waves responsible for mental focus (64).

One of the primary deficit in ADHD, especially the hyperactive symptoms, causes learning difficulties. (65). In addition, another study found that DVD-deficient mice had reduced learning performance where they underperformed in an operant conditioning challenge (66). According to some studies, vitamin D supplementation does not improve all the symptoms of the patient with ADHD, but it is useful for the improvement of some symptoms. These findings suggest that vitamin D supplements may help certain symptoms of ADHD, but are ineffective in treating them as a whole (67).

Conclusions

Vitamin D is an essential hormone that acts to maintain the phenotypic stability of cell signaling systems, particularly the Ca^{2+} and redox pathways. Changes in these pathways during development may explain neurodevelopmental diseases. Numerous studies reveal the role of Vitamin D in brain development, synaptic plasticity, neuroprotection and dopaminergic system physiology, as well as in the transmission and connectivity certain neural circuits. Studies in humans have reported an association between low vitamin D levels and the prevalence of neurodegenerative, neuroinflammatory, and neuropsychological disorders. However, there is no certainty about the potential role that vitamin D plays in these pathologies because both its causal role in pathogenesis and its usefulness as a serum biomarker remain unproven.

Conducted systematic review did not confirm the positive effect of vitamin D supplementation on mental health in healthy adults. It has only been supported by some studies that mainly included depression and not other mental health problems. Due to the heterogeneity of the studies, it was not possible to determine the duration and adequate dose of vitamin D supplementation to improve mental health. Among the mental health problems, there are more studies showing that vitamin D supplementation is beneficial, especially in depression.

While some studies have indicated that this supplement should be combined with physical activity to achieve effective results, some studies have found that the supplement is less effective than obtaining vitamin D from food sources.

Randomized controlled trials focusing on the effects of vitamin D on well-defined mental disorders in childhood and adolescence are of great importance to elucidate the therapeutic potential of vitamin D supplements. Some studies have supported the potential positive effect of vitamin D on mental health in children. These studies say that taking vitamin D as a supplement in a balanced diet or outside of safe sunbathing supports mental health in children.

Vitamin D deficiency may potentially be a risk factor for ADHD, depression, schizophrenia and autism, but data are mixed and more studies and longitudinal studies are needed. The role of vitamin D in the pathogenesis of these diseases is unclear. Randomized controlled trials are needed to properly place vitamin D in the context of brain diseases in terms of prevention and treatment.

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VITAMIN D AND DRUGS

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Although labeled for use in various diseases, its primary use is the treatment and prevention of vitamin D deficiency and rickets. One of the fat-soluble vitamins is vitamin D. There are two main forms of vitamin D, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Both are equally well absorbed from the small intestine and metabolized in the same way. Although cholecalciferol and ergocalciferol are equally well absorbed, a meta-analysis shows that cholecalciferol is more effective at raising and maintaining circulating vitamin D levels (1, 2). Although animal experimental studies have shown that there may be differences in toxicity between cholecalciferol and ergocalciferol, human studies have not supported this. Vitamin D's active forms, ergocalciferol or cholecalciferol, are found in dietary supplements frequently in fortified milk and cereal products. For vitamin D to be converted to its active form, it must undergo hydroxylations in the liver and kidney. So, calcitriol (1,25-dihydroxy vitamin D), active vitamin D, is produced. Vitamin D activity is dependent on this active metabolite (3).

Vitamin D is involved in metabolism by a mixed-function oxidase system (CYP) as the hepatic-renal pathway. The first conversion occurs via hepatic 25-hydroxylases, including cytochrome P450 (CYP) enzymes 2R1, 27A1 and 3A4. Both in the kidney and at the local tissue level, 25(OH)D is one more hydroxylated by 1α-hydroxylase (CYP27B1) to form calcitriol, the active form of vitamin D. On the other hand, the catabolism of vitamin D metabolites is mediated by 24-hydroxylase (CYP24A1) (4). CYP24A1 protects against vitamin D toxicity by catabolism of 1,25-(OH)2D3 to inactive metabolites excreted as calcitroic acid in urine and feces (3).

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One of the multifunctional microsomal enzymes is CYP3A4. It is found in excess in the liver and is involved in the xenobiotic conversion of many endogenous substances and drugs. It has a dual effect, providing 25-hydroxylation and 24-hydroxylation of vitamins D2 and D3 (3). Therefore, the inhibitory or inducing effect on CYP3A4 may affect vitamin D levels. 25-hydroxylase CYP3A4 is a phase I biotransformation enzyme for some drugs (5). As shown by in vitro studies, half of the drugs are metabolized by CYP3A4 and that other drugs can induce CYP3A4 activity as well as inhibit it. (6). Examples of drugs that are activated by CYP3A4 or that activate or inhibit CYP3A4 activity are given in Table 1 (1). It has been shown that when drugs such as carbamazepine, hyperforin, phenobarbital, and rifampin are used for a long time, only CYP3A4 is stimulated, while other CYP enzymes such as CYP27B1 and CYP24A1 are not affected. This interaction with CYP3A4 suggests that as a result of this enzyme-mediated inactivation and rapid clearance of vitamin D metabolites, it will be an important factor in the etiology of insufficient bone mineral density (BMD) and drug-induced osteomalacia (7, 8).

CYP3A4 is known to be active not only in hepocytes but also in intestinal mucosa. (9). Therefore, interaction with oral intake of vitamin D is likely to be more significant than with drugs administered intravenously (10). It may also play a role in other mechanisms for drug-vitamin D interactions (1, 10). For example: 1) Taking it with drugs that inhibit the absorption or increase the elimination of dietary fat will alter the intestinal absorption of vitamin D, and 2) The risk of developing hypercalcemia will increase if taken together with calcium-sparing drugs (1, 10).

It has been shown that some endogenous substances and/or drugs that are substrates of CYP3A4 may or may not affect active vitamin D (1,25-(OH)2D3) by inhibiting or inducing it. However, it has also been shown that its effects on active vitamin D are achieved at a defined substrate concentration. For example, tacrolimus is a substrate of CYP3A4 at normal doses and does not inhibit/induce CYP3A4, but at higher concentrations has an inhibitory effect on CYP3A4 and alters vitamin D metabolism (11). Thus, dose-dependent induction/inhibition by CYP3A4 substrate inhibitors/inducers can have sometimes beneficial and sometimes not beneficial effects on clinical situations. Therefore, vitamin D levels can be changed in treatments focused on CYP3A4 based on substrate concentration, site specificity, substrate specificity, and dose (12).

Measurement of serum 25(OH)D3 is a reliable marker for clinically evaluating the vitamin D status of the organism. However, an adequate 25(OH)D3 level in terms of bone health is still open to discussion. some institutions suggest that 25(OH)D levels ≥20 ng/mL in serum will be sufficient, while others suggest levels ≥30–32. Again, there may be interindividual differences in the interpretation of serum concentrations. For example, it has been suggested that 25(OH)D levels may be lower in obese individuals than in normal-weight individuals due to the sequestration of the vitamin stored in adipose tissue. (10, 13).

Table 1. Examples of drugs that are activated by CYP3A4 or that activate or inhibit CYP3A4 activity

Drug Group	Effect on Vitamin D Status	Findings/Recommendations
Enzyme-Inducing Anti-Epileptic Drugs (EIAEDs)	Increase the need for vitamin D, affect calcium absorption	Regular monitoring of serum calcium and vitamin D status is required in high-risk patients with epilepsy who have been taking carbamazepine or other enzyme-inducing anticonvulsants for 6 months or more. Vitamin D and calcium supplementation may be needed, with the required dosage varying between 400-4000 units depending on individual and sociocultural characteristics.
Laxatives	High doses and long-term use may adversely affect fat-soluble vitamin absorption, including vitamin D	High doses and long-term use of laxatives may interfere with the absorption of fat-soluble vitamins such as vitamin D.
Metformin	Lower serum 25(OH)D concentrations in users compared to nonusers	Cohort studies have found a negative association between oral metformin use and vitamin D status.
Thiazide Diuretics	Theoretical risk of hypercalcemia, but studies do not support this data	Concomitant use of thiazide diuretics and vitamin D theoretically may cause hypercalcemia, but studies do not support this data.
Calcium Channel Blockers	May cause lower serum 25(OH)D values by inhibiting CYP3A4	Verapamil and diltiazem, as well as nifedipine, may lower serum 25(OH)D values due to their effects on CYP3A4.
Statins	Possible interaction with vitamin D; lower levels associated with myalgia and statin myopathy	Some statins, such as lovastatin, atorvastatin, and simvastatin, may have interactions with vitamin D. Low vitamin D levels in patients taking statins have been associated with myalgia, and there is a reversible association. Statin myopathy may also increase with vitamin D deficiency.
Antimicrobials	Rifampicin may induce CYP3A4, isoniazid may inhibit it; can alter vitamin D status	Rifampicin and isoniazid can alter vitamin D status due to their effects on CYP3A4.
Sunscreens	Excessive use may decrease vitamin D synthesis	Frequent or excessive use of sunscreens may lead to a decrease in vitamin D synthesis, potentially resulting in lower plasma vitamin D levels.
Bile Acid Sequestrants	Uncertain effect on vitamin D status	These drugs may bind to fat-soluble vitamins, including vitamin D, reducing its absorption. However, the overall effect on vitamin D status is uncertain based on available studies.
Lipase Inhibitors	Blocks absorption of dietary fats, including fat-soluble vitamins like vitamin D	Orlistat blocks the absorption of dietary fats, including fat-soluble vitamins like vitamin D. This can potentially lead to lower vitamin D levels. It is recommended to monitor vitamin D status in individuals taking orlistat and consider vitamin D supplementation if deficiency is detected.

Drugs Affecting Vitamin D Status

Many drugs can affect vitamin D status. here we review and summarize the findings on key drug groups in the light of key studies.

Enzyme-Inducing Anti-Epileptic Drugs (EIAEDs): Enzyme-inducing antiepileptics such as phenobarbital, carbamazepine and phenytoin increase the need for vitamin D by increasing hepatic catabolism of vitamin D to inactive metabolites and adversely affect calcium absorption (10, 14). Patients with epilepsy who have been taking carbamazepine or other enzyme-inducing anticonvulsants for 6 months or more are more likely to need vitamin D and calcium supplementation. The daily vitamin D requirement needed in these cases may vary between 400-4000 units depending on individual and sociocultural characteristics (10). Therefore, regular monitoring of serum calcium and vitamin D status is required in high-risk patients.

Data on new antiepileptic drugs are still limited. In a retrospective study of more than 15,000 patients, a high risk of bone fracture was reported for gabapentin, but not for levetiracetam, lamotrigine or oxcarbazepine (15). On the other hand, a study on topiramate, gabapentin, and levetiracetam showed no harmful effects (16).

Laxatives: Laxatives speed up bowel movement and reduce food transit time. Therefore, high doses and long-term use of laxatives may adversely affect the absorption of fat-soluble vitamins such as vitamin D (10).

Metformin: In a cohort study, serum 25(OH)D concentrations were found to be 7.3 nmol/L lower in oral metformin users than in nonusers (17). However, a study of more than 10,000 patients found a negative association between oral antidiabetic use and vitamin D status, and another study found a specific effect due to metformin only (18, 19).

Thiazide Diuretics: Vitamin D increases intestinal calcium absorption, while thiazide diuretics decrease urinary calcium excretion. Therefore, theoretically, their concomitant use may cause hypercalcemia, especially in patients with hyperparathyroidism or impaired renal function. However, studies do not support this data (10, 20).

Calcium Channel Blockers: Verapamil and diltiazem may cause lower serum 25(OH) D values by inhibiting CYP3A4(3, 10). It has also been reported that nifedipine, another calcium channel blocker, can induce vitamin D catabolism due to its ligand for the Nuclear Pregnan X Receptor (PXR) (17).

Statins: Lovastatin, atorvastatin and simvastatin are known to be metabolized by CYP3A4 (review, last). One study evaluated the relationship between vitamin D and atorvastatin levels and found lower levels of atorvastatin in those who took 800 IU vitamin D supplements for 6 weeks (21). A meta-analysis of 2420 patients shows that low vitamin D levels in those taking statins are associated with myalgia, and there is a reversible association (22). It has also been suggested that statin myopathy may increase with vitamin D deficiency (10).

Antimicrobials: Vitamin D both increases the production of cathelicidin with antimicrobial activity and regulates the activity of macrophages. As rifampicin induces CYP3A4 and isoniazid inhibits it, vitamin D status can be altered by these drugs (23, 24).

Sunscreens: Sunscreens may cause a decrease in vitamin D synthesis, so a decrease in plasma vitamin D levels may occur as a result of widespread, frequent use or excessive use of sunscreen (25, 26).

Warnings on the use of vitamin D

Some oral solution/drop formulations of vitamin D contain propylene glycol. Propylene glycol is a food additive that is generally considered safe. However, the European Medicines Agency states that exceeding 1 mg/kg/day may cause side effects. Taking it together with another drug containing propylene glycol or any alcohol dehydrogenase substrate such as ethanol may cause serious side effects in children under 5 years old and especially in neonates and premature neonates (27). Therefore, when choosing oral liquid preparations for young children, it should be ensured that the correct vitamin D formulation is selected to avoid propylene glycol toxicity. Excessive propylene glycol can cause central nervous system toxicity (e.g., seizures, intraventricular hemorrhage) lactic acidosis, tachycardia, tachypnea, hyperosmolality, and diaphoresis (28).

It should not be forgotten that there is a risk of vitamin D-induced hypercalcemia even at normal doses in patients with kidney disease, especially kidney failure; Close clinical monitoring should be performed to ensure adequate supplementation. In patients with stage 3 or higher kidney disease, the use of a vitamin D analog appears to be more appropriate, according to the recommendations of the National Kidney Foundation. (29).

Patients with cystic fibrosis, celiac disease, some liver diseases' forms, biliary cirrhosis or biliary tract disease gallbladder that reduce the absorption of vitamin D will need to be given higher doses of vitamin D (30-34). Again, patients using certain drugs (such as some anticonvulsants, and glucocorticoids) may also need higher doses. In such cases, it may be necessary to prescribe active vitamin D analogs (34, 35).

Side effects that may occur as a result of the recommended daily intake of vitamin D in pregnant women have not been reported. 25-hydroxyvitamin D passes into breast milk according to maternal serum concentration. Infants who are exclusively breastfed and do not take other vitamin D supplements will have vitamin D deficiency, especially if the breast milk concentration is also low (without maternal supplementation). The recommended daily vitamin D requirement for the mother during breastfeeding is 600 International Units/day and the Tolerable Upper Intake Limit is 4000 International Units/day. Since hypercalcemia has been reported with maternal use of high doses of vitamin D, infant serum calcium concentrations should be closely monitored, usually when high doses of vitamin D are prescribed to a nursing mother (36).

Conclusion

There are medications that have the potential to affect the level of vitamin D in the blood, but there is insufficient data on the subject and further studies are needed. More importantly, until definitive data is obtained, all healthcare professionals should be aware of the potential effects of medications on vitamin D status. In risky situations, serum 25(OH)D concentrations should be monitored. If necessary, vitamin D supplements should be recommended to achieve adequate vitamin D levels, optimize the effectiveness of treatment, and limit medication side effects and toxicity.

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VITAMIN D INTOXICATION

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Introduction

Childhood intoxications are a public health issue which is among significant cause of pediatric emergency unit admissions worldwide. According to American Association of Poison Control Centers (AAPCC) data, approximately one million of exposure to poison among children younger than six years of age is being reported annually. Since not all cases of intoxication are reported to poison control centers, this may be underestimated. Majority of intoxications occurs at home. Intoxications are more common in males during preadolescence and in females during postpuberty (1). Intoxications are predictable and preventable events, with most pediatric intoxications being unintended. Cases of intoxication are accidental and heuristic in children younger than six years of age, whereas suicidal attempt or drug abuse may be encountered in older children and adolescents. A significant proportion of these cases do not give a history of intoxication at admission. The clinical presentation of the patients ranges from being asymptomatic to very severe clinical deterioration, depending on the substance exposed (1,2). Although the patient or his/her relative does not give a history of intoxication, a possible exposure to toxic substances should be considered in conditions such as acutely impaired consciousness, respiratory distress or insufficiency, cardiac insufficiency, refractory seizures, arrhythmia, and unexplained metabolic acidosis. Approach to an intoxicated child begins with stabilization of airway, respiration and circulation. It proceeds with identification of agent(s), decontamination, prevention of absorption of toxic substance, elimination, antidote administration, supportive treatment and process of stabilization. Stages of the approach are determined by type and amount of toxin(s), duration of exposure, route of exposure, and severity of intoxication (2). The most common toxic substances in pediatric age group include cosmetic/personal care products (12%), household cleaners (11%), analgesics (8%), foreign bodies/toys (7%), and dietary supplements/plants/homeopathic drugs (6%). Intoxications with vitamin preparations are common, though fatal cases associated with vitamins are extremely rare (1).

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Vitamins are substances which exist in natural foods in small amounts, are necessary for growth, development and normal metabolism, and may lead to various health problems when deficient (3). Supplementary substances should be determined by a physician in accordance with patient's age, gender, health status, risk factors and family history. In 2015 Council for Responsible Nutrition report, it was found that 98% of food supplement users take vitamins and minerals. It was observed that, of the supplements used, 78% were multivitamins, 32% vitamin D, 27% vitamin C, 24% calcium, and 18% vitamin B/B complex. Need for supplement use is found to include maintenance of general health, fitness and replacement of dietary food deficiencies. The current data demonstrate that healthy individuals have a minimal risk associated with use of multivitamins and multiminerals at recommended daily doses (4). In clinical practice, vitamin deficiencies are more common than intoxications. Unfortunately, there is vitamin use at doses much higher than the recommended doses due to false beliefs that vitamin preparations provide extra energy and promotes muscle growth. Some vitamins may produce adverse effects when taken at quite high doses (5). Vitamins are divided into two groups: water-soluble and lipid-soluble. Water-soluble vitamins have low toxicity rates, as they are stored in the body in scent amounts. Among water-soluble vitamins, thiamine, riboflavin, pantothenic acid, folic acid, biotin and vitamin B12 have not reported to cause any toxicity (3). Nevertheless, ascorbic acid (vitamin C), nicotinic acid (vitamin B3) and pyridoxine (vitamin B6) may cause toxicity, although they are water-soluble vitamins. Lipid-soluble vitamins have a higher toxic potential, as they biologically accumulate. Among lipid-soluble vitamins, cases of intoxication caused by overdose or chronic overuse of vitamins A, D and E. Vitamin K, however, may lead to severe anaphylactoid reactions following rapid intravenous administrations (5,6).

Vitamin D

Vitamin D is an important pro-hormone which is lipid-soluble, plays a vital role in bone and calcium metabolism (7). It is considered to play an important role not only in calcium homeostasis and bone mineralization, but also in immune system, cardiovascular health and prevention of cancer. Vitamin D stores deplete especially during winter. In deficiency state, osteoporosis and rickets develop in adults and children, respectively. Oral vitamin D supplementation is therefore necessary in case of lack of exposure to sunlight. The recommended maintenance dose of vitamin D is 600 IU/day over 1 year of age and 400 IU/day under 1 year of age. These doses aim to keep 25-hydroxyvitamin D [25(OH) D] levels at 20 ng/mL. High-dose vitamin D is used for rickets, hypoparathyroidism and chronic kidney failure-induced renal osteodystrophy in children (8,9).

Increased vitamin D supplementation in the absence of medical supervision or prescription of very high doses without medical follow-up may lead to exogenous hypervitaminosis D, also known as vitamin D toxicity. Although vitamin D toxicity resulting in hypercalcemia is rare, it may be life-threatening unless diagnosed early and treated. Uncontrolled use of supplementary products or accidental, unintended ingestions in pediatric age group are the most common causes of vitamin D toxicity (10).

Vitamin D Intoxication

Hypervitaminosis D is a clinical condition which may occur as a result of use of inappropriately high doses of vitamin D and may lead to severe health problems. Vitamin D toxicity may be acute or chronic, depending on the duration of exposure (11).

In the literature, data regarding toxic dose of vitamin D vary. The Institute of Medicine defined tolerable upper intake limits of vitamin D. Based on this; while the tolerable limit for those at and older than 9 years of age is 4000 IU/day, it is 3000 IU/day for children aged 4-8 years and 2500 IU/day for children aged 1-3 years. It has been reported that intoxications may occur at doses over 1000 IU/day in infants younger than 6 months of age. Furthermore, vitamin dose should be readjusted in patients with possible malabsorption such as celiac disease, gastrectomy or inflammatory bowel disease. In such situations, these cases should be followed-up more closely to avoid intoxication, as they may need higher doses to replenish vitamin D stores (12).

Etiology

In healthy individuals, vitamin D intoxication may be due to use at higher doses than prescribed or prescription errors. This may be observed in patients who require higher doses for treatment of disorders, including osteoporosis, rickets, renal osteodystrophy, celiac disease or inflammatory bowel disease. In addition, in medical conditions like granulomatous disorders and lymphomas, endogenous hypervitaminosis D may occur due to overproduction of 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] (5).

Epidemiology

According to United States Poison Control Centers, it is revealed that annually, 66,599 (2.59%) individuals are intoxicated with vitamin preparations and that vitamins have become one of 25 most common cause of intoxications in humans. 45,537 (4.92%) of more than 900,000 pediatric admissions to AAPCC was found to occur due to vitamin-induced intoxications. It was also reported that 10,178 of vitamin-induced intoxications were caused by vitamin D, 4604 of which occurred in children younger than 5 years of age. It was determined that three cases among all vitamin D intoxications had severe clinical presentation, with no fatal cases reported (1).

Pathophysiology:

Vitamin D is not a biologically active substance. It therefore requires enzymatic transformation to active metabolites in liver and kidneys. Vitamin D is synthesized from 7-dehydroxycholesterol in a reaction catalyzed by ultraviolet B irradiation. It is metabolized to 25(OH)D in the liver by the enzyme 25-hydroxylase. It is transformed into $1,25(OH)_2D$ in kidneys by the enzyme 1-alpha-hydroxylase and re-enters into circulation. Major function of vitamin D is to regulate calcium homeostasis. It enhances absorption of calcium from intestines by increasing production of calcium-binding proteins and plasmalemmal calcium pump proteins. In the bones, it stimulates osteoclastic precursors to differentiate into mature osteoclasts. Mature osteoclasts, together with parathyroid hormone, causes mobilization of calcium stores form bones, increasing serum calcium concentrations (5, 13).

The enzyme 1-alpha-hydroxylase is also expressed in extrarenal sites. The most common clinical picture of extrarenal synthesis of $1,25(OH)_2D$ is hypercalcemia and hypercalciuria seen in patients with granulomatous diseases. In such cases, extrarenal synthesis of $1,25(OH)_2D$ from activated macrophages, independently of parathyroid hormone, in lungs and lymph nodes occurs (5, 13).

In case of exposure to high-dose vitamin D, the enzyme 25-hydroxylase tries to prevent hepatic toxicity via a negative feedback mechanism. However, it this compensatory mechanism fails, vitamin D-binding receptors saturate and vitamin D begins to accumulate in liver and adipose tissue. This causes increased concentrations of 25(OH)D and other vitamin D metabolites. Exceeding the capacity of vitamin D-binding protein leads, in turn, to increased release of $1,25(OH)_2D$ (14).

Symptoms and Signs

Clinical symptoms of vitamin D intoxication varies and may present most commonly with nonspecific symptoms such as exhaustion, fatigue, weakness, headache, dizziness, loss of appetite and bone pain. Elevated vitamin D concentrations lead to increased calcium absorption and development of hypercalcemia. Clinical symptoms of vitamin D toxicity primarily results from effects of hypercalcemia (15).

Calcium affects nervous, musculoskeletal and digestive systems, as well as kidneys. Neuropsychiatric symptoms, including altered consciousness, confusion, depression, psychosis, stupor and coma, may occur. Gastrointestinal symptoms of vitamin D intoxication, however, include nausea, vomiting, abdominal pain, polydipsia, anorexia, constipation, peptic ulcer and pancreatitis. Cardiovascular symptoms include hypertension and arrhythmias. While hypercalciuria is the earliest sign of renal symptoms, polyuria, nephrocalcinosis and kidney failure may also occur. Increasing calcium concentrations lead to polyuria and polydipsia. Diuresis causes loss of salt and water, further impairing calcium excretion. Physical examination of patients with evidence of toxicity may reveal reduced skin turgor and dry mucosae due to dehydration. On physical examination, abdominal tenderness, without evidence of rebound tenderness or guarding, may be observed. Sometimes, physical examination may be completely normal, with no clinical findings (5,10). Clinical symptoms and signs of vitamin D intoxication are represented in Table 1.

Diagnosis

A comprehensive history and physical examination are crucial for diagnosis of vitamin D toxicity. Issues such as medications, including over-the-counter vitamin supplements, chronic diseases, dietary history, and, particularly, overuse of vitamin D-enriched milk or other products should be comprehensively questioned (16).

Evaluation of serum calcium, parathyroid hormone and 25(OH)D levels is recommended. Patients with symptomatic exogenous toxicity due to use of high-dose vitamin D are found to have hypercalcemia, hyperphosphatemia, very low PTH, elevated 25(OH) D concentration, and normal or elevated $1,25(OH)_2D$ concentrations. If endogenous active metabolite intoxication exists, suppressed PTH, reduced or normal 25(OH)D, and elevated $1,25(OH)_2D$ concentrations are found (17).

Despite of several discussions, current guidelines indicate that serum inactive 25(OH) D levels >100 ng/mL (250 nmol/L) have been defined as hypervitaminosis, whereas serum levels >150 ng/mL (375 nmol/L) have been proposed to define vitamin D intoxication (10,11). Since, in such patients, hypercalcemia-related kidney dysfunction, dehydration and electrolyte imbalances due to excessive vomiting may develop, the patients should have electrolytes, as well as liver and kidney function tests checked (5). All patients should have an electrocardiogram obtained due to risk of arrhythmia. Acute hypercalcemia directly shortens myocardial action potential, which is reflected on ECG as a shortened QT interval. Although moderate hypercalcemia seems to not have a significant clinical impact on cardiac conduction system, and prevalence of supraventricular or ventricular arrhythmias, arrhythmias have been defined in patients with severe hypercalcemia. An ST segment elevation mimicking myocardial infarction has been reported in hypercalcemic patients. Moreover, a shortened ST segment and flattened T-waves may also be seen (5).

Table 1: Clinical symptoms and signs of vitamin D intoxication

Neuropsychiatric	Altered consciousness Poor concentration Confusion Apathy Depression Psychosis Stupor Coma
Gastrointestinal	Nausea Vomiting Abdominal Pain Polydipsia Anorexia Constipation Peptic ulcer Pancreatitis
Cardiovascular	Hypertension Bradycardia Arrhythmia Shortening of QT interval ST segment elevation Flattened T-waves
Urinary	Polyuria Polydipsia Hypercalciuria Nephrolithiasis Nephrocalcinosis Oliguria Anuria
Musculoskeletal	Bone pain Muscle weakness

Treatment

The main objective in management and treatment of vitamin D intoxication includes lowering of serum calcium levels and supportive treatment for elimination of hypercalcemia-induced problems. Vitamin D has a long half-life, as it is lipid-soluble and stored in liver and adipose tissue (5). This is due to slow release of stored vitamin D from fat depositions.

The first thing to do in exogenous vitamin D intoxication is discontinuation of vitamin D supplementation and reduction of dietary calcium intake (14). If there is endogenous toxicity, the patients are recommended not to be exposed to sunlight and other ultraviolet B light sources.

Intravascular fluid resuscitation with isotonic fluids provides restoration of intravascular volume, increases glomerular filtration rate, and urinary calcium excretion. After establishment of rehydration, loop diuretics are administered. Loop diuretics increases urinary calcium excretion through inhibition of calcium reabsorption from loop of Henle. Hypercalcemia-induced renal dysfunction, and dehydration and electrolyte imbalance due to excessive vomiting may develop. Therefore, fluid resuscitation should be made in case of dehydration and efforts should be provide supportive treatment for correction of electrolyte imbalances. Kidney functions should be closely monitored, and promptly treated in case of impairment.

Use of glucocorticoids in vitamin D intoxication is controversial and they are usually used for vitamin D toxicity due to granulomatous diseases. Glucocorticoids decrease plasma calcium levels by increasing urinary calcium excretion and reducing intestinal calcium absorption (5).

Antiresorptive therapy with calcitonin, biphosphonates or both may be beneficial in severe cases of hypercalcemia caused by increased osteoclastic bone resorption due to direct effect of $1,25(OH)_2D$ on bone tissue. It inhibits bone resorption by affecting osteoclast function. It is clinically impossible to know whether osteoclastic bone resorption increases, however, increased bone resorption can be assumed so in the presence of significant hypercalcemia (10).

Phenobarbital may be a beneficial treatment for vitamin D intoxication by reducing 25(OH)D concentrations through induction of hepatic microsomal enzyme (18). Ketoconazole nonspecifically reduces synthesis of $1,25(OH)_2D$ from activated mononuclear cells by inhibiting cytochrome P450 and CYP27B1, though it is not recommended for long-term use as it inhibits many other important CYPs (19).

Hemodialysis is recommended for refractory cases of hypercalcemia (5).

Prognosis

Most cases of vitamin D toxicity usually recover without any severe complication or sequelae. When the literature is reviewed, it is observed that, although rare, there are cases in which severe hypercalcemia causes acute kidney failure requiring hemodialysis (20).

Conclusion

Vitamin D preparations are supplements which are commonly prescribed or used as over-the-counter medication by patients. Usage rates of vitamin D supplements have recently been significantly increased. Therefore, patients should definitely be informed about detrimental effects of over or unnecessary use and about adherence to prescription regimen. Healthcare professionals, however, should closely follow patients requiring high-dose regimen and pay attention to recommended daily requirements to avoid prescription errors.

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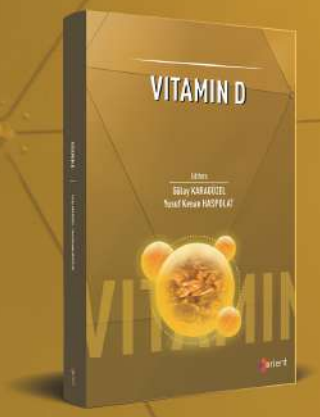

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