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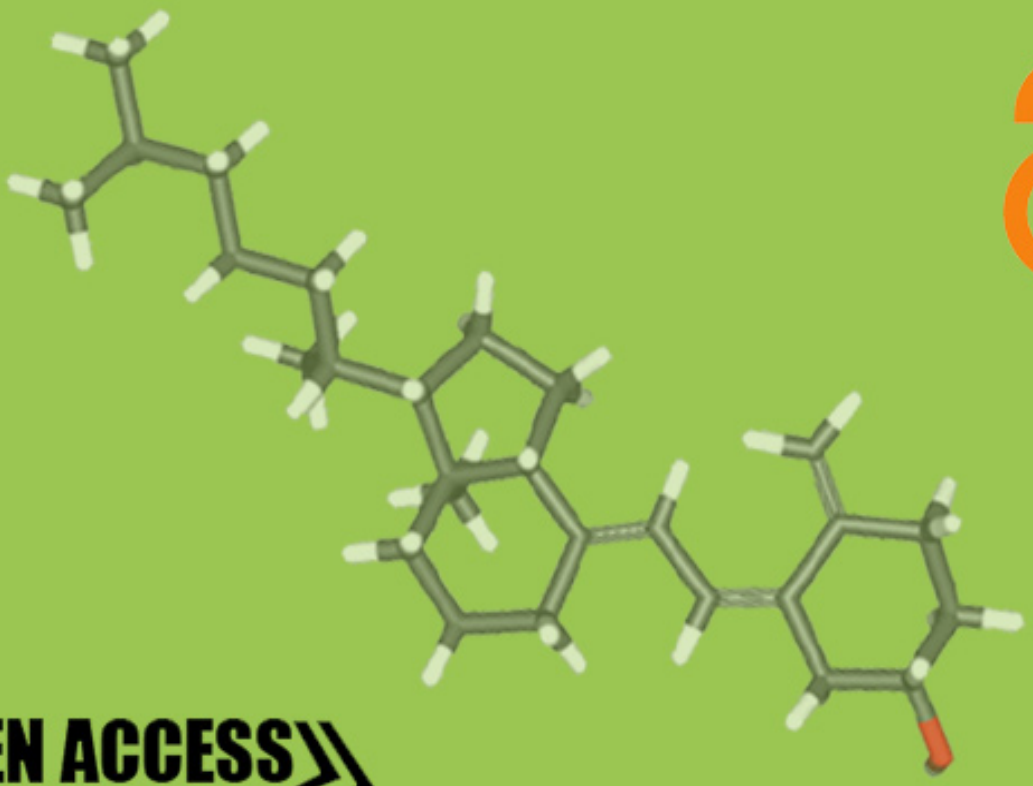
Vitamin D deficiency : Causes & Treatment



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Vitamin D Deficiency: Causes & Treatment

Chapter 1

Causes and Treatment of Vitamin D Deficiency

Karunakaran Poongkodi

Assistant Professor of Endocrine Surgery, Government Mohan Kumaramangalam Medical College, Salem, Tamilnadu, India – 636030.

Mobile No: +91 94 4311 9743; Email: poongkodithesurgeon@gmail.com

Abstract

Vitamin D deficiency is a worldwide public health problem across all age groups including infants, children, adults, and elderly. Very few foods naturally contain or are fortified with vitamin D. The major source of vitamin D is from sunlight exposure. Vitamin D, the sunshine vitamin is synthesized from 7-dehydocholesterol present in the skin of humans by the action of ultraviolet B radiation (290 – 315 nm). The major cause of vitamin D deficiency is lack of adequate sunlight exposure. In utero and during childhood, vitamin D deficiency can cause growth retardation and skeletal deformities and may increase the risk of hip fracture later in life. Vitamin D deficiency in adults can precipitate or exacerbate osteopenia and osteoporosis, cause pain producing osteomalacia and muscle weakness, and increase the risk of fall and fracture. Vitamin D deficiency is associated with increased risk of common cancers, autoimmune diseases, infectious diseases, and cardiovascular mortality. Serum 25-hydroxy vitamin D (25-OHD) is the reliable marker of vitamin D status and a level below 20 ng/ml defines deficiency. However, an optimal level above 30 ng/ml is required to maximize the bone health and non-skeletal benefits of vitamin D. A sensible sun exposure for 5 – 15 min between 1000 and 1500 hours in the spring, summer and autumn and supplementation of atleast 400 IU, 600 IU and 800 IU of vitamin D/day among infants and children, adults and elderly respectively shall guarantee vitamin D sufficiency in at risk population.

1. Sources of Vitamin D

Humans get vitamin D from sunlight exposure, dietary sources and supplements [1]. "Vitamin D" (calciferol) refers to both cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). Cholecalciferol is produced by the action of ultraviolet B light (UVB; wavelength,

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290–320 nm) on 7- dehydrocholesterol in the skin of humans, and is the form of vitamin D found in oily fish. Ergocalciferol is formed when ultraviolet (UV) light irradiates the fungal steroid, ergosterol. Few foods naturally contain vitamin D_3 or D_2 [1-3]. Few foods are fortified with Vitamin D. Some of the sources of Vitamin D are given in the **Table 1**.

Table 1: Sources of vitamin D_2 and vitamin D_3

Natural sources	
Cod liver oil	400–1,000 IU/teaspoon vitamin D_3
Salmon, fresh wild caught	600–1,000 IU/3.5 ozvitamin D_3
Salmon, fresh farmed	100–250 IU/3.5 oz vitamin D_3, vitamin D_2
Salmon, canned	300–600 IU/3.5 oz vitamin D_3
Sardines, canned	300 IU/3.5 oz vitamin D_3
Mackerel, canned	250 IU/3.5 oz vitamin D_3
Tuna, canned	236 IU/3.5 oz vitamin D_3
Shiitake mushrooms, fresh	100 IU/3.5 oz vitamin D_2
Shiitake mushrooms, sun-dried	1,600 IU/3.5 oz vitamin D_2
Egg yolk	20 IU/yolk vitamin D_3or D_2
Sunlight/UVB radiation	20,000 IU equivalent to exposure to 1 minimal erythemal dose (MED) in a bathingsuit. Thus, exposure of arms and legs to 0.5 MED is equivalent to ingesting3,000 IU vitamin D_3
Fortified foods	
Fortified milk	100 IU/8 oz, usually vitamin D_3
Fortified orange juice	100 IU/8 oz vitamin D_3
Infant formulas	100 IU/8 oz vitamin D_3
Fortified yogurts	100 IU/8 oz, usually vitamin D_3
Fortified butter	56 IU/3.5 oz, usually vitamin D_3
Fortified margarine	429 IU/3.5 oz, usually vitamin D_3
Fortified cheeses	100 IU/3 oz, usually vitamin D_3
Fortified breakfast cereals	100 IU/serving, usually vitamin D_3
Pharmaceutical sources in the United States	
Vitamin D_2 (ergocalciferol)	50,000 IU/capsule
Drisdol (vitamin D_2) liquid	8,000 IU/cc
Supplemental sources	
Multivitamin	400, 500, 1,000 IU vitamin D_3 or vitamin D_2
Vitamin D_3	400, 800, 1,000, 2,000, 5,000, 10,000, and 50,000 IU

IU=25 ng. [Reproduced from M.F.Holick et al. J Clin Endocrinol Metab, July 2011, 96(7):1911–1930]

2. Photosynthesis of Vitamin D_3

During exposure to sunlight, UVB radiation (290–315 nm) is absorbed by Pro-vitamin D, 7-dehydrocholesterol (relatively rigid 4 – ringed structure) that is present in the lipid bilayer of plasma membranes of both epidermal keratinocytes and dermal fibroblasts [4-6]. The energy is absorbed by the double bonds in the B ring, which results in rearrangement of the double bonds and opening of the B ring to form previtamin D_3. The opening of B ring during the formation of previtamin D_3 by UVB radiation makes it less rigid and increases the membrane permeability to Calcium and various ions. Once formed, previtamin D_3, which is entrapped within the plasma membrane lipid bilayer, rapidly undergoes rearrangement of its double bonds to form the more thermodynamically stable vitamin D_3. During this transformation process, vitamin D_3 is ejected from the plasma membrane into the extracellular space. The vitamin D-binding protein in the dermal capillary bed has an affinity for vitamin D_3 and draws it into the circulation. On excessive exposure to sunlight, previtamin D_3 and vitamin D_3 that has formed and not escaped into the circulation continues to absorb UV radiation and isomerizes into inactive photoproducts, namely tachysterol and lumisterol, thereby preventing Vitamin D intoxication.

3. Metabolism of Vitamin D in The Regulation of Calcium, Phosphorus Homeostasis and Skeletal Metabolism

Vitamin D_2 and vitamin D_3 obtained from dietary and supplementary sources are incorporated into chylomicrons and transported by the lymphatic system into the venous circulation. Vitamin D made in the skin (D_3) or ingested (D_2 or D_3) in the diet can be stored in and then released from fat cells [1,7,8]. Vitamin D (here after "D" represents D_2 or D_3) in the circulation is bound to the vitamin D–binding protein, which transports it to the liver, where vitamin D is converted by vitamin D-25-hydroxylase to 25-hydroxyvitamin D [25-OHD]. Although this is the major circulating form of vitamin D, 25-OHD is biologically inactive and must be converted in the kidneys by 25-hydroxyvitamin D-1α- hydroxylase (1α-OHase) (CYP27B1) to the biologically active form -1,25-dihydroxyvitamin D [1,25 $(OH)_2D$]. Serum phosphorus, calcium, fibroblast growth factor 23 (FGF-23), and other factors can either increase (+) or decrease (–) the renal production of 1,25 $(OH)_2D$. Fibroblast growth factor 23, secreted from the bone, causes the sodium–phosphate cotransporter to be internalized by the cells of the kidney and small intestine and also suppresses 1,25-dihydroxyvitamin D synthesis. 1,25 $(OH)_2D$ decreases its own synthesis through negative feedback and decreases the synthesis and secretion of parathyroid hormone by the parathyroid glands. 1,25 $(OH)_2D$ increases the expression of 25-hydroxyvitamin D-24- hydroxylase (24-OHase) (CYP24) to catabolize 1,25 $(OH)_2D$ to the water-soluble, biologically inactive calcitroic acid, which is excreted in the bile. The free form of 1,25 $(OH)_2D$, a steroid hormone upon entering the target cell interacts with specific nuclear Vitamin D receptor (VDR), which is phosphorylated (Pi). The 1,25 $(OH)_2D$-VDR

complex combines with the retinoic acid X receptor (RXR) to form a heterodimer, which in turn interacts with the vitamin D-responsive element (VDRE), causing enhancement or inhibition of transcription of vitamin D-responsive genes. 1,25 $(OH)_2D$ enhances intestinal calcium absorption in the small intestine by enhancing the expression of the epithelial calcium channel (transient receptor potential cation channel, subfamily V, member 6 [TRPV6]) and calbindin 9K, a calcium-binding protein (CaBP). 1,25 $(OH)_2D$ is recognized by its receptor in osteoblasts, causing an increase in the expression of the receptor activator of nuclear factor-κB ligand (RANKL). RANK, the receptor for RANKL on preosteoclasts, binds RANKL, which induces preosteoclasts to become mature osteoclasts. Mature osteoclasts mobilize calcium and phosphorus from the bone, maintaining calcium and phosphorus levels in the blood. Adequate calcium (Ca^{2+}) phosphorus ($HPO4^{2-}$) product (product of blood concentrations of calcium and phosphorus) is necessary for the mineralization of the skeleton.

4. Non-Calcemic Actions of Vitamin D

The revelation of vitamin D receptor (VDR) and the local production of active form of vitamin D- 1,25 $(OH)_2D$ from the circulating 25-OHD by 1-α hydroxylase activity in almost all the nucleated cells and the tissues of the body has provided insight onto the multitude of biological functions of vitamin D [3,5,6,9]. The VDR is present in the small intestine, colon, osteoblasts, activated T and B lymphocytes, islet cells, parathyroid cells and most organs in the body, including brain, heart, skin, gonads, prostate, breast, and mononuclear cells. It has been reported that the blood concentrations of 25-OHD above 78 nmol/L (30 ng/mL), is necessary for extra renal production of 1,25$(OH)_2D$. 1,25 $(OH)_2D$ is known to control over 200 genes involved in various physiological functions including control of cellular proliferation and differentiation, apoptosis, inhibition of angiogenesis, modulation of immune cells, cathelicidin production against infectious agents, increased insulin production by the pancreas, decreased renin production by the kidneys, increased myocardial contractility, prevention of inflammatory bowel disease, and promotion of thyroid-stimulating hormone secretion [10,11]. The locally produced 1,25 $(OH)_2D$ is converted to inactive calcitroic acid and does not enter circulation. Therefore, it does not influence calcium metabolism. The locally produced 1,25$(OH)_2D$ in parathyroid cells inhibits the expression and synthesis of parathyroid hormone. In addition, skeletal muscle possesses VDR. Performance speed and proximal muscle strength improves markedly when 25-OHD levels increases above 30 ng/ml [8,12].

5. Definition of Vitamin D Deficiency

Serum circulating level of 25- OH vitamin D is the most reliable indicator of Vitamin D status of the body [1,12-18]. 25-OHD is the major circulating form of vitamin D with half-life of about 2 weeks. 25-OHD is measured by various methods such as Radioimmunoassay, High Performance Liquid Chromatography but the gold standard is Liquid Chromatography- Tan-

dem Mass Spectrometry. Adherence of assay methodology to National Institute of Standards and Technology should reduce bias. Although 1,25 $(OH)_2D$ is the active form of vitamin D, it is not used to assess the vitamin D status as it has a short half-life of less than 4 hours in circulation. More importantly, during vitamin D deficiency, there is a compensatory increase in the parathyroid hormone secretion which stimulates the kidney to produce more 1,25 $(OH)_2D$. Therefore, the levels of 1,25 $(OH)_2D$ may be normal or even elevated when the patient is severely vitamin D deficient. However, the measurement of 1,25 $(OH)_2D$ is useful in acquired and inherited disorders in the metabolism of vitamin D and phosphate, including chronic kidney disease, hereditary phosphate-losing disorders, oncogenic osteomalacia, pseudovitamin D-deficiency rickets, vitamin D-resistant rickets, as well as chronic granuloma forming disorders such as sarcoidosis and some lymphomas. 25-OHD is inversely related to parathyroid hormone levels (PTH). The PTH decreases with increase in 25-OHD level and reaches a nadir when 25-OHD levels are between 30 and 40 ng/ml when maximum bone health and non-skeletal benefits are observed. As vitamin D deficiency progresses, parathyroid gland is maximally stimulated leading to secondary hyperparathyroidism. The PTH data with reference to 25-OHD has been used by Institute of Medicine (IOM) and other research committees to define vitamin D deficiency status and inform treatment decisions.

Vitamin D deficiency is defined as serum 25-OHD concentration less than 20 ng/ml. Insufficiency as 25-OHD level between 21 and 29 ng/ml. Vitamin D in toxication occurs when 25-OHD exceeds 150 ng/ml.

Status	Serum 25 OH	Vitamin D Concentration
Severe Deficiency	<10 ng/ml	<25 nmol/liter
Deficiency	<20 ng/ml	<50 nmol/l
Insufficiency	21 – 29 ng/ml	50 – 74 nmol/l
Sufficiency	30 – 100 ng/ml	75 – 250 nmol/l
Optimal	30 – 60 ng/ml	75 – 150 nmol/l
Toxic	>150 ng/ml	>375 nmol/l

6. Assessment of Vitamin D Deficiency

Screening the blood levels of 25-hydroxyvitamin D of the population at risk in reputed laboratory enrolled in Vitamin D External Quality Assessment Scheme (DEQAS) proficiency program is recommended. Screening the general population is not currently recommended [2,14].

7. Causes of Vitamin D Deficiency

The major source of Vitamin D for the human kind is exposure to sunlight. More than 90% of the vitamin D requirement is obtained from casual exposure to sunlight [4-6,19]. The

skin has a large capacity to produce vitamin D. Exposure to 1 Minimal Erythemal Dose (MED) of 54mJ/sq.cm (which imparts light pinkness after exposure) among young adults in bathing suits increases blood concentrations of vitamin D equivalent to that observed with the doses of 10,000 to 20,000 IU of vitamin D. Therefore, 1 MED is equivalent to 10- 50 times the recommended dietary in takes of 400 IU, 600 IU and 800 IU of vitamin D among infants, adults and elderly aged 70 years or above respectively. Lack of adequate exposure to sunlight is the most important cause of vitamin D deficiency.

Any factor that either influences the number of solar UVB photons that penetrate the skin or alters the amount of 7-dehydrocholesterol in the skin influences the cutaneous production of vitamin D_3. The amount of 7-dehydrocholesterol in the epidermis is relatively constant and begins to decline only later in life. Melanin is an effective natural sunscreen and efficiently absorbs UVB photons. Therefore, dark skinned people with increased melanin pigmentation (such as an African American who never burns and always tans with skin type V) requires 5 to 10 times longer exposure to sunlight compared to light skinned individuals (skin type III- always burns, always tans) to produce same amount of vitamin D. Sunscreen absorbs UVB radiation and some UVA (321–400 nm) radiation before it enters the skin. Therefore, a sunscreen with a sun protection factor (SPF) of 8 reduces vitamin D_3 synthesis in the skin by 95% and a SPF of 15 by 98%. Time of day, season, and latitude also dramatically influences vitamin D_3 synthesis by skin. Although the sun is closest to the earth in winter, the sun's rays strikes the earth surface at a more oblique angle (zenith angle). Due to the oblique angle, UVB photons must pass through the ozone for a greater distance and therefore, more UVB photons are efficiently absorbed by the ozone layer. In addition, with the more oblique angle there are fewer photons per unit area striking the earth. Time of day, season, and latitude all influence the zenith angle of the sun [5,6]. Above 37° latitude during the months of November through February, there are marked decreases (80-100%, depending on latitude) in the number of UVB photons reaching the earth's surface. Therefore, very little if any vitamin D_3 is produced in the skin during the winter. However, below 37° latitude and closer to the equator, more vitamin D_3 synthesis occurs in the skin through-out the year. Similarly, in the early morning or late afternoon, the zenith angle is so oblique that very little if any vitamin D_3 is produced in the skin even in the summer.

Thus, sun exposure for 5-15 minutes is safe and sensible between the hours of 1000 and 1500 in the spring, summer, and autumn, because this is the only time when enough

UVB photons reach the earth's surface to produce vitamin D_3 in the skin. This is 25% of what would cause a minimal erythemal response.

Chronic excessive sun exposure is known to cause skin damage, skin wrinkling and skin cancers [20,21]. In New Zealand and Australia, sun exposure causes 99% of non-melanoma

skin cancers such as basal and squamous cell carcinoma and 95% of melanoma [22]. At the same time, a reasonable sun exposure is required to prevent vitamin D deficiency. Therefore, in these region, an application of a sunscreen with a SPF of 15 is recommended after sensible exposure, to prevent the damaging effects of chronic excessive exposure to sunlight. Veiled woman or individuals who remain covered outside for cultural reasons, institutionalized individuals and elderly confined to indoors who have in adequate sun exposure are prone to vitamin D deficiency. The most important determinant of vitamin D deficiency in infants is maternal 25-OHD status. Infants born to veiled, dark skinned mother have vitamin D deficiency. Breast milk is a poor source of vitamin D containing less than 20 IU/liter. Infants who are exclusively breast-fed for prolonged time are prone to vitamin D deficiency.

Malabsorption, celiac disease, cystic fibrosis and Whipple's surgery interferes with the absorption of the vitamin D from dietary sources. Anticonvulsants, glucocorticoids, rifampicin, highly active anti- retroviral therapy and immuno suppressive agents increase the metabolism of 25-OHD. Chronic liver failure and renal failure decrease the synthesis of vitamin D.

Vitamin D is inversely associated with body mass index more than 30 kg/sq.m [12].

Vitamin D is fat soluble and is stored in the body fat. Any excess vitamin D_3 that is produced during exposure to sunlight can be stored in the body fat and used during the winter, when little vitamin D_3 is produced in the skin. However, in obese individuals, vitamin D is sequestered in the abdominal fat and this fat can be an irreversible sink for vitamin D, increasing the risk of vitamin D deficiency [23,24]. Inherited disorders in the metabolism of vitamin D and phosphate also leads to vitamin D deficiency. Polymorphisms in the genes for vitamin D-binding protein, 7- dehydrocholesterol reductase (which affects the amount of substrate 7-dehydrocholesterol in skin) and 25-hydroxylase may contribute to variation in 25-OHD levels. The causes and effect of vitamin D deficiency are summarized in the **table 3**.

CAUSE	EFFECT
Decreased skin synthesis	
Sunscreen use — absorption of UVB radiation by sunscreen	Decreases vitamin D_3 synthesis — SPF 8 by 95%, SPF 15 by 98%
Skin pigment — absorption of UVB radiation by melanin	Decreases vitamin D_3 synthesis by as much as 99%
Aging — reduction of 7-dehydrocholesterol in the skin	Decreases vitamin D_3 synthesis by about 75% in a 70-year-old
Season, latitude, and time of day — number of solar UVB photons reaching the earth depending on zenith angle of the sun (the more oblique the angle, the fewer UVB photons reach the earth)	Above about 35 degrees north latitude (Atlanta), little or no vitamin D_3 can be produced from November to February
Patients with skin grafts for burns — marked reduction of 7-dehydrocholesterolin the skin	Decreases the cutaneous production of vitamin D_3
Decreased bioavailability	

Malabsorption — reduction in fat absorption, resulting from cystic fibrosis, celiac disease, Whipple's disease, Crohn's disease, bypass surgery, medications that reduce cholesterol absorption, and other causes	Impairs the absorption of vitamin D
Obesity — sequestration of vitamin D in body fat	Reduces availability of vitamin D
Increased catabolism	
Anticonvulsants, glucocorticoids, HAART (AIDS treatment), and antirejection medications — binding to the steroid and xenobiotic receptor or the pregnane X receptor	Activates the destruction of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D to inactive calcitroic acid
Breast-feeding	
Human breast milk – Poor source of Vitamin D	Increases infant risk of vitamin D deficiency when exclusively breast-fed
Decreased synthesis of 25-hydroxyvitamin D	
Liver failure	
Mild-to-moderate dysfunction	Causes malabsorption of vitamin D, but production of 25-hydroxyvitaminD is possible
Dysfunction of 90% or more	Results in inability to make sufficient 25-hydroxyvitamin D
Increased urinary loss of 25-hydroxyvitamin D	
Nephrotic syndrome — loss of 25-hydroxyvitamin D bound to vitamin D–binding protein in urine	Results in substantial loss of 25-hydroxyvitamin D to urine
Decreased synthesis of 1,25-dihydroxyvitamin D	
Chronic kidney disease	
Stages 2 and 3 (estimated glomerular filtration rate, 31 to 89 ml/min/1.73 m^2) Hyperphosphatemia increases fibroblast growth factor 23, which decreases 25-hydroxyvitamin D-1α hydroxylase activity	Causes decreased fractional excretion of phosphorus and decreased serum levels of 1,25-dihydroxyvitamin D
Stages 4 and 5 (estimated glomerular filtration rate <30 ml/min/1.73 m^2) In ability to produce adequate amounts of 1,25-dihydroxyvitamin D	Causes hypocalcemia, secondary hyperparathyroidism, and renal bone disease
Heritable disorders — rickets	
Pseudovitamin D deficiency rickets (vitamin D–dependent rickets type 1) — mutation of the renal 25-hydroxyvitamin D-1α-hydroxylase gene (CYP27B1)	Causes reduced or no renal synthesis of 1,25-dihydroxyvitamin D
Vitamin D–resistant rickets (vitamin D–dependent rickets type 2) — mutation of the vitamin D receptor gene	Causes partial or complete resistance to 1,25-dihydroxyvitamin D action, resulting in elevated levels of 1,25-dihydroxyvitamin D
Vitamin D–dependent rickets type 3 — over production of hormone responsive- element binding proteins	Prevents the action of 1,25-dihydroxyvitamin D in transcription, causing target-cell resistance and elevated levels of 1,25-dihydroxyvitamin D
Autosomal dominant hypophosphatemic rickets — mutation of the gene for fibroblast growth factor 23, preventing or reducing its breakdown	Causes phosphaturia, decreased intestinal absorption of phosphorus, hypophosphatemia, and decreased renal 25-hydroxyvitaminD-1α-hydroxylase activity, resulting in low-normal or low levels of 1,25-dihydroxyvitamin D

X-linked hypophosphatemic rickets — mutation of the PHEX gene, leading to elevated levels of fibroblast growth factor 23 and other phosphatonins	Causes phosphaturia, decreased intestinal absorption of phosphorus, hypophosphatemia, and decreased renal 25-hydroxyvitamin D-1α-hydroxylase activity, resulting in low-normal or low levels of 1,25-dihydroxyvitamin D
Acquired disorders	
Tumor-induced osteomalacia — tumor secretion of fibroblast growth factor 23 and possibly other phosphatonins	Causes phosphaturia, decreased intestinal absorption of phosphorus, hypophosphatemia, and decreased renal 25-hydroxyvitamin D-1α-hydroxylase activity, resulting in low-normal or low levels of 1,25-dihydroxyvitamin D
Primary hyperparathyroidism — increase in levels of parathyroid hormone, causing increased metabolism of 25-hydroxyvitamin D to 1,25-hydroxyvitamin D	Decreases 25-hydroxyvitamin D levels and increases 1,25-dihydroxyvitamin D levels that are high-normal or elevated
Granulomatous disorders, sarcoidosis, tuberculosis, and other conditions, including some lymphomas — conversion by macrophages of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D	Decreases 25-hydroxyvitamin D levels and increases 1,25-dihydroxyvitamin D levels
Hyperthyroidism — enhanced metabolism of 25-hydroxyvitamin D	Reduces levels of 25-hydroxyvitamin D

8. Consequences of Vitamin D Deficiency

Signs and symptoms of vitamin D deficiency include hypocalcemic seizures, tetany in infants, rickets in toddlers and children, osteomalacia and osteoporosis in adults. In the presence of $1,25(OH)_2D$, intestinal calcium absorption increases from 10 - 15% to 30% - 40% and phosphorus absorption from 60% to 80%. Vitamin D deficiency causes compensatory increases in parathyroid hormone synthesis and secretion. PTH stimulates 1 alpha-hydroxylase in the kidney and increases the conversion of 25-OHD to $1,25(OH)_2D$, there by enhancing intestinal calcium absorption while worsening vitamin D deficiency. Increased PTH enhances the tubular reabsorption of calcium by the kidney. As the vitamin D deficiency progresses the parathyroid glands are maximally stimulated resulting in secondary hyperparathyroidism. Increased PTH levels enhances phosphaturia resulting in low serum phosphorus or hypophosphatemia. In adequate calcium-phosphorous product causes diminished mineralization of skeleton resulting in classic signs of rickets and osteomalacia. Rickets results from poor osteoid mineralization adjacent to the growth plate and is only seen during growth, with a peak incidence during the periods of rapid growth in early infancy and early puberty [14]. On the other hand, Osteomalacia results from inadequate osteoid mineralization at sites of bone modelling and remodeling and is common to both children and adults. Some of the osseous signs of Vitamin D deficiency are listed in the **Table 4**. PTH also induces transformation of pre-osteoclasts to osteoclasts. Mature osteoclasts dissolve the mineralized collagen matrix in the bone and mobilizes calcium from skeleton causing osteoporosis and increased risk of fracture. Unlike osteoporosis, osteomalacia is associated with bone pain. It is believed that the hydration of the demineralized matrix beneath the periosteum elevates the periosteum and pushes it outwards [1]. The stretching of the periosteum stimulates the sensitive nerve endings to cause pain in osteomalacia.

Table 4. Osseous signs of vitamin D deficiency (common to less common)

• Swelling of wrists and ankles
• Rachitic rosary (enlarged costochondral joints felt lateral to the nipple line)
• Genu varum, genu valgum or windswept deformities of the knee
• Frontal bossing
• Limb pain and fracture
• Craniotabes (softening of skull bones, usually evident on palpation of cranial sutures in the first 3 months)
• Hypocalcemia - seizures, carpopedal spasm
• Myopathy, delayed motor development
• Delayed fontanelle closure
• Delayed tooth eruption
• Enamel hypoplasia
• Raised intracranial pressure
• Brown tumor secondary hyperparathyroidism

Radiological features

• Cupping, splaying and fraying of the metaphysis of the ulna, radius and costochondral junction
• Coarse trabecular pattern of metaphysis
• Osteopenia
• Fractures

Biochemical features of vitamin D deficiency include hypocalcemia, secondary hyperparathyroidism, hypophosphatemia and elevated alkaline phosphatase titers.

9. Non-Skeletal Consequences of Vitamin D Deficiency

Non-osseous features of vitamin D deficiency include cardiomegaly and marrow fibrosis with pancytopenia or microcytic hypochromic anemia. Vitamin D deficiency is associated with increased risk of common cancers in the prostate, colon, breast possibly related to dysregulation in cellular proliferation and differentiation [1,5,11]. Similarly, dysregulation in immune function results in increased risk of autoimmune diseases like multiple sclerosis, type 1 diabetes mellitus and rheumatoid arthritis and many infectious diseases like tuberculosis [23,10,24]. Vitamin D deficiency also causes muscle weakness, gait abnormality and increased risk of fall [11,25].

Table 4: Vitamin D intakes recommended by the IOM and the Endocrine Practice Guidelines Committee

Life stage group	IOM recommendations				Committee recommendations for patients at risk for vitamin D deficiency	
	AI	EAR	RDA	UL	Daily requirement	UL
Infants 0 to 6 months 6 to 12months	400 IU(10 µg) 400 IU (10 µg)			1,000 IU (25 µg) 1,500 IU (38 µg)	400 -1,000 IU 400-1,000 IU	2,000 IU 2,000 IU
Children 1-3 yr 4-8 yr		400 IU(10 µg) 400 IU (10 µg)	600 IU(15 µg) 600 IU(15 µg)	2,500 IU(63 µg) 3,000 IU(75 µg)	600-1,000 IU 600-1,000 IU	4,000 IU 4,000 IU
Males 9-13 yr 14-18yr 19-30yr 31-50yr 51-70yr >70yr		400 IU(10 µg) 400 IU (10 µg) 400 IU(10 µg) 400 IU (10 µg) 400 IU(10 µg) 400 IU (10 µg)	600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg) 800 IU(15 µg)	4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg)	600-1,000 IU 600-1,000 IU 1,500-2,000 IU 1,500-2,000 IU 1,500-2,000 IU 1,500-2,000 IU	4,000 IU 4,000 IU 10,000 IU 10,000 IU 10,000 IU 10,000 IU
Females 9-13 yr 14-18yr 19-30yr 31-50yr 51-70yr >70yr		400 IU(10 µg) 400 IU (10 µg) 400 IU(10 µg) 400 IU (10 µg) 400 IU(10 µg) 400 IU (10 µg)	600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg) 800 IU(15 µg)	4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg)	600-1,000 IU 600-1,000 IU 1,500-2,000 IU 1,500-2,000 IU 1,500-2,000 IU 1,500-2,000 IU	4,000 IU 4,000 IU 10,000 IU 10,000 IU 10,000 IU 10,000 IU
Pregnancy 14-18yr 19-30yr 31-50yr		400 IU(10 µg) 400 IU (10 µg) 400 IU(10 µg)	600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg)	4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg)	600-1,000 IU 1,500-2,000 IU 1,500-2,000 IU	4,000 IU 10,000 IU 10,000 IU
Lactation[a] 14-18yr 19-30yr 31-50yr		400 IU(10 µg) 400 IU (10 µg) 400 IU(10 µg	600 IU(15 µg) 600 IU(15 µg) 600 IU(15 µg)	4,000 IU(100 µg) 4,000 IU(100 µg) 4,000 IU(100 µg)	600-1,000 IU 1,500-2,000 IU 1,500-2,000 IU	4,000 IU 10,000 IU 10,000 IU

Abbreviations: AI: adequate intake; EAR: estimated average ewquirement; UL: tolerable upper intake level; [a]mother's requirement, 4,000-6,000 IU/d (mothers's intake for infant's requirement if infant is not receiving 400 IU/d). Reproduced from M. F. Holick et al. J Clin Endocrinol Metab, July 2011, 96(7):1911–1930.

10. Treatment of Vitamin D Deficiency

Cholecalciferol (25-OHD_3) is preferable over ergocalciferol (25-OHD_2) for treatment of vitamin D deficiency [15], although both are efficient. Vitamin D has a high therapeutic index. Vitamin D intoxication manifests only beyond 150 ng/ml (375 nmol/l) of 25-OHD. In general, for every 100 IU of vitamin D taken in, serum 25-OHD concentration increases approximately by 1 ng per milliliter (3 nmol per liter). The treatment and prevention strategies for vitamin D

deficiency across different age groups and specific etiology are summarized in the **Table 5**.

The most serious consequence of Vitamin D deficiency is hypocalcemic seizures and are common in infants less than 6 months of age. Aim of the therapy is to prevent seizures [14]. Intravenous bolus of 10 ml of 10% calcium gluconate over 20 minutes is recommended for seizures. If seizures recur, a repeat bolus or calcium infusion up to 4mmol/kg/day is administered until serum calcium is over 1.8 mmol/L. 1α-hydroxyvitamin D_3 or calcitriol at 60–120 ng/kg/day shall be co-administered with oral calcium until the serum calcium concentration is over 2.1 mmol/L. Australian Pediatrics endocrine group -Consensus statement recommends the following treatment protocol for vitamin D deficiency, depending on the age group. Neonates are given 1000 IU/day for 3 months and a maintenance dose of 400 IU/day. Infants aged 1 to 6 months are given 3000 IU/d for 3 months or 300000 IU over 1- 7 days followed by maintenance dose of 400 IU/day. Children more than 1 year are given 5000 IU for 3 months or 500000 IU over 1- 7 days and a maintenance dose of 600 to 1000 IU/day. High dose Stoss therapy is administration of the total requirement of vitamin D as a single dose either orally or by intramuscular injection. However, a few cases of hypercalcemia and nephrocalcinosis has been reported with such therapy.

Table 5: Treatment and prevention strategies for Vitamin D deficiency

Age/ Cause	Treatment with vitamin D (D_2 or D_3)	End point	Prevention and maintenance dose of vitamin D	Monitoring
0-1 year	2000 IU/d D x 6 weeks Or 50000IU/week x 6 + calcium 50 mg/kg/d x 1-2 week	Repeat if 25-OHD <30 ng/ml	400 – 1000 IU/d	Serum calcium and alkaline phosphatase monthly Serum calcium, magnesium, phosphorus, alkaline phosphatase q 3 months; Wrist X ray- to assess the healing of rickets; 25-OHD annually
1 – 18 years	2000 IU/d x 6 weeks Or 50000IU/week x 6	Repeat if 25-OHD <30 ng/ml	600 – 1000 IU/d	
Adults	6000 IU/d or 50000 IU/week x 8	Repeat if 25-OHD <30 ng/ml	1500 – 2000 IU/d	
Pregnant & Lactating woman	4000 – 6000 IU/d Or 50000 IU/week x 8	Repeat if 25-OHD <30 ng/ml	1500 – 2000 IU/d	
Nephrotic syndrome	50000 IU/week x 8	Repeat if 25-OHD <30 ng/ml	1000 – 2000 IU/d	
Obese	6000 – 10000 IU/d	Repeat if 25-OHD <30 ng/ml	3000 – 6000 IU/d	

Malabsorption	300000 IU per month intramuscularly x 3		300000 or 600000 IU i.m every year	
Extrarenal production of 1,25 $(OH)_2D$; Granulomatous disease or some lymphoma	50000 IU/week x 4	maintain 25-OHD between 20 – 30 ng/ml to prevent hypercalcemia	400 IU/d	Serial 25-OHD and serum Calcium
Primary Hyperparathyroidism and vitamin D deficiency	50000 IU/week x 8	Repeat if 25-OHD <30 ng/ml	800 – 1000 IU/d	Serial level of serum calcium
Chronic kidney disease Stage 2 & 3	50000 IU/week x 8	Repeat if 25-OHD <30 ng/ml	1000 IU/d	Control serum phosphate
Chronic kidney disease Stage 4 & 5	0.25 – 1 mcg/day of 1,25 $(OH)_2$ D Calcitriol or Doxecalciferol 10 -20 mcg PO three times per week		1000 IU/d + calcitriol	

During pregnancy, daily regimen should at least include a prenatal vitamin containing 400 IU vitamin D with a supplement that contains at least 1000 IU vitamin D. Lactating women may need to take a minimum of 1400–1500 IU/d, and to satisfy the requirements of an infant who is exclusively breast fed, the mother requires 4000 to 6000 IU/d to transfer enough vitamin D into her milk.

In adults, Vitamin D deficiency is treated with oral dose of 50,000 IU of D_2 or D_3 weekly once for 8 weeks or until 25-OHD is over 30 ng/ml and maintenance dose of 1500 – 2000 IU/day. Supplementation may be required for long-term in many individuals as the risk factors predisposing to vitamin D deficiency persist life long. In elderly people aged 70 years or more, Level I evidence indicates Calcium supplementation 1000 to 1300 mg/day combined with vitamin D 800- 1000 IU/d reduces the risk of falls and fracture [26,27]. However, long-term calcium therapy is not recommended as it predisposes to hypercalcemia, hypercalciuria, renal stones and non- adherence due to unpalatability.

In the subset of patients with reduced bioavailability of vitamin D (due to malabsorption, post- biliary surgery, Whipple's surgery), a dose of 300000 IU of D_2 or D_3 intramuscular (i.m) route every month for 3 months or until 25-OHD is over 30 ng/ml is recommended. A maintenance dose of 30000 or 60000 IU i.m every year.

In subjects with granulomatous disease or some lymphoma, wherein macrophages or immune cells convert 25-OHD into 1,25-$(OH)_2D$, Vitamin D deficiency is treated judiciously

with 50000 IU every week for 4 weeks maintaining the serum levels of 25-OHD between 20 and 30 ng/ml. A maintenance dose of 400 IU/day is sufficient. These patients should be closely monitored for hypercalcemia and hyperuricemia.

The Endocrine Practice Guidelines Committee suggest that the maintenance tolerable upper limits (UL) of vitamin D, should be 1000 IU/d for infants up to 6 months, 1500 IU/d for infants from 6 months to 1 year, 2500 IU/d for children aged 1–3yr, 3000 IU/d for children aged 4–8yr, and 4000 IU/d for everyone over 8yr and should not exceed the above without medical supervision. However, higher levels of 2000 IU/d for children 0–1 year, 4000 IU/d for children 1–18 yr, and 10,000IU/d for children and adults, 19yr and older may be needed to correct vitamin D deficiency. Treatment and prevention strategies for vitamin D deficiency is summarized in the **Table 3**. Treatment with calcitriol (1,25-$(OH)_2$D) is only indicated for certain cases of hypocalcemia and chronic kidney failure.

11. Conclusion

Vitamin D deficiency is a pandemic. The major cause of vitamin D deficiency is inadequate sun exposure. The serum level of 25-hydroxy vitamin D is the best indicator of vitamin D status. 25-OHD above 20 ng/ml is required to prevent rickets in children and osteomalacia in adults. An optimal range of 25-OHD between 30 and 60 ng/ml not only maximizes bone health but is also necessary for the extra-renal production of 1,25 $(OH)_2$D and are believed to provide preventive as well as therapeutic benefits in a wide variety of common cancers, autoimmune disorders, and infectious diseases. Sun exposure for 5 – 15 min between 1000 and 1500 hours in the spring, summer and autumn is safe and sensible. A minimum of 400 IU, 600 IU and 800 - 1000IU/day from dietary and supplementary sources among infants and children, adults, and elderly respectively, should guarantee vitamin D sufficiency in population at risk.

12. Refernces

1. Holick MF. Vitamin D Deficiency. N Engl J Med. 2007; 357(3): 266–281.

2. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011; 96(7): 1911–1930.

3. Holick MF, Chen TC. Vitamin D deficiency: A worldwide problem with health consequences. Am J Clin Nutr. 2008; 87(4).

4. Holick, Michael F, Wacker M. Sunlight and Vitamin D. Dermatoendocrinol. 2013; 5(1): 51–108.

5. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Vol. 80, The American journal of clinical nutrition. 2004.

6. Holick MF. Vitamin D: A D-Lightful health perspective. In: Nutrition Reviews. 2008.

7. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. Chem Biol. 2014; 21(3): 319–329.

8. Rosen CJ. Vitamin D Insufficiency. World Health. 2011; 248–254.

9. Zhang R, Naughton DP. Vitamin D in health and disease: current perspectives. Nutr J. 2010; 9(1): 65.

10. Bikle D. Nonclassic actions of vitamin D. Vol. 94, Journal of Clinical Endocrinology and Metabolism. 2009. p. 26–34.

11. Rosen CJ, Adams JS, Bikle DD, Black DM, Demay MB, Manson JAE, et al. The nonskeletal effects of vitamin D: An endocrine society scientific statement. Endocr Rev. 2012; 33(3): 456–492.

12. Rosen CJ. Vitamin D Insufficiency. N Engl J Med. 2011; 364: 248–254.

13. Nowson CA, McGrath JJ, Ebeling PR, Haikerwal A, Daly RM, Sanders KM, Seibel MJ, Mason RS; Working Group of Australian and New Zealand Bone and Mineral Society ES of A and OA. Vitamin D and health in adults in Australia and New Zealand: a position statement. Med J Aust. 2012; 196: 1–7.

14. Munns C, Zacharin MR, Rodda CP, Batch J a, Morley R, Cranswick NE, et al. Prevention and treatment of infant and childhood vitamin D deficiency in Australia and New Zealand: a consensus statement. Med J Aust. 2006; 185(5): 268–272.

15. Brit. Assoc. Derm. Consensus Vitamin D position statement 2011. Nutrition. 2011.

16. Pearce SHS, Cheetham TD. Diagnosis and management of vitamin D deficiency. BMJ. 2010; 340: b5664.

17. Kennel K a, Drake MT, Hurley DL. Vitamin D deficiency in adults: when to test and how to treat. Mayo Clin Proc. 2010; 85(8): 752–758.

18. Paxton G a, Teale GR, Nowson C a, Mason RS, McGrath JJ, Thompson MJ, et al. Vitamin D and health in pregnancy, infants, children and adolescents in Australia and New Zealand: a position statement. Med J Aust. 2013; 198(3): 142–143.

19. Prentice A. Vitamin D deficiency: A global perspective. In: Nutrition Reviews. 2008.

20. Veierod MB, Weiderpass E, Thorn M, Hansson J, Lund E, Armstrong B, et al. A Prospective Study of Pigmentation, Sun Exposure, and Risk of Cutaneous Malignant Melanoma in Women. JNCI J Natl Cancer Inst. 2003; 95(20): 1530–1538.

21. Contet-Audonneau JL, Jeanmaire C, Pauly G. A histological study of human wrinkle structures: Comparison between sun-exposed areas of the face, with or without wrinkles, and sun-protected areas. Br J Dermatol. 1999; 140(6): 1038–1047.

22. Armstrong BK, Kricker A. The epidemiology of UV induced skin cancer. J Photochem Photobiol B Biol. 2001; 63(1–3): 8–18.

23. Schwalfenberg GK. A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. Mol Nutr Food Res. 2011; 55(1): 96–108.

24. The odoratou E, Tzoulaki I, Zgaga L, Ioannidis JP. Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials. BMJ. 2014; 348: g2035.

25. Murad MH, Elamin KB, Abu Elnour NO, Elamin MB, Alkatib A a, Fatourechi MM, et al. Clinical review: The effect of vitamin D on falls: a systematic review and meta-analysis. J Clin Endocrinol Metab. 2011; 96(10): 2997–3006.

26. IOM I of M (US). Dietary reference intakes for calcium and vitamin d. Vol. 130, IOM. 2011.

27. Ross A, Taylor C, Yaktine A. Dietary Reference Intakes for Calcium and Vitamin D. Inst Med. 2010.

Vitamin D Deficiency: Causes & Treatment

Chapter 2

Enviromental Factors and Multiple Sclerosis

Georgi Slavov*

Department of Neurology, Medical University, Plovdiv, Bulgaria.

***Correspondence to: Georgi Slavov,** Department of Neurology, Medical University, Plovdiv, Bulgaria.*

Email: georgi.slavov.15130@gmail.com

1. Introduction

Multiple sclerosis (MS) is a socially significant immune-mediated disease with unknown etiology. The first symptoms are usually registered in the reproductive age-between the 20th and 40th year, with first peak between the 21st and 25th year, and the second peak between the 41st and 45th year. In less than 5 to 10% of the cases the onset of the disease is before the 10th and the after the 50th year. Results from different studies of the disease evolution show that women suffer from it more frequently than men-ration women/ men 2:1, 3:1 [1,2,3,4,5]. The three main forms of progress of the disease: relapsing-remitting (RRMS), secondary-progressive (SPMS) andprimary-progressive (PPMS), reflect the chronic development of the process, disabling the patients for a different period of time. The relapsing-remitting MS is observed in 85-90% of all patients; typical are oligoclonal bands in the cerebrospinal fluid, the clinical development in on average of 90% of the cases correlates with the MRI findings. The fast development of new symptoms or deterioration of old ones, with duration of over 24 hours, is followed by remission and recovery to a different degree. The relapsing-remitting phase progresses into the therapeutically unfavorable secondary-progressive phase in 50-70% of the patient after 10 years after the onset of the disease, on the average [6,7,8,9]. A joint study of the WHO and the World Bank, *Global Burden of Disease Study* (1998), shows that "MS is one of the hundred most severe diseases in the world, and thus it is listed among the ovarian and prostate cancer, trachoma and leprosy" [10]. A comprehensive study of the aspects of the immune-mediated process of CNS, the proving of scientific facts on the participation of specific factors in the pathogenesis of the disease are a premise for the optimization of the therapeutic methods, through inclusion of new immune-effective drugs.

2. Immunology

Multiple sclerosis (MS) is an immune-mediated disease, which is characterized by demyelination, axonal transection and oligodendropathy in the central nervous system (CNS). The modern vision on the immune-mediated genesis is based on the analyses of the changes in the immunological indices in the blood and the CSF of the patients, in the focal lesions and on the results obtained during experimental allergic encephalomyelitis (EAE). The interaction between peripheral autoreactive antigenic cells (ag) of unknown origin causes clonal expansion of proinflammatory populations and mediators of the inflammation, which pass the blood-brain barrier (BBB) and cause perivascular demyelination, neurodegeneratuion and axonal disorders in the CNS [11,12].

Today MS is considered as organ-specific disease with the following characteristics:

- Specificities of the etiology, including assumed viral agent, genetic factors and environmental factors;

- Specificity of the autoantigens from the target organ: myelin basic protein (MBP), myelin-oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG);

- Aberrant immune response from Th1- and Th17-mediated, disseminated inflammation of the central myelin structures with cytokine production imbalance from Th1,Th17,Th2 и CD4+CD25+FoxP3 subpopulations in the CNS and the periphery [11,13,14,15,16].

2.1. Activation of peripheral antigen-specific immunocompetent cells

In healthy individuals there are low concentrations of autoreactive T and B lymphocytes, which due to suppression remain tolerant to the individual's own antigens. In MS, it is assumed that the impaired immune tolerance and molecular mimicry is the reason for the immune-mediated reactivity in the peripheral circulation and the CNS [17]. Another hypothesis suggests transportation of antigens from the CNS to the peripheral flow after injury and/or infection [18,19,20]. This belief is supported by experimental results-inoculation of MBP in the brain and the CSF of animals causes cellular and hormonal responses in the peripheral flow. It is believed, that the cerebrospinal and the interstitial fluid carry the antigens from the CNS, expressed on the membranes of the antigen-presenting cells (APC) [11]. APC monocytes, macrophages, B-lymphocytes and dendritic cells and parenchymal microglia cells express on their surface highly immunogenic part of the antigen, costimulatory molecules and class I/ II molecule of the major histocompatibility complex (MHC). The T-lymphocytes recognize the antigens through two types of T-cell receptors (TCR)–αβ/γδ [21]. The trimolecular complex (MHC class I/II molecule, antigen and TCR) is the first signal that induces immune

response, however, it is not sufficient for complete T-cell activation. Complete sensitization occurs after interaction between costimulatory molecules, expressed on the membranes of T-lymphocytes(CD28, CTLA-4, CD40 ligand) and APC (B7.1(CD80), B7.2(CD86), CD40 (22). After complete T-cell sensitization, the aberrant immune response is maintained by the APC cytokine secretion-IL12, IL23, which stimulates the T-cells to secrete inflammation inductors IL2, IL17, IFNγ and proteins with key role for their transfer through the BBB-sphingosine-1-phosphate receptor [22]. In MS it is assumed that the Th1-dependent synthesis of IFNγ, TNFα, IL2, IL12 andthe Th17-mediated production of IL17, IL22, are inflammation inductors, while the Th2-mediated secretion of IL4, IL5, IL6, IL13 has anti-inflammatory effect [18,23,24,25,26,27].

2.2. Blood-brain barrier-transfer of immunocompetent cells and inflammatory antigens from the periphery to the CNS

The blood-brain barrier, composed of endothelial cells and extracellular matrix, is impermeable for large molecules and for the majority of cells. The transfer of immunocompetent cells and other inflammatory antigens through the BBB determines immune inflammation in the CNS with subsequent destruction of the myelin [28].

The influx of inflammatory cells through the BBB includes several stages:

- Attachment of the leukocytes to the vascular endothelium through adhesion molecules-E-selectin and P-selectin, integrin, expressed on their surface. The process is realized through the connection-chemokine receptor on the leukocyte surface / ligand on the endothelial membrane;

- Activation of integrins, mediated by G-protein production;

- Adhesion of the leukocytes to the endothelium after bonding of the leukocyte receptors with own ligands on the membranes of endothelial adhesion molecules-ICAM1, ICAM2; increased leukocyte adhesion following repeated connection of late leukocyte activating factor-VLA-4 with the receptor of the endothelial molecule VCAM1;

- Influx of leukocytes in the CNS [29].

2.3. Immune-inflammatory process and neurodegeneration in CNS

The influx of CD4+ T-cells into the CNS is followed by intensive immune inflammation. The process is triggered after presentation of autoantigens in complex with MHC molecule from local cells-microglial cells and dendritic, as well as from macrophages and monocites in the CNS [17,22,30]. The last stage finishes with tissue damage in the CNS. The tissue disorders have different mechanism: immune-inflammatory, degenerative (Wallerian degeneration),

ischemic, oxidative. Recent studies show results for axonal disorders in the early stages of the disease. Axonal transection is found in patients with disease progression from 2 weeks to 27 years [31]. The number of axons with transection in the lesion areas exceeds 11000 mm^3 [32]. The registered correlation between the immune-inflammatory activity in the focal lesions and the axonal damage is a reason to assume immune mechanism of the axonal transection, however, currently there is no proof for direct disorders caused by specific immune mediators [22]. Immunopathomorphological analyses show heterogeneity of the focal lesions with respect to the inflammatory infiltrate, the degreeof remyelination and the oligodendrocyte damages. Today, there are 4 models of myelin destruction. Samples 1 and 2 are typical for the experimental disease with classical autoimmune inflammation-T-cell and antibody-mediated destruction of myelin. Sample 3 is observed in the virus-induced experimental disease and suggests viral etiology, cytotoxic T-cell and proinflammatory cytokines, which mediate the cell death. Sample 4 is found during autopsy of patients with primary-progressive MS. It is assumed that this is caused by genetic disorders or metabolic defect. Sample 1 and 2 are present in all types of MS, 3 in aggressive progression, sample 4-in primary-progressive progression [33,34,35]. The notion of dependence between the sample of the myelin destruction, the clinical manifestations and the therapeutic response potential, is a ground for studying the participation of different factors in the immune-mediated process-genetic, hormonal and environmental, in order to improve the control of the disturbed immune regulation.

3. Results from Epidemiological and Population-Genetic Studies, Which Favor the Hypothesis on the Participation of Environmental Factors in the Etiopathogenesis of the Disease

3.1. Epidemiological studies results

3.1.1. Geographical areasof distribution of the disease:

In 1982, J. Kurtzke et al define geographical areas with high, average and low frequency of the MS disease. The areas of high frequency, disease rate over 30/ 100 000 people, are regions in Northern Europe (north latitude from 44º to 64º), Northern USA, South Canada, Southeast Australia, New Zeeland, etc. The areas with average frequency, disease rate between 5 to 20/ 100 000 (north latitude 32º-47º), include territories around the Mediterranean basin, the south regions of the USA and Africa and vast areas of Australia. Low disease rate, under 5/ 100 000 people, is observed in the regions with tropical and subtropical climate –Latin America, Japan, China, etc [22,36].

Bulgaria is located between 41,5º and 44º north latitude and the index “geographical area” suggests average disease rate. Epidemiological study conducted in the country by O. Kalafatova in 1983, shows disease rate of 21.3/ 100 000 people. The most recent study, N. Topalov 1999, registers double increase of this index for the last 17 years – 44.5/ 100 000 people

[37,38,39]. The correlation coefficient between the disease rate and the geographical area is high (r = 0.79), however, the disease rate in some countries like Korea and China, is significantly lower compared to the same geographical latitudes in Europe and North America [37]. The MS disease rate in France is lower than some European territories at the same latitude, such as Spain and Italy. In the northern regions of Europe, the rate is higher compared to the southern areas [36,39].

The MS distribution in the world is characterized with north-south gradient. Moving away from the Equator the disease rate increases, affecting larger groups of the population and reaching 100/ 100 000 in some northern regions. The epidemiological studies show decrease of the gradient in the northern hemisphere, in north-south direction and increase of the index in the same direction in Australia [22]. A study conducted in 1979 in the USA among 5305 war veterans, shows south-north gradient of the disease rate on the territory of the country. The results indicate variation of the index in the area of the 37th parallel – increase and decrease of the disease rate above and below the 37th parallel. In the eastern regions of the country, the 39th parallel divides the areas with high disease rate from those with average rate [40,41].

3.1.2. Migration and MS:

A study of the migration factor shows dependence between the area of residence during the years of adolescence (10-15 years of age) and the risk of developing the disease. If individuals from one geographical area move, before they reach 10 or 15 years of age, to area with different disease rate, the migrants would assume the risk rate of the new area. This index does not change in case of migration after that age. This finding is supported also by a study among population of European immigrants in South Africa. In case of permanent resettlement before the age of 15 years, the immigrants adopt the risk of the new area-13/ 100 000 people, if the immigration occurs after that age, the individuals keep the risk of the previous area-30-80/ 100 000 people [42]. These results suggest etiological significance of exogenic factors during the childhood years, with subsequent long latent period until the first onset of the disease in later years.

3.2. Data from genetic studies

The genetic etiology of the disease has been a subject to numerous studies. The first studies with families with MS date back from the end of the 19th century. Charcot presents evidence of genetically determined risk of the disease, confirmed by modern studies. In the first generation of the family with MS proband, the risk is 20 to 40% higher compared to the general population. The risk evaluation in twins from families with MS, there is concordance of 20% to 30% in the monozygotic twins, and 2-5% in the dizygotic twins. In genetically identical individuals, the discordance in the onset of the disease reaches 70% in both individuals. These results suggest that the effect of certain environmental factors plays a key role for the onset of

MS. [8,43,44,45,46]. The arguments in favor of the genetic predisposition are based on epidemiological observations of racial clustering of MS cases, and prevalence of the disease among ethnic groups from high-risk areas. There is low disease rate among black Africans, American Indians, Asian, etc [22,41]. In North Europe, the representatives of European ethnicities are at higher risk of developing the disease, than the individuals with different descent [36,43]. In Bulgaria, I. Milanov et al, 1999, establish low disease rate (18.4/100 000) among the Roma population, compared with the average for the country (44.5 / 100 000) [47]. Today, we believe that the genes of the major complex of the tissue compatibility (Major Histocompatibility Complex, MHC) in chromosome 6p21.3 control the response to the different antigens, as well as the hereditary risk in 10 to 50% of the MS cases in North Europe [48,19]. Three large-scale genetic studies among families with MS in USA, Canada and Australia identify 13 regions in chromosomes 5,6,17 and 19, associated with susceptibility to the disease; however, there is no definition for the term "risk population" [43]. The genetic tests confirm the polygenic nature of MS, and show intra-individual heterogeneity, which determines the variation in the severity of the immune respons, the type of demyelination and the response to the immune-stimulation therapy [29]. Today, the general believe is that MS is a disease with moderate hereditary risk, modifies to a different extend by environmental factors. The majority of the results are indicative of the participation of exogenic factors in the etiopathogenesis of the disease. In the last years, subject of scientific interest is the role of Vitamin D, as environmental factor, in the distribution of MS.

4. Synthesis and Metabolic Activation of Vitamin D

Vitamin D is secosteroid hormone. In the human body, there are two forms: endogenous, Cholecalciferol (Vit D_3), and exogenous Ergocalciferol (Vit D_2). The serum concentration is a result of: synthesis of cholecalciferol (D_3) from 7-dehydrocholesterol in the skin after UV irradiation, 70-80% of the total amount; synthesis ofergocalciferol (D_2) from plant plant sterol-ergosterol, following UV irradiation, 10-20% of the total amount [50,51,52,53,54]. In the circulation, there are metabolites: 25-hydroxyvitamin D (25(OH)D, calcidiol),1,25dihydroxyvitamin D (1,25$(OH)_2$D, calcitriol) and 24,25-dihydroxyvitamin D (24,25$(OH)_2$D). The serum levels of 25(OH)D are result of the Vitamin D_2 and D_3 hydroxylation in the liver, through cytochrome P450 (CyP) 27A1. 24,25$(OH)_2$D has high concentration in the circulation, insufficiently known biological properties and results from the metabolizing of 25(OH)D in the kidneys. 1,25$(OH)_2$D is biological active form with properties of hormone, obtained after hydroxylation (1α-hydroxylase, CyP 27B1) of 25(OH)D in the kidneys. Vitamin D and the metabolites are inactivated in the liver, through conjugation or oxidation, to glucuronides and sulfates [50]. They are transported to the target cells by serum glycoprotein. The biological effects are mediated by nuclear receptor VDR-a member of the nuclear hormone receptor superfamily [26]. More than 30 tissues in the human body express VDR: parathyroid, bone, renal, the B-cells of the

pancreas, keratinocytes, oligodendrocytes, astrocytes, macrophages, neurons, lymphocytes, etc [50,54,55,56,57]. The scientific evidence shows that 3% of the human genome depends on the Vitamin D Receptor, which explains the pleiotropic effects and the possible participation in the pathogenesis of different diseases (demyelinating, cardiovascular, colorectal carcinoma, etc). The pleiotropic effects, autocrine/ paracrine, occur at 10 nmol/l fold higher concentrations of $1,25(OH)_2D$ than the physiological levels, maintaining the Ca balance. Recent studies find intracellular synthesis of $1,25(OH)_2D$ through local hydroxylation of the 25(OH)D entering the target cells. The local synthesis provides high concentrations of the active metabolite, necessary to achieve the pleiotropic effects. The active 1α-hydroxylase is contained in the monocytes and macrophages, the renal and parathyroid cells and with intracellular synthesis $1,25(OH)_2D$ achieves immune-modulating effects [58].

5. Exogenic Factors Effecting the Vitamin D metabolism and their Relation to the Disease Rate of MS

5.1. Solar exposure/UV radiation

The main source for Vitamin D synthesis is the sunlight. After twenty minutes exposure to the sunlight, the synthesized Vitamin D is between 15-20 000 IU (199). In geographical areas with sunlight of less than 2000 hours annually, the disease rate of MS is higher, compared to areas with sunlight of more than 2000 hours [55].

Study results show that after maximum exposure to sunlight, the amount of the synthesized Vitamin D is comparable to oral intake of 250 μg Vitamin D [59,60].

The intensive exposure to sunlight between the age of 6 and 15 years reduces the risk of MS [54]. The intensity of the UV radiation and the Vitamin D synthesis depend on number of factors: geographical latitude, the altitude above sea level, seasonality, ozone saturation of the atmosphere, the Solar Zenith Angle (SZA), cloudiness, etc.

5.2. Latitude

The Vitamin D deficiency correlates to the geographic latitude and the daily hours of sunlight. In Northern European countries – Denmark, Finland, Ireland, there is deficiency in 50% of the children aged 12.5 yearsincluded in a study, with 50-75 nmoll/serum l25(OH)D being considered as sufficient. In South America there is deficiency in 53.4% of the studied individuals, in comparison to the general population [61,62].

5.3. Altitude

Study results show that the disease rate of MS varies depending on the altitude above the sea level of the place of residence-in reduces in areas at more than 1000 m above the sea level,

and increases in areas below 1000 m [59]. This suggests a correlation between the amount of synthesized Vitamin D and the changes in the intensity of the sunlight and UV irradiation. There are indicative results showing increased with 41% risk in white American adolescents with low serum levels of Vitamin D, as well as evidence of risk decrease when the serum concentrations are increased with 50 nmol/l [59,63]. According to D. Pierrot et al, the risk of MS is associated with low serum levels of Vitamin D in the childhood years [64]. In our country, in 1989 B. Yordanov analyzes the MS distribution in flat and mountain regions and finds lower rate in the flat regions. The results of N. Topalov (1999) regarding the geographical features of the patient's place of residence, until the first symptoms of the disease, confirm that the flat terrain is a "protective" factor (L:OR-0,55; 55% CI-0,30 до 0.99), [37,39].

5.4. Seasonality

There are scientific observations showing seasonal fluctuations in the clinical activity of MS-higher during the spring months compared to the winter. It is assumed that the reduced Vitamin D synthesis in the autumn and winter, caused by the less days of sunlight during these seasons, is the reason for the activation of the disease in the spring [55,65]. A study in Canada including 40 000 MS patients shows much higher number of individuals born in May than in November. CJ Willer et al report higher frequency of MS episodes during the warm months compared to the cold [66,67].

5.5. Ozone

The ozone saturation of the atmosphere is a specific factor determining the geographical area. The ozone reduces the UV rays reaching the Earth, and decreases the Vitamin D synthesis [68].

5.6. Solar Zenith Angle (SZA)

The size of the solar zenith angle changes the intensity of the UVradiation. When the zenith angle is big, the amount of the UV rays reaching the Earth surface is less, as they travel longer distance through the atmosphere with significant absorption/ dispersion. SZA is biggest early in the morning and during the winter, and it is smallest in the summer and at the Equator [68].

5.7. Cloudiness

The clouds absorb and reflect the UV rays and in some circumstances they cause high intensity of UV radiation. With the increase of the altitude above sea level, the UV radiation and the Vitamin D synthesis also increase [68].

Epidemiological and ecological studies show reverse causality between the UV rays and

the MS rate-at the Equator the disease has the lowest rate, the SZA there is the smallest and the UV radiation intensity is the highest [56,69].

5.8. Diet

The European Action on Nutrition and Health Survey, 1996, a study of adults from Southern and Northern Europe, shows lower serum level of 25(OH)D (20-30 nmol/l) in the residents of the South-European territories, than in those from the northern latitudes (40-50 nmol/l). The unexpected difference is associated with the diet habits-consumption of fish oil and cod liver (400 IU or 10μg daily) in Northern Europe, as well as the light skin pigmentation, increase the Vitamin D synthesis [50]. An interesting finding is the difference in the MS rate in Norway – lower compared to the other Scandinavian countries. It is assumed that this is a result of the specific diet habits in the country-high consumption of foods containing Vitamin D [56]. There are various factors effecting the synthesis and the metabolic activation of Vitamin D: limited exposure to sunlight, due to the geographical features of the region, or traditional clothing; the skin pigmentation; diet; slow metabolism with the advancement of age; gene polymorphism of 7-dehydrocholesterol reductase in the skin; cytochrome P450 25-hydroxylase in the liver; the Vitamin D-binding protein in the circulation; renal and liver diseases, etc [70]. Today it is believed, that the individual characteristics are a key factor in the correct evaluation of the multiple effects of the endo/exogenic factor in the synthesis and metabolism of Vitamin D. In this respect, there is an interesting study of Hawaiian surfers, following 15 hours/ week exposure to sunlight, for a period of 3 months. The studied population showed great variation of the serum concentrations-from 15 to 75 ng/ml. It is assumed that this difference depends on the individual characteristics [70].

6. Vitamin D Status Evaluation

An indicator of the Vitamin D status in the body, is the serum concentration of 25(OH) D, which correlates to the biologically active metabolite with the properties of hormone $1,25(OH)_2D$ [71]. The following criteria are used:

- The level necessary for maximum intestinal Ca absorption;
- The level necessary for highest bone mineral density;
- The level of minimum bone loss and reduced fracture risk [50,60].

In 2011 the US Institute of Medicine (IOM) accepts as optimal 25(OH)D concentration levels of ≥ 50 nmol/l, as insufficient 30-50 nmol/l, and as deficit 30 nmol/l [72]. Based on epidemiological observations, the American Endocrine Society recommends serum 25(OH)D concentrations from 75 to 110 nmol/l, as beneficial for the public health-reducing the risk of autoimmune, cardiovascular and infectious diseases, type 2 diabetes, etc [72]. A multicenter

research in Bulgaria, conducted in 2012 by A.M. Borisova et al, defines for the Bulgarian population the following concentrations: ≥ 50 nmol/l-sufficient; 25-49,99 nmol/l-insufficient, and < 25 nmol/l-deficit. The accepted levels are identical with those specified in the Consensus Conference in Germany in 2012, which allows comparative analysis of the study results of Bulgarian and international teams and cohorts [61]. The study includes 2032 individuals aged 20 to 80 years. Of them 47% men and 53% women, all residing in regions located from 41^{O} to 44^{O} northern latitude. In the studied population, the serum 25(OH)D concentrations ≥ 50 nmol/l reach optimum-PTH suppression and are defined as sufficient level, necessary for maintaining the optimal health status of the skeletal system. In 54.5% of the studied individuals, the results show Vitamin D deficiency at 24.2% insufficiency. Severe deficiency is observed two times more often in the women, compared to the men, within the age range 20 to 44 years ($p<0.007$) [37]. These facts suggest gender differences, modulated by the estrogens [73,74]. The results are of great scientific interest, following comparison with the last epidemiological survey in the country: twofold increase of the MS disease rate for the period 1983-1999; ratio women/men-1.9/1; average age of first symptoms 30.1 years [39].

The analysis of the results from the multicenter study brings the following conclusions: the gender is leading factor for the Vitamin D synthesis; second is the lifestyle and third is the place of residence, related to the environmental conditions (smog, number of sunny days in the year, dust, etc). The evaluation of the seasonal dynamics of Vitamin D in the studied group, shows significant differences in one season compared to the other three-winter (24.14 nmol/l, 95% CI : 25.70-28.58), spring (43.56 nmol/l, 95 CI: 41.96-45.17), summer (61.74 nmol/l, 95% CI: 58.95-64.55), autumn (52.75 nmol/l, 95% CI: 50.63-54.88),($p<0,001$) [50]. These results suggest the necessity of year-round prophylactics of the individuals with deficiency or insufficiency, so the negative consequences for the health of the population could be prevented. The literature review shows that one the discussed topics is the Vitamin D replacement dose [75,76,77,78,79,80,81]. For adults, the American Endocrine Society recommends daily intake of 1500-2000 IU Vitamin D, for serum 25(OH)D levels >75 nmol/l, necessary for the mineralization of the skeletal system. Many researchers believe, that the definition of sufficiency, insufficiency and deficiency of Vitamin D, must be determined according to the health consequences [50].

7. Experimental Evidence Substantiating the Hypothesis of Vitamin D Participation in the Pathogenesis of MS

There are experimental data proving the immune-modulating activity of Vitamin D in the CNS and the peripheral organs of the immune system. There is established expression of Vitamin D receptor (VDR) on APCs, dendritic cells, T and B-lymphocytes, macrophages, $1,25(OH)_2D$ synthesis from astrocytes, involvement of the vitamin in the myelin production in the CNS [25,82]. The treatment of suitable test animal with Vitamin D metabolites prevents

the development of experimental allergic encephalitis (EAE), if applied before the induction of myelin protein, and delays the progressive course when applied after the first clinical symptoms. In respect to EAE, M. Cantonara et al (1996) find the following: the application of $1,25(OH)_2D$ 24 hours before the induction with myelin protein, terminates the development of the pattern; the treatment of EAE with $1,25(OH)_2D$ in dose of 300 ng after the first symptoms, holds the progressive development [83]. An experiment of M. Cantorna et al (2000) with IL4-/IL4+ mice, finds: the treatment of IL4-mice with $1,25(OH)_2D$ has lower efficiency in holding the progressing clinical symptoms, compared with IL4+ test animals. The interruption of the pattern in the IL4- mice by high doses of $1,25(OH)_2D$ does not change the IL4 levels, but causes intensive production of TGFβ1. These results are grounds to assume that the high $1,25(OH)_2D$ doses are key factor for the differentiation of the regulatory subpopulation, associated with the production of the anti-inflammatory cytokine TGFβ1 [84].

The treatment of EAE with $1,25(OH)_2D$ registers reduction of the clinical symptoms, decrease of the infiltrates in some areas of the focal lesions, suppressed expression of MHC class II molecules, reduced number of APCs (monocytes, macrophages, microglial cells), reduction of the CD4+ lymphocytes and NOSII-Nytric oxide synthase [85]. After treating EAE mice with $1,25(OH)_2D$, JH. Chang et al discover: reduced number of lymphocytes in the CNS, suppressed expression of CCR6-CC120, facilitating the transfer of Th17-lymphocytes from the periphery to the CNS; increased production of IL10, mediated by TGFβ1; suppressed differentiation of Th17 phenotype by VDR. In vitro studies show: $1,25(OH)_2D$ inhibits the Th1 proliferation and the production of the anti-inflammatory cytokines IL2, IL6, IL12, TNFα, IFNγ; suppresses the expression of MHC class II molecules, suppresses the functional activity and holds the maturation of the dendritic cells; increases the expression of IL4. It is believed that $1,25(OH)_2D$ suppresses the differentiation to Th1/Th2 phenotype and induces the synthesis of regulatory T cells population. Some authors maintain that the immune-regulatory potential of $1,25(OH)_2D$ dominates the suppressive effects [27]. In case of combined application of Dexamethazone with $1,25(OH)_2D$, the CD4+ T-cells differentiate to Th2 phenotype with intensive production of IL5, IL10, and inhibited synthesis of IFNγ. The treatment with Dexamethazone alone suppresses the IFNγ secretion, without changing the levels of IL10 [86]. A research from 2007 studies combined treatment with IFNβ, cyclosporine and TX 527-hypocalcemic analogue of $1,25(OH)_2D$. The combination TX 527/ IFNβ has synergic and immune-modulating activity-inhibition of the antigen presentation, induction of the Th2-mediated cytokine secretion. The clinical effects-delay of the clinical symptoms, extending the paralysis-free period is significantly improved, compared to the application of TX 527 and IFNβ separately [87]. Based the results from experimental model with mice, A. Boonstra et al (2001) assume that $1.25(OH)_2D$ directly transforms the ratio Th1/Th2 subpopulations to domination of the Th2-mediated secretion of IL4. After stimulation with T-cell receptor, $1,25(OH)_2D$ affects the CD4+ T-cell activity, and causes suppression of the polarization to Th1 phenotype and in-

duction of the differentiation to Th2 phenotype. This and other results suggest that CD4+ T-cells are targets for 1,25$(OH)_2$D in the process of Th2 differentiation. The mechanism of the immune-modulating action of the Vitamin D metabolites is yet to be explained. Today, we assume that the biologically active metabolite with characteristics of hormone 1,25$(OH)_2$D achieves its effects through VDR, however, some authors find non-genomic mechanisms of its action. In this respect, there is an interesting study of two groups of test animals-VDR+/VDR- mice. The model includes 54% VDR+ mice, and 27% VDR- mice. Following treatment with 1,25$(OH)_2$D, the VDR+ animals do not develop experimental disease, while EAE is registered in 35% of the VDR- animals [88].

The experimental evidence substantiate the hypothesis for CD4+ T-cell mediated myelin destruction with imbalance of the cytokine secretion of TH1, TH17, TH2 subpopulations. During the exacerbation, the imbalance in the periphery is characterized by decrease of the regulatory CD4+CD25+FoxP3 subpopulation, associated with the production of the anti-inflammatory cytokines TGFβ1, IL10, inhibited IL4 synthesis and increased secretion of the proinflammatory cytokinesIFNγ, IL17, TNFα.

8. Evidence from Clinical Observations of the Participation of the Vitamin D Metabolites the MS Pathogenesis

In MS, large portion of the evidence of the immune potential of the Vit-D metabolites is obtained through experimental observations, and smaller portion from studies of MS patients. M Soilu-Hanninen et al (2005) compare the serum 25(OH)D concentrations during the summer and winter season, in the relapse-remission phases, in 40 RRMS patients and 40 clinical healthy individuals. During the winter there are no significant differences between the levels of patients and the controls. In the summer, the patients show significantly lower serum levels of 25(OH)D (58nmol/L), compared to the healthy controls (85 nmol/L). During MS relapse, the serum concentrations of 25(OH)D tend to decrease, compared with the remission phase, but remain within referent limits (196). In 15 RRMS patients, treated with 25(OH)D in increasing doses from от 0,5 μg/day to 2.5 μg/day and oral intake of Ca up to 800 mg/day for 48 weeks, there is significant decrease of the relapse rate, compared to previous period, and there is no hypercalcemia in dose of 2.5 μg/ day [69].

In 2006, in the USA is conducted a prospective case-control study among 7 million military personnel. 257 MS patient are registered, selected according to gender, race and age. Two groups are formed based on race including patients and controls. The comparison of the serum 25(OH)D concentrations between the two groups (patients and controls), shows lower serum levels of 25(OH)D in the representatives of the black race. The MS risk evaluation of the two groups with different ethnicity finds significant decrease of the index when the serum levels of 25(OH)D are increased with 50 nmol/L, only in the representative of the white race

[63]. J.Smolders et al (2008) study the connection of the serum concentrations of 25(OH)D, 1.25$(OH)_2$D with the severity of the neurological deficit /EDSS/ and the episode rate in 267 patients with relapsing-remitting, primary and secondary progressive MS. The serum Vitamin D levels are significantly lower in the progressive forms of the disease, compared to the relapsing-remitting form. There is no statistically significant connection between the serum levels of the metabolites and the episode rate. The serum concentrations of 25(OH)Dand 1.25$(OH)_2$D are lower in individuals in relapse, compared to the episode-free patients. No significant connection was found between the 1.25$(OH)_2$D levels and the severity of the neurological deficit. The analysis of the relative risk of relapse in patients with and without exacerbation, shows increase with 51% of the relapse-free patients with increase of the 25(OH)D levels in the serum with 10 nmol/L [60]. A study of J.Kragt et al (2009) evaluates the serum concentrations of 25(OH)D and 1,25$(OH)_2$D during the summer and the winter months, and according to the gender in 110 controls and 103 RRMS patients. Subject of interest are the differences related to the gender, and probably modulated by the estrogens: in the women there is negative correlation between the deficit /EDSS/ and the serum concentrations of 25(OH)D, unlike the men; only in the women the increase of the serum concentrations of 25(OH)D in the serum with 10 nmol/L, reduces the risk of MS with 19% [89]. D. Pierrot et al (2012) study the connection between the relapse rate and the changes in the 25 (OH)D serum after treatment with Cholecalciferol/amp.100 000 IU (average dose 3010 IU/ day in 156 RRMS patients). Until the moment of registration, 76 of the studied patient had been on therapy with drugs modifying the disease course – Copaxone 20 mg. s.c/ day; Avonex 30 μgr. i.m.once per week; βIFN1a 22/44 MIUs.c three times per week. In the other 80 patients, the therapy with Cholecalciferol starts simultaneously with medication modifying the disease course. The serum levels of 25(OH)D in the studied patients increase on average with 49-110 nmol/L. There is negative significant connection between the relapse rate and the serum concentration of 25(OH)D. Each increase of the serum concentrations with 10 nmol/L reduces the risk of relapse with 13.7%. This trends is kept until concentrations of 110 nmol/L are reached, then maintained unchanged above levels of 120 nmol/L. In MS patients with Vitamin D deficit, the authors recommend therapy, which can ensure 25(OH)D serum levels of 100 nmol/L [90]. Studies conducted among nurses (NIHS I, NHS II (2004), evaluate the risk of MS depending on the Vitamin D intake as poly-vitamins with average daily dose of 400 IU. The results show 40% reduction of the risk in the group with intake of poly-vitamins, compared to the other [91]. H.Derakhshand et al (2013) conduct double-blind, randomized study of 30 patients with optic neuritis and 25(OH)D serum levels under 30 nmol/L. The patients were grouped in two: 15 patients take Calcitriol50 000 IU per week for a period of 12 months; the other 15 patients take placebo. Subject to evaluation are: the dynamics of the MRI findings and the risk of another MS relapse. The individuals treated with Vitamin D show significantly lower risk of relapse (RR=0,316, p=0,007) and statistically important smaller number of lesions, both new and Gd enhanced lesions, compared to the placebo patients [92].

D. Golan et al (2013) conduct double-blind, randomized study of 45 MS patients treated with IFNβ in two dose regimens of Vitamin D_3. In 21 patients the daily intake of Vitamin D_3 is 800IU, and in 24 patients 4380 IU. Subject of evaluation are: the serum concentrations of Ca, 25(OH)D, IL17, IL10, IFNγ, the relapse rate, the severity of the neurological deficit (EDSS). In both groups the levels of 25(OH)D significantly increase (48/68 nmol/L: 48/122.6 nmol/L). In the patients with higher dose Vitamin D_3, statistically significant is the increase of IL17, unlike the patients with lower dose [93]. M. Soilu-Hänninen et al (2012) conduct double-blind, randomized study of 66 RRMS patients, treated with IFNβ 1b and additional therapy with Cholecalciferol 20 000 IU once per week. Two groups are formed: 34 patients take Vitamin D_3 in the mentioned dose, 32 patient take placebo. The serum levels of 25(OH)D in the patients treated with Vitamin D_3 reach 110 nmol/L and are significantly higher than the placebo group. There was no hypercalcemia. There were no significant differences between the two groups with respect of: new T2 lesions, number of active lesions, the relapse rate and the severity of the neurological deficit [94]. Study of G. Mossayebi et al (2011) analyzes the effect of Cholecalciferol therapy on the immune reactivity, MRI and clinical indications of the disease activity in 62 RRMS patients treated with IFNβ1b. Two groups are formed: 28 patients receive additional monthly dose of Vitamin D_3-300000 IU i.m.; in 34 patients the therapy is placebo. At the end of the 6th month after the beginning of therapy, there were no significant differences between the groups with respect of: severity of the neurological deficit and Gd enhanced lesions. The patients treated with Vit-D_3 show statistically significant increase of IL10, TGFβ1, compared to the placebo group. In identical comparison, the IFNγ serum concentrations remain unchanged [95]. In open randomized study in vitro, K.Samanta et al (2011) evaluate the potential of 1.25$(OH)_2$D to modulate the immune imbalance in 49 MS patients. The first group includes 25 patients treated with Cholecalciferol 14 000 IU/ day and Ca 120 mg/day, for one year. In the second group of 24 patients, the therapy is placebo. At the end of the first year, in the patients treated with Vit-D_3 the serum levels of 25(OH)D are significantly higher than the placebo group, and there is reduction of the abnormal activity of the antigen-specific peripheral mononuclear cells, without change in the T-cell reactivity, compared to the placebo [96]. Mahon et al (2003) conduct double-blind, placebo-controlled study of 39 RRMS patients and initial serum level of 25(OH)D under 20 ng/ml. A group of 17 patients is treated daily, for a period of six months, with Cholecalciferol and Ca 800 mg. The other 22 patients are treated with Ca 800 mg and placebo for the same period. At the end of the 6th month, the patients treated with Vit-D_3 and Ca show significant increase of the 25(OH) levels and the serum TGFβ1, unlike the patients treated with Ca and placebo. In both groups, the serum concentrations of TNFα, IL13, IFNγ remain unchanged. The authors believe that the Vit-D_3 has the potential to influence the immune-regulatory imbalance in RRMS patients, however, the doses needed for immune tolerance, are subject of further studies [97].

The clinical observations do not present one-directional results on the connection be-

tween the dynamics in the serum concentrations of the Vit-D metabolites and changes of the immune indexes, the severity of neurological deficit, Gd enhanced lesions during MRI test.

Under discussion are questions such as: subgroup of MS patients suitable for treatment with Vitamin D; the doses of application, duration of the treatment; serum levels of 25(OH)D causing suppression of the immune reaction.

Proving scientific facts for the cause-and-effect relationship of the changes in the 25(OH) D levels in the serum, with the dynamics of the immune and clinical indexes of the disease activity, would enrich the available scientific data, and would optimize the therapeutic approach through treatment with Vitamin D.

In Bulgaria, the MS disease rate has increased two fold over a period of 17 years. In this respect, studies of the interaction between environmental factors and their role in the pathogenesis of the disease are substantiated and necessary.

In 2016 was completed a study of 86 individuals from the white race-46 RRMS patients and 40 controls. The variations in the serum concentrations of 25(OH)D, IFNγ, IL17A, TGFβ1, IL4, IL10 were evaluated during the relapse and remission phases. In the patients with relapse and severe neurological deficit, the 25(OH)D levels are reliably lower, compared with the healthy individuals. During the remission, the levels are statistically significantly increased, but they do not reach those of the controls. In the studied population, the 25(OH) D deficit increases the general risk of MS 3.43 times. There is significant negative correlation between the 25(OH)D levels and the severity of the neurological deficit during exacerbation. The comprehensive analysis of the effect of the studies indexes on the degree of EDSS during relapse, shows dependence between the severity of the deficit and the serum levels of 25(OH) D, IL17A, TNFα during that period [98].

10. Conclusion

The multiple sclerosis is one of the scientific problems for the modern neurology. The regulatory immune imbalance has a key role for the chronic-progressive course of the immune-mediated myelin destruction, and for the treatment effect. Many studies are being conducted today, aiming to identify new factors, participating in the aberrant immune response, in order to achieve optimal suppression by combining appropriate immune-efficient agents. The studies of the role of Vitamin D in the pathogenesis of MS are an aspect of the modern conception of the comprehensive control of the pathological process.

The evaluation of the clinically significant changes depending on the degree of impairment of the Vitamin D status-insufficiency/ deficit, is crucial for the prophylactics and treatment of conditions with proven participation of Vit-D in the pathological process.

The persisting immune imbalance is associated with impaired immune tolerance, resulting from insufficient number of known factors, including environmental. The reported evidence on the participation of 25(OH)D, an environmental factor, in the pathogenesis of MS, gives grounds for studying of the therapeutic potential of the metabolite to control the impaired immune regulation and clinically manifested disease activity. Finding scientific proof on the benefits of using new immune-effective drugs in optimal and safe dose, alone or in combination, will increase the potential of the modern methods of therapeutic intervention to modify the outcome of the disease.

11. References

1. Georgiev D. Multiple Sclerosis and other Demyelinating diseases. In: Milanov I, Iancheva S. Neurology.Sofia: Medicine andgymnastics, 2007.

2. Chang JH, Cha HR, Lee DS, Seo KY, Kweon MN. 1,25-Dihydroxyvitamin D_3 Inhibits the Differentiation and Migration of TH17 Cells to Protect against Experimental Autoimmune Encephalomyelitis. PLoS ONE. 5(12): 10.

3. Duquette P. Hormonal factors in multiple sclerosis. Int MS J. 2001; 9(1): 17-25.

4. Luetic G. MS in Latin Americ. Int MS J. 2008; 15(1): 6-11.

5. Whitcare CC, Reingold SC, O`Looney PA. A gender gap in autoimmunity. Science. 1999; 283: 1277-1278.

6. Leray E, Yaouanq J, Page E L, Coustans M, Laplaud D, Oger J, and Gilles Edan. Evidence for a two-stage disability progression in multiple sclerosis. Brain 2010; 133(7): 1900–1913.

7. Pirko L, Hoseworthy J.H. Demielinated Disorders of the Central Nervous System In: Textbook of Clinical Neurology, Eds.: Goetz CG. Philadelphia. Saunders Elsevier, 2007: 1103-1133.

8. Riley SR, Tullman MJ. Multiple Sclerosis. In: Merritt`s Neurology, Eds.: Rowland LP, Pedley TA. Phildelphia. Wolters Kluwer, 2010: 903-918.

9. Thompson AJ. Treatment of progressive multiple sclerosis. In: Multiple sclerosis 2, Eds.: McDonald WI, Hoseworthy JH. Philadelphia. Elsevier Science. 2003: 341-359.

10. Ustun TB. Introduction form the World Health Organization . Eur J Neurol. 1998; 5(2): 3.

11. Bar-Or A, Darlington PJ. The immunology of multiple sclerosis. In: Multiple Sclerosis therapeutics, Eds:Cohen AJ, Rudick RA. New York. Cambridge University Press, 2011: 20-33.

12. Birnbaum G, Antel J. The immunology of multiple sclerosis. In: Clinical Neuroimmunolgy, Eds: Antel J, Birnbaum G, Hartung H-p, Vincent A. New York. Oxford University Press 2005: 185-194.

13. Hartung H-P, Kieseier BC, Hemmer B. Purely systemically active anti-inflammatory treaatments are adequate to control multiple sclerosis. J Neurol. 2005; 252(5): 30-37.

14. Korn T, Oukka M, Bettelli E. Th17 cells: effectors T cells with inflammatory properties. Semin Immunol. 2007; 19(6): 362-371.

15. Sospedra M, Martin M. Immunology of multiple sclerosis. Annu Rev Immunol. 2005; 23: 683-747.

16. Wegner C. Pathological differences in acute inflammatory demyelinating disease of the central nervous system. Int MS J. 2005; 12: 12-9.

17. Bar-Or A. The immunology of multiple sclerosis. Semin Neurol 2008;28(1):29-45.

18. Christense T. The Role of EBV in MS Pathogenesis. The International MS Journal . 2006; 13: 52–57.

19. Krone B, Oeffner F, Grange JM. Is the risk of multiple sclerosis related to the 'biography' of the immune system? J Neurol 2009; 256(7): 1052-1060.

20. Owens GP, Bennett JL. Trigger, pathogen, or bystander: the complex nexus linking Epstein- Barr virus and multiple sclerosis. Mult Scler. 2012; 18(9): 1204-1208.

21. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KHG. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. ClinExpImmunol. 2010; 162(1): 1-11.

22. Milanov I. Multiple Sclerosis and Demyelinatingdiseases. Sofia: Medicine and gymnastics -Sofia.; 2014.

23. Cantorna MT, Snyder L, Lin YD, Yang L. Vitamin D and 1,25(OH)2D regulation of T cells. Nutrients. 2015; 7(4): 3011-3021.

24. Cantorna MT, Woodward WD, Hayes CE, DeLuca HF. 1,25-dihydroxyvitamin D3 is a positive regulator for two anti-encephalitogenic cytokines TGFB1 and IL4. J Immunol 1998; 160: 5314-5319.

25. Cantorna MT. Vitamin D and multiplse sclerosis: an update. Nutr Rev. 2008; 104: 182-191.

26. Carlberg C, Quack M, Herdick M, Bury Y, Polly P, Toell A. Central role of VDR conformations for understanding selective actions of vitamin D_3 analogues. Steroids 2001; 66: 213-221.

27. Chang JH, Cha HR, Lee DS, Seo KY, Kweon MN. 1,25-Dihydroxyvitamin D3 Inhibits the Differentiation and Migration of TH17 Cells to Protect against Experimental Autoimmune Encephalomyelitis. PLoS ONE 5(12): 10.

28. Hartung H-P,Bar-Or A, Zonkas I . What do we know about the mechanism of action of disease-modifying treatment in MS. J Neurol. 2004; 251(5): 12-19.

29. Piccio L, Cross AH., Immunology of Multiple Sclerosis In: Giesser BS. Primer on Multiple Slcerosis.. New York: Oxford University Press; 2011; 47-56.

30. Wu G.F, Alvarez E. The immunopathophysiology of Multiple Sclerosis. NeurolClin. 2011; 29: 257-278.

31. Mahad D , Trapp B , Ransohoft M. Tissue pathology of multiple sclerosis In : Antel S, Birnbaum G, Hartung H -P, Vincent A , eds. Clinical Neuroimmunology. New York: Oxford University Press: 2005: 173-183

32. Dutta R, Trapp BD. The pathology of multiple sclerosis. In:Multiple Sclerosis Therapeutics, Eds:Rudick RA, Cohen JA, New York. Cambridge University Press, 2011: 12-19.

33. Bruck W. Clinical implications of neuropathologic findings in MSS. In: Kappos L, Hartung H-P. International Schering Symposium 10 years Betaferon, 2003, March 15.

34. Lassman H. Pathology of Multiple Sclerosis. J Neurol Sci. 2001; 187(1): 320.

35. Pathology and Clinical Features of Multiple Sclerosis. In: Clanet M, Fredikson S. The pathology of Multiple Sclerosis, Proceeding of the MS Forum Modern Management Workshop, 2000: 6-8.

36. Pugliatti M, Sotgiu S, Rosati G. The worldwide prevalence of multiple sclerosis. Clin Neurol Neurosur. 2002; 104: 182- 191

37. JordanovB. Multiple sclerosis.Sofia:Medicine andgymnastic -Sofia.; 1989.

38. Kalafatova O. Clinical-epidemiological study of disseminated sclerosis (dissertation). Sofia: Medical University, 1983.

39. Topalov N. Clinical-epidemiological study of disseminated sclerosis (dissertation). Sofia: Medical University, 1999.

40. Kurtzke JF, Beebe GW, Norman JE Jr. Epidemiology of multiple sclerosis in United States veterans: I. Race, sex, and geogrpaphic distribution. Neurology. 1979; 29: 1228-1235.

41. Kurtzke JF, Wallin MT. Epidemiology. In: Burks JS, Johnson KP. Multiple Sclerosis: diagnosis medical management, and rehabilitation. New York: Demos Medical Publishing; 2000: 49-70.

42. Kurtzke JF, Dean G, Botha DPJ. A method of estimating age at immigration of white immigrants to South Africa, with an example of its importance. S Arf Med J. 1970; 44: 663-669.

43. Baranzini SE, Oksenberg JR. Role of genomics in studying MS. Int MSJ. 2002; 9(3): 91-98.

44. Lincoln JA, Cook SD. An overview of gene-epigenetic-environmental contributions to MS causation. J Neurol Sci. 2009; 286: 54-57.

45. Munger KL, Zhang SM, O'Reilly E, Hernán MA, Olek MJ, Willett WC, Ascherio A. Vitamin D intake and incidence of multiple sclerosis. Neurology. 2004; 62(1): 60-65.

46. Orton S-M, Herrera BM, Yee MI, W. Valdar, SV Ramagopalan, AD Sadovnick, GC Ebers .Sex ratio of multiple sclerosis: a longitudinal study. Lancet Neurol. 2006; 11(5): 932-936.

47. Mellanby RJ, Thomas DC, Lamb J. Role of regulatory T-cells in autoimmunity. ClinSci (Lond) 2009; 116(8): 639-649.

48. Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. Lancet Neurol 2004; 3: 104-110.

49. Laroni A, Calabrese M, Perini P, Albergoni MP, Ranzato F, Tiberio M, Battistin L, Gallo P. Multiple sclerosis and autoimmune diseases Epidemiology and HLA-DR association in North-east Italy. J Neurol. 2006; 253: 636-639.

50. Borisova A-M, Bojanov M, KolarovZ, PopivanovP, Shinkov A, Stoilov R, Svinarov D, Petranova C, Pilosof V. Recommendations for Diagnosis, Prevention and Treatment of Vitamin D Deficiency and Deficiency D. Sofia: Bulgarian Society of Endocrinology, 2013: 5-7.

51. Vankova M, Boyanov M. Vitamin D deficiency and its correction - a brief review of the literature. Medical Review. 2013; 49 (3): 5-11

52. Deluca HF, Cantorna MT. Vitamin DL its role and uses in immunology. FJ. 2001; 15: 2579-2584.

53. Kulie T, Groff A, Redmer J, Hounshell J, Scbrager S. Vitamin D: An Evidence-Based Review. J Am Board Fam Med. 2009; 22(6): 698-704.

54. Lips P. Vitamin D physiology. Prog Biophys Mol Biol. 2006; 92: 4-8.

55. Brown SJ. The Role of Vitamin D in Multiple Sclerosis. Ann Pharmacother. 2006; 40(6): 1158-1161.

56. Matusevicius D, Kivisäkk P, He B, Kostulas N, Ozenci V, Fredrikson S, Link H. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. MultScler. 1999; 5(2): 101-104.

57. van Etten E, Gysemans C, Branisteanu DD, Verstuyf A, Bouillon R, Overbergh L, Mathieu C. Novel insights in the immune function of the vitamin D system: synergism with interferon-beta. J Steroid Biochem Mol Bioln. 2007; 103(3-5): 546-551.

58. Holick MF. Vitamin D deficiency. N Engl J Med. 2007; 357: 266-281.

59. Siva S, Kantarci O. An introduction to the clinical spectrum of inflammatory demyelinating disorders of the central nervous system. In: Siva A, Kesselring J, Thompson AJ. Frontiers in Multiple Sclerosis. London: Martin Dunitz Ltd,

1999: 4-6.

60. Smolders J, Damoiseaux J, Menheere P, Hupperts R. Vitamin D as an immune modulator in multiple sclerosis, a review. J Neuroimmunol. 2008; 194(1-2): 7- 17.

61. Borisova A-M, Shinkov A, Vlahov J, Dakovska L, Todorov T, Svinarov D, Kassabova L. Deficiency, insufficiency and sufficiency of vitamin D in the Bulgarian population. Endocrinology. 2012; 3, 3, 122-134.

62. Miller DM, Weinstock-Guttman B, Béthoux F, Lee JC, Beck G, Block V, Durelli L, LaMantia L, Barnes D, Sellebjerg F, Rudick RA. A meta-analysis of methylprednisolone in recovery from multiple sclerosis exacerbations. Mult Scler. 2000; 6(4): 267-273.

63. Moyad M. Vitamin D: A Rapid Review. Dermatol Nurs. 2009; 21(1): 1-13.

64. Pierrot-Deseilligny C1, Rivaud-Péchoux S, Clerson P, de Paz R, Souberbielle JC. Relationship between 25-OH-D serum level and relapse rate in multiple sclerosis patients before and after vitamin D supplementation. Ther Adv Neurol Disord. 2012; 5(4): 187-98.

65. Sharrack B, Hughes RAC. Clinical scales for multiple sclerosis. J NeurolSci. 1996; 135: 1-9.

66. Racke MK, Dhib-Jalbut S, Cannella B, Albert PS, Raine CS, McFarlin DE. Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-beta 1. J Immunol. 1991; 146(9): 3012-3017.

67. Wilkins A , Scolding N. Protecting axons in multiple sclerosis. Multiple Sclerosis 2008; 14: 1013-1025.

68. Kimlin MG. Geographic location and vitamin D synthesis. Mol Aspects Med. 2008; 29(6): 453-461.

69. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expended disability status scale (EDSS). Neurol. 1983; 33: 1444-1452.

70. Sospedra M, Martin M. Immunology of multiple sclerosis. Annu Rev Immunol. 2005; 23: 683-747.

71. Binkley N, Krueger D, Cowgill CS, Plum L, Lake E, Hansen KE, DeLuca HF, Drezner MK. Assay variation confounds the diagnosisof hypovitaminosis D: a call for standartization. J Clin Endocrinol Metab 2004; 89: 3152-3157.

72. Gilman A. The Pharmacological Basis of Therapeutics.In: Friedman PA, Brunton LL. Updated Vitamin D and Calcium Recommendations. New York: McGraw-Hill Medical; 2011.

73. Correale J, Ysrraelit MC, Gaitán MI. Gender differences in 1,25 dihydroxyvitamin D3 immunomodulatory effects in multiple sclerosis patients and healthy subjects. J Immunol 2010 ; 185(8): 4948-4958.

74. Eikelenboom MJ1, Killestein J, Kragt JJ, Uitdehaag BM, Polman CH. Gender differences in multiple sclerosis: cytokines and vitamin D. J Neurol Sci 2009; 15; 286(1-2).

75. Gonzalez Chr. Vitamin D Supplementation: An Update. US Pharmacist 2010; http://www.medscape.com/viewarticle/731722.

76. Grimnes G, R. Joakimsen, Y. Figenschau, P. A. Torjesen, B. Almås, R. Jorde. The effect of high-dose vitamin D on bone mineral density and bone turnover markers in postmenopausal women with low bone mass—a randomized controlled 1-year trial. Osteoporos Int 2011 2012; 23(1): 201-211.

77. Holick MF, Binkle NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney R P, Murad M H, Weaver CM. Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline. Clin Endocrinol Metab. 2011; 96(7): 1911-1930.

78. Leidig-Bruckner G., H. J. Roth, T. Bruckner, A. Lorenz, F. Raue, K. Frank-Raue. Are commonly recommended dosages for vitamin D supplementation too low? Vitamin D status and effects of supplementation on serum 25-hydroxyvi-

tamin D levels—an observational study during clinical practice conditions. Osteoporos Int. 2011; 22(1): 231-240.

79. Mosayebi G, Ghazavi A, Ghasami K, Jand Y, Kokhaei P. Therapeutic effect of vitamin D3 in multiple sclerosis patients. Immunol Invest 2011; 40(6): 627-639.

80. Panitch HS, Hirsch RL, Schindler J, Johnson KP. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. Neurology 1987; 37(7): 1097-102.

81. Waterhouse P, Penninger JM, Timms E. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. Sciense 1995; 270: 985-988.

82. Siva S, Kantarci O. An introduction to the clinical spectrum of inflammatory demyelinating disorders of the central nervous system. In: Siva A, Kesselring J, Thompson AJ. Frontiers in Multiple Sclerosis. London: Martin Dunitz Ltd, 1999: 4-6.

83. Cantorna M T, Hayes C E, DeLuca H F. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. Proc Natl Acad Sci U S A 1996; 93(15): 7861–7864.

84. Cantorna MT, Humpal-Winter J, DeLuca HF. In vivo upregulation of interleukin-4 is one mechanism underlying the immunoregulatory effects of 1,25-dihydroxyvitamin D(3). Arch Biochem Biophys. 2000; 377(1): 135-138.

85. Garcion E, Sindji L, Nataf S, Brachet P, Darcy F, Montero-Menei CN. Treatment of experimental autoimmune encephalomyelitis in rat by 1,25-dihydroxyvitamin D3 leads to early effects within the central nervous system. Acta Neuropathol 2003 ; 105(5): 438-448. Epub 2003 Jan 31.

86. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, de Waal-Malefyt R, Coffman RL, Hawrylowicz CM, O'Garra A. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med 2002; 195(5): 603-616.

87. van der Mei IA, Ponsonby Al, Dwyer T, Blizzard L, Taylor BV, Kilpatrick T, Butzkueven H, McMichael AJ. Vitamin D levels in people with multiple sclerosis and community controls in Tasmania, Australia.JNeurol2007; 254: 581-590.

88. A, Schadt E, Garren H, Langer-Gould A, Strober S, Cannella B, Allard J, Klonowski P, Austin A, Lad N, Kaminski N, Galli SJ, Oksenberg JR, Raine CS, Heller R, Steinman L. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat Med. 2002; 8(5): 500-508.

89. Kragt JJ, Amerongen BM, Killestein J, Dijstra CD, Uitdehaag BMJ, Polman CH, Lips P. Higher levels of 25-hydroxyvitamin D are associated with a lower incidence of multiple sclerosis only in women. Mult Scler. 2009; 15: 9-15.

90. Piccio L, Cross AH., Immunology of Multiple Sclerosis In: Giesser BS. Primer on Multiple Slcerosis.. New York: Oxford University Press; 2011; 47-56.

91. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-Hydroxyvitamin D levels and Risk of Multiple Sclerosis. JAMA 2006; 296(23): 2832-2838

92. Derakhshandi H, Etemadifar M, Feizi A, Abtahi SH, Minagar A, Abtahi MA, Abtahi ZA, Dehghani A, Sajjadi S, Tabrizi N. Preventive effect of vitamin D3 supplementation on conversion of optic neuritis to clinically definite multiple sclerosis: a double blind, randomized, placebo-controlled pilot clinical trial. Acta Neurol Belg. 2013; 113(3): 257-263.

93. Golan D, Halhal B, Glass-Marmor L, Staun-Ram E, Rozenberg O, Lavi I, Dishon S, Barak M, Ish-Shalom S, Miller A. Vitamin D supplementation for patients with multiple sclerosis treated with interferon-beta: a randomized controlled trial assessing the effect on flu-like symptoms and immunomodulatory properties. BMC Neurol 2013; 14; 13: 60.

94. Soilu-Hänninen M1, Airas L, Mononen I, Heikkilä A, Viljanen M, Hänninen A. 25-Hydroxyvitamin D levels in serum at the onset of multiple sclerosis. Mult Scler 2005 ;11(3): 266-271.

95. Mokry LE, Ross S1, Ahmad OS, Forgetta V, Smith GD, Leong A, Greenwood CM, Thanassoulis G, Richards JB. Vitamin D and Risk of Multiple Sclerosis: A Mendelian Randomization Study. PLoS Med. 2015; 12(8): e1001866.

96. Kimball S, Vieth R, Dosch HM, Bar-Or A, Cheung R, Gagne D, O'Connor P, D'Souza C, Ursell M, Burton JM. Cholecalciferol plus calcium suppresses abnormal PBMC reactivity in patients with multiple sclerosis. J Clin Endocrinol Metab. 2011; 96(9): 2826-2834.

97. Mahad D , Trapp B , Ransohoft M. Tissue pathology of multiple sclerosis In : Antel S, Birnbaum G, Hartung H -P, Vincent A , eds. Clinical Neuroimmunology. New York: Oxford University Press: 2005: 173-183

98. SlavoGS. Clinical-laboratory study of serum vitamin D levels and cytokines in patients with relapsing-remitting multiple sclerosis.(дissertation). Plovdiv: Medical university, 2016.

Vitamin D Deficiency: Causes & Treatment

Chapter 3

Analytical Aspects of Vitamin D

Luque de Castro, MD*[1,2,*]*; Castillo-Peinado LS [1,2]

[1]*Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, Córdoba, Spain.*

[2]*Maimónides Institute of Biomedical Research (IMIBIC), Reina Sofía University Hospital, University of Córdoba, Córdoba, Spain.*

[*]***Correspondence to: Luque de Castro, MD ,*** *Department of Analytical Chemistry, Annex Marie Curie Building, Campus of Rabanales, University of Córdoba, Córdoba, Spain.*

Email: qa1lucam@uco.es

Abstract

This chapter deals with vitamin D and its metabolites from the point of view of the steps involved in their analytical determination. Thus, after a brief description of the characteristics and metabolism of vitamin D to introduce the target metabolites, the analytical aspects of these compounds are addressed. First, we discuss the suitability of the types of samples (*e.g.,* fresh and dry blood, urine, saliva, cerebrospinal fluid) in which vitamin D and its metabolites are determined; then, the stability of the most used sample (blood as both serum and plasma) under different storage conditions is considered in the light of the different metabolites to be measured. The half-life and levels in blood of these compounds, the sources and supplementation with vitamin D, as well as the role of vitamin D and its metabolites in human health are also matters of this chapter prior to discuss in depth the sample preparation and derivatization steps and emphasize a key aspect as sample volume. The description and discussion of the analytical methods for determination of vitamin D and its metabolites encompass, as the most important, immunoassays methods and mainly those involving chromatographic separation prior to detection, with mass spectrometry and the gold technique for identification and determination.

Abbreviations: ACN: Acetonitrile; AD: Ampliflex™ Diene; ALTM: All-laboratory trimmed mean; APCI: Atmospheric pressure chemical ionization; CPBA: Competitive protein binding; CV: Coefficient of variation; DAD: Diode array detector; DBP: Vitamin D binding protein; DBS: Dried blood spots; DEQAS: International Vitamin D External Quality Assurance Scheme; FI: Flow-injection; GC: Gas chromatography; IAE: Immunoaffinity extraction; LC: Liquid chromatography; LLE: Liquid–liquid extraction; LLOQ: Lower limit of quantitation; NIST: National Institute of Technology; PTAD: 4-phenyl-1,2,4-triazole-3,5-dione; QqQ-MS: triple quad mass spectrometry detector; RIA: Radioimmunoassay; RRB: Radioreceptor binding; SIL-IS: Stable isotopically labeled internal standard; SPE: Solid-phase extraction; SRM: Selected reaction monitoring; UV-B: Ultraviolet B

1. Vitamin D: Characteristics and Metabolism

Vitamin D is a prohormone that comes in many forms, but the two major physiologically relevant ones are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Technically, vitamin D is classified among secosteroids in which one of the rings has been broken **(Figure 1).** As derived from a steroid, the structure of vitamin D retains its numbering from its parent compound cholesterol: the 9,10 carbon–carbon bond of ring B is broken, as indicated by the inclusion of "9,10-*seco*" in the official nomenclature. The configuration of the double bonds are notated *E* for "entgegen" or *trans*, and *Z* for "zuzammen" or *cis* [1]. Thus, the official name of vitamin D_3, by relation to cholesterol, is 9,10-*seco* (5Z,7E)-5,7,10(19) cholestatriene-3β-ol; while the official name of vitamin D_2 is 9,10-s*eco* (5Z,7E)-5,7,10(19),22-ergostatetraiene-3β-ol. The differences between both are a double bond between C22 and C23, and a methyl group on C24 for vitamin D_2.

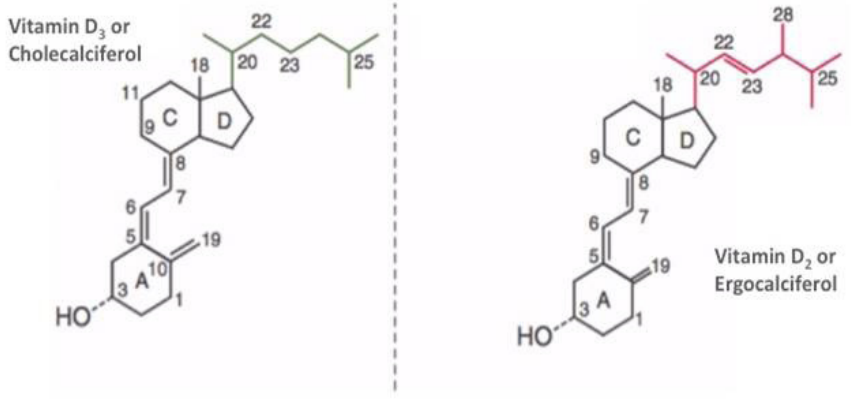

Figure 1: Important nutritional forms of vitamin D.

Vitamin D_3, the naturally occurring form, originates from dermal synthesis by conversion of 7-dehydrocholesterol, a precursor of cholesterol, into pre-vitamin D_3 under ultraviolet UV-B radiation, on the plasmatic membrane of skin cells. This process is followed by fast thermal isomerization to vitamin D_3 **(Figure 2)**. Approximately 50% of pre-vitamin D is converted into vitamin D in 2 h, formed in the cell membrane and then transported to the extracellular space. From this, vitamin D is transported in blood bound mainly by the transporter vitamin D binding protein DBP to reach the liver. There, vitamin D-25-hydroxylasa of cytochrome P450 produces the 25-hydroxylation in the liver to yield the main circulating form, 25(OH)D. D-25-hydroxylasa is not strictly regulated in the liver; therefore, an increase of cutaneous production of vitamin D or its intake increase the level of circulating 25(OH)D —both 25(OH)D_3 and

25(OH)D_2. Therefore, the joint measure of both monohydroxylated vitamin D metabolites is used to determine the status of vitamin D.

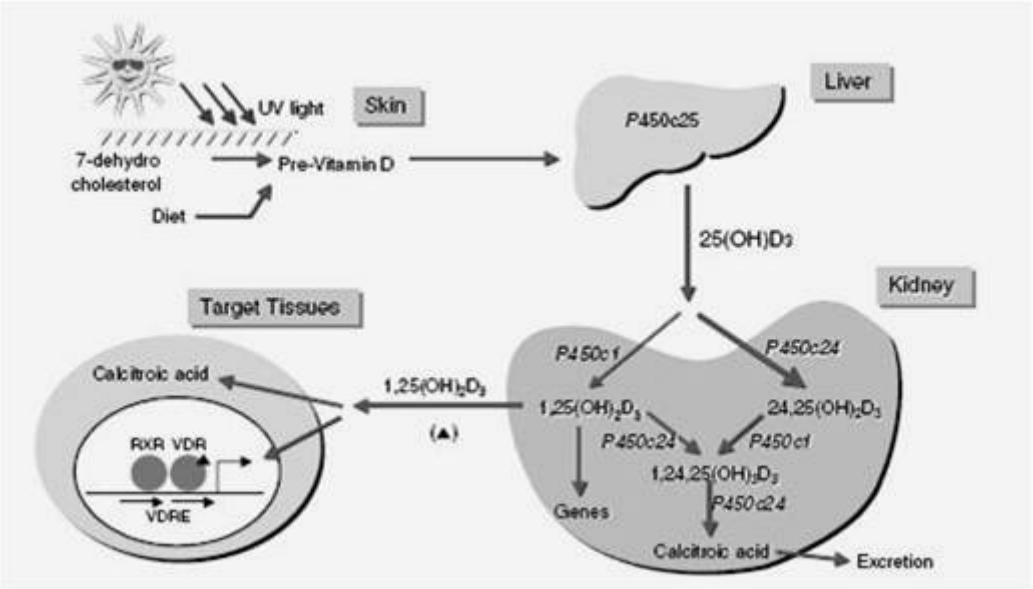

Figure 2: Activation and molecular pathways for vitamin D: integrative schematic synthesis, metabolism and molecular action.

Further 1α-hydroxylation of 25(OH)D both in the kidney and extrarenal sites gives place to the hormonal form, 1,25(OH)$_2$D, which mediates its pleiotropic effects through the ubiquitous vitamin D receptor that binds to vitamin D response elements in target genes to regulate their transcription [2]. 1,25(OH)$_2$D has potent antiproliferative and cell differentiation-inducing activities in addition to its role in calcium homeostasis [3]. The inactivation of vitamin D is carried out by side chain oxidation by the mitochondrial 24-hydroxylase, which catalyzes the conversion of both 25(OH)D_3 and 1,25(OH)$_2D_3$ into a series of 24- and 23-hydroxylated products targeted for excretion, culminating in the water-soluble biliary metabolite 26,23-lactone and calcitroic acid [4]. C3-epimerization is a second biochemical pathway, via which the major vitamin D metabolites —25(OH)D_2, 24,25(OH)$_2D_3$ and 1,25(OH)$_2D_3$— are converted to their respective epimeric forms and are further metabolized through the C-24 oxidation pathway [5].

Even though 1,25(OH)$_2$D is in fact the biologically active form of vitamin D, serum 1,25(OH)$_2$D provides no information about vitamin D status and is often normal or even elevated due to secondary hyperparathyroidism associated with vitamin D deficiency. On the contrary, 24,25(OH)$_2D_3$ is the major circulating dihydroxylatedvitamin D metabolite in human serum with concentrations between 0.7–40 nmol/L [6,7]. Serum 24,25(OH)$_2D_3$ concentrations show a strong positive correlation with serum 25(OH)D_3 levels, and are approximately 10% of 25(OH)D_3 levels [7–14].

2. Analytical Aspects of Vitamin D

This section involves subjects such as the clinical samples used for vitamin D determination, the stability of the most frequently used samples, half-life and normal levels in

humans of vitamin D and its metabolites, main sources of vitamin D and supplements on this vitamin, as well as its role in human health.

2.1. Clinical samples for determination of vitamin D

Biological matrices for determination of vitamin D and its metabolites in human include dried blood spots (DBS) [15–22], urine [23], saliva [24], cerebrospinal fluid [25], and mainly blood.

DBS collection is a minimally invasive sampling to obtain blood samples on cards or filter paper. Therefore, it is a practical way to screen for vitamin D deficiencies in large epidemiological studies or in at risk populations where venipuncture is impractical, including pediatric populations, rural clinics, and developing countries, or where blood volume is limited as in neonates [19]. Calibration of DBS analysis is difficult owing to the absence of blank samples in the case of endogenous analytes, and the fact that current reference analytics is performed using plasma or serum samples and not dried blood [20].

Measurement of vitamin D in saliva is interesting from the perspective that it is likely to reflect the free, non-protein-bound hormone in plasma [26], and may serve as a better indicator of vitamin D status [27]. Concentrations of salivary 25(OH)D_3, however, are at the picomolar ranges, less than one-thousandth of that in plasma, and require sensitive assay technology for measurement [28]. A good correlation was found between the serum and salivary 25(OH)D_3 levels [24]

Urine is a more complicate sample for vitamin D analysis as most of its metabolites are conjugated; therefore, in addition to volume normalization, if required, sample preparation involves hydrolysis (usually catalyzed by β-glucuronidase, followed sometimes by derivatization). Ogawa *et al.* found the amount of 24,25$(OH)_2D_3$ in urine 2–3 times greater than that of 25(OH)D_3, contrary to the 1:10 ratio of 24,25$(OH)_2D_3$ to 25(OH)D_3 in serum [23]. Sulfate conjugated vitamin D metabolite 25(OH)D_3 3-sulfate has also been found in human plasma at levels similar to or higher than that of 25(OH)D_3 in adults or infants, respectively, but lower in preterm infants [29]. This conjugate has not been proven to have significant biological activity [30].

Cerebrospinal fluid from multiple sclerosis patients was used to study the controversial information on the effect of vitamin D metabolites on the disease. The concentration of 25(OH)D in this biofluid from the target patients was not significantly different as compared with patients with other inflammatory neurological diseases, but the cerebrospinal fluid/serum ratio was significantly lower in multiple sclerosis patients [25]. The authors of this study stated that the results they obtained do not support that 25(OH)D is actively transported to the cerebrospinal fluid, or that the cerebrospinal fluid or serum levels or their ratio exert a

major impact on multiple sclerosis activity. A comprehensive metabolomics study of vitamin D metabolites in multiple sclerosis patients has not been developed so far [31].

Blood, either as serum or plasma is, with a high difference, the most used biofluid for clinical studies of vitamin D and its metabolites in such a way that the reference values for the status of vitamin D are established in one of them.

Blood (as serum or plasma) has been the clinical sample preferred by the authors' research team for development of methods for determination of vitamin D and its metabolites. The team has worked on the metabolism of vitamin D since 1997, when metabolomics had not been defined as such omics discipline, and published 7 articles between 1997–1999 dealing with methods to improve the determination of vitamin D and metabolites using plasma as clinical sample [32–38]. Also a review on the state-of-the-art and trends on the determination of vitamin D [39] was published by the authors. Further studies on vitamin D metabolites developed by the research team (between 2007 and 2013) were based on serum as clinical sample, and mainly devoted to reduce the sample size, automation of sample preparation, improvement of sensitivity and cross-sectional studies [40-48]. More recently, the team has developed a study on the suitability of serum or plasma for the determination of vitamin D and its key mono- and dihydroxymetabolites [49].

2.2. Stability of serum/plasma samples for analysis of vitamin D

Vitamin D and its metabolites have traditionally been considered unstable compounds sensitive to light and temperature [50]; therefore, studies on their stability have been developed, but not in depth. A study published in 2004 was devoted only to the 25(OH)D metabolite, which was determined by a chemiluminescence immunoassay method in serum and plasma samples both fresh and frozen [51]. Comparison of the results showed *p* values greater than 0.7, indicating no significant difference between fresh and frozen samples, the latter subjected to five freeze–thaw cycles; thus demonstrating that the samples did not need to be frozen if they are assayed within 5 days from sampling. The equivalent cross-reactivity of 25(OH)D_2 and 25(OH)D_3 in the target immunoassay used was also demonstrated. Cross-reactivity to the dihydroxy vitamin D metabolites was accepted, but without clinical concern due to the approximately 1000-fold concentration difference between these metabolites and the 25(OH)D in the circulation. As the authors stated, concentrations of 1,25$(OH)_2$D at five times normal values would contribute less than 0.05 nmol/L to the measured 25(OH)D concentration. Thus, pathological 1,25$(OH)_2$D concentrations would be expected to contribute less than 0.1% to the overall imprecision. A further study in serum involving only the 25(OH)D metabolite and RIA analysis showed stability of this compound at –25 ºC in storage between 6 and 24 years [52].

In a more recent study on stability of the 25(OH)D metabolite in serum ,the levels determined by LC–MS/MS were compared with those obtained by conventional radioimmunoassay

(RIA) –no distinction between 25(OH)D_3 and 25(OH)D_2— [53]. Note that 1,25(OH)$_2$D, less concentrate than the monohydroxy metabolites, was not determined. The values obtained by the RIA method exhibited a mean bias of about 8.35 ng/mL, most likely as a result of cross-reaction of the antibody with low-abundant metabolites, including 24,25(OH)$_2D_3$. Various preanalytical factors, such as long sample sitting prior to serum separation, repeated freeze–thaw cycles, and the presence of anticoagulants had no significant effects on the determinations.

A more comprehensive stability study was developed by the authors' team using serum and plasma samples subjected to: (1) room temperature, 25 ºC preserved from sunlight; (2) refrigeration, 6 ºC, and freezers, (3) at –20 ºC and, (4) at –80 ºC. The subsequent monitoring of vitamin D and its mono- and dihydroxymetabolites, the influence of freeze/thaw cycles on the stability of the target compounds, as well as the behavior of lyophilized serum and plasma have been the subject of the study [54].

2.3. Half-life and levels of vitamin D metabolites in humans

The parent sterol vitamin D has a half-life close to 24 h [55], relatively short as compared with 25(OH)D, which has a half-life of 2–3 weeks [56,57]. Therefore, 25(OH)D measurement is a better indicator of vitamin D storage, whether obtained from sunlight (UV exposure) or dietary sources. The most potent physiologically active circulating metabolite produced by humans is 1,25(OH)$_2$D, which has a half-life of 4–8 h [58–60]. While 25(OH)D circulates at the nmol/L concentrations, 1,25(OH)$_2$D is present at the pmol/L concentrations; at a 1000-fold lower concentration than 25(OH)D (with the reference interval for healthy adults being 38–134 pmol/L [61]), which means that this dihydroxymetabolite represents the greater challenge in assay development.

The levels of vitamin D metabolites that define normal, abnormal or pathological values in adult humans are established with respect to the circulating form. There is virtually unanimous agreement that a serum level of 25(OH)D less than 30 nmol/L defines vitamin D deficiency, but there are different opinions regarding the 25(OH)D levels that define vitamin D insufficiency which may differ from 50 nmol/L [62] to 75 nmol/L [63]. The Institute of Medicine recommends that deficiency corresponds to <30 nmol/L, and places a person risk relative to bone health; 30–50 nmol/L places some, but not all, persons at risk for inadequacy, which can be considered as insufficiency; while sufficiency (adequate) is established for ≥50 nmol/L that meets the needs of 97.5 % of the population. About 1 billion people worldwide are estimated to have 25(OH)D levels of less than 75 nmol/L [64]. The Institute of Medicine also states that levels higher than 75 nmol/L are not consistently associated with increased benefit [65]. **Table 1** summarizes the levels of both vitamin D and 25(OH)D as expressed by K. Poongkodi [66] including extreme values.

Table 1. Levels of vitamin D and 25(OH)D which define normal, abnormal and pathological values in serum from human adults [66].

Status	25(OH)D, ng/mL	Vitamin D, nmol/L
Severe Deficiency	<10	<25
Deficiency	<20	<50
Insufficiency	21 – 29	50 – 74
Sufficiency	30 – 100	75 – 250
Optimal	30 – 60	75 – 150
Toxic	>150	>375

It has also been proposed that free or bio-available 25(OH)D provides a better assessment of vitamin D sufficiency than total 25(OH)D [27]. Free or bio-available 25(OH)D can be calculated taking measured DBP and albumin into account [67] or can be directly measured by immunoassay [68]. The 24,25$(OH)_2D_3$ to 25$(OH)D_3$ ratio may also be a predictor of serum 25$(OH)D_3$ response to vitamin D_3 supplementation [11,14,69].

Differentiation between the concentration of 25(OH)D and its epimer 3-epi-25(OH) D in infants is of interest in this population as studies in up to 1 year of age have shown significant 3-epi-25(OH)D concentrations as high as 200 nmol/L with relative contribution to total 25(OH)D as high as 55% [70–76]. This behavior can be attributed to either transference of the epimer in the utero or to postnatal formation.

2.4. Sources of and supplementation with vitamin D

Vitamin D is derived from two major sources in humans, with approximately 80–90% produced on the skin resulting in cholecalciferol (D_3), and the other 20% is derived from dietary sources, which can be animal cholecalciferol (D_3) or plant derived ergocalciferol (D_2). The latter originates from the yeast and plant sterol ergosterol and is obtained from diet or supplements.

Small amounts of vitamin D can also be obtained by nutritional intake of either vitamin D_3 or by foods fortified with vitamin D_2. Both forms undergo a substrate-dependent liver hydroxylation to 25-hydroxyvitamin D [25$(OH)D_2$ and 25$(OH)D_3$], as stated in **Figure 2**.The perceived conception that vitamin D supplementation can prevent, improve or cure chronic disorders has caused over the last years a massive rise in demand for measurement of 25(OH) D in blood as surrogate marker of vitamin D status.

There is much interest in intermittent dosing for patient convenience, and long term adherence, but caution is warranted when using supraphysiological bolus doses as these may be counterproductive leading to transient vitamin D intoxication in the immediate post-dosing period [77–79] or adverse effects in the longer term [80,81]. There are not conclusive results

on vitamin D supplementation. Thus, interventional studies on vitamin D supplementation (1.200 IU vitamin D per day) in patients with Parkinson disease showed no conclusive results when compared with individuals who received placebo during the same period [82]. On the contrary, 5 studies of vitamin D supplementation in rodents on the effect of vitamin D therapy on substantianigra dopamine neurons resulted in: one study with a significantly higher dopamine level and two a significantly higher concentration of the enzyme promoting conversion to dopamine (tyrosine hydroxylase), in the substantianigra of the vitamin D supplemented rodent group [83–85]. One of these studies also demonstrated that oxidative injury of the substantianigra was significantly lower after vitamin D supplementation [83]. This result was in line with another study reporting higher neuronal survival in the substantianigra when $1,25(OH)_2D$ was injected [86]. However, very high concentrations of $1,25(OH)_2D$ enhanced neurotoxity, as also found in Klotho-insufficient mice that resemble human aging and exhibit abnormal levels of serum $1,25(OH)_2D$ caused by abnormally high vitamin D metabolism in the kidney [86,87].

In infant studies, vitamin D supplementation (2.000 IU/day) in the first year of life increased the risk of developing atopy, allergic rhinitis, and asthma late in life [88].

2.5. The role of vitamin D and its metabolites in human health

Over the past decade, more than 1600 studies have been conducted on vitamin D, and more than half of them are cohort or observational studies demonstrating an association between deficits in vitamin D and a litany of acute and chronic disorders (cardiovascular disease, cancer, diabetes, fractures, depression and respiratory tract infections, to name a few) [89]. These findings have fueled the hypothesis that vitamin D supplementation—a widely available, low-cost and mostly harmless intervention—might treat or even prevent these disorders. Association, however, is not causation.

At present, evidence supports vitamin D supplementation to help prevent fractures (particularly if given with calcium), and possibly to prevent falls and slightly reduce mortality (particularly in older patients [>70 years of age]). No other effects have been proven. For many other conditions, the evidence for vitamin D supplementation is plagued by the use of small, poor-quality trials. Lastly, testing of 25(OH)D levels in the general population is not necessary, and very high doses should be avoided.

Clinical interest in Vitamin and its purported roles not only in calcium and bone metabolism but in several other medical conditions (diabetes, cardiovascular disease, multiple sclerosis, cancer, psychiatric disorders, neuro-muscular disease) has led to a surge in laboratory requests for 25(OH)D and $1,25(OH)_2D$ measurement, but also their ratio and ratios of other vitamin D metabolites. The search for vitamin D metabolites-based biomarkers that could be associated to given diseases is other of the open fields for interesting research, which the authors' team

has contributed. First, a proper use of the metabolomics in the field of vitamin D as diseases biomarkers started with the publication of a review on "the analytical process to search for metabolomics biomarkers", to establish the basis for use of the proper analytical steps in each case [90]. Other contributions have constituted calls of attention on the unclarified action of the different metabolites of vitamin D on multiple sclerosis and the role of metabolomics to clarify the vitamin D–multiple sclerosis relationship [31,91].

3. Sample Preparation for Determination of Vitamin D and its Metabolites

3.1. Sample preparation

Sample preparation for determination of vitamin D in the clinical field has been the workhorse of most analytical chemists working in this field, who have looked for interferents removal, automation and preconcentration steps to decrease sample volume as much as possible, increase sensitivity, selectivity and the number of analytes to be determined, and shorten the analysis time. Therefore, traditional sample preparation steps have consisted of:

(1) Deproteination or removal of protein and related macromolecules, a common step in dealing with serum or plasma samples. The step involves mixing equal volumes of sample and precipitant reagent such as acetonitrile (ACN) [92–98], ammonium sulfate [99,100] or ethanol [101,102]. This is the only preparation step prior to selective quantitation (*e.g.*, RIA).

(2) Saponification, mainly used with samples containing high contents of lipids such as infant formulae [102,103], enriched milk [104–107], eggs [108], fish oil [108–111] or margarine [112]. This step substitutes deproteination and is followed by liquid–liquid extraction (LLE). Saponification and subsequent removal of lipids involve losses of vitamin D and its metabolites by dragging; losses that have been traditionally evaluated by a recovery study using radioactive isotopes.

(3) LLE constitutes an alternative to the two previous sample preparation steps. It can be simple or multiple LLE. The most general manual procedure has been that of Bligh and Dyer [113].

(4) Solid-phase extraction (SPE), used since the earliest methods for quantitation of vitamin D and its metabolites, underwent a remarkable expansion with commercialization of SPE cartridges, which virtually substituted manual column packing.

The improvement of sample preparation achieved by SPE can be summarized in: lower amount of neutral lipids in the extract as compared with LLE; higher protection of the equipment used for subsequent individual separation (LC or GC) or by direct quantitation by competitive protein binding (CPBA) radioreceptor binding (RRB) or RIA; effective decrease in costs in terms of cartridges and solvents; high preconcentration factors by final elution

with small eluent volumes; and, availability for selective separation of the analytes based on polarity differences.

Nevertheless, SPE also involves some drawbacks such as: necessity for calibration procedures to minimize the variability among commercial cartridges; potential introduction of contaminants that can remain in the sorbent and might be eluted in subsequent steps; excess of confidence in the cleanup capacity of the procedures, with absence of checking and control steps.

(5) Liquid chromatography preparation as step previous to individual chromatographic separation. Molecular exclusion [114,115], solid–liquid partitioning either by normal [116–118] or reverse-phase chromatography [114,119,120] or a combination of them [115,119,120] have been used prior to RIA [121] or CPBA [94].

An overview of the contributions to sample preparation for determination of vitamin D and its metabolites by the authors' research team are as follows:

A first contact of the authors' team by the last decade of the XX century with its new research line on vitamin D was to improve a previous sample preparation SPE procedure for plasma found in the literature [122]. Keeping protein precipitation by ACN, the SPE step was improved by more efficient mixtures used for washing and elution from two subsequent cartridges (Bond-Elut C18 and Bond-Elut silica)[32]. A drastic decrease of interferents (a cleaner LC–UV-chromatogram) that allowed the determination of 24,25$(OH)_2$D, 1,25$(OH)_2$D, in addition to 25(OH)D, the only determined in the previous procedure [122].

An on-line coupling of a flow-injection (FI) manifold to the loop of the injection valve of an LC–UV detector arrangement allowed partial automation of the SPE step by locating a C18 minicolumn in the loop of the FI injection valve and selecting sequential passage through it of conditioning solution, sample, washing solution, and eluant [33]. The continuous method provided as main advantages a single and miniaturized SPE (smaller amount of sorbent), reusability of the minicolumn, drastic preconcentration factor and simple automation. The method thus developed was improved by a more effective protein precipitation by changing ACN to isopropanol, then compared advantageously with an RIA method for 25(OH)D [34]. The use of an aminopropyl-silica sorbent and the same continuous arrangement showed the following advantages provided by this polar sorbent instead of the nonpolar C18 sorbent [35]: lower detection and quantitation limits and better CV % values and recoveries than the previous methods. A new application of the arrangement allowed the determination of vitamins D_2, D_3, K_1 and K_3, and also the 25$(OH)D_3$, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ metabolites [36]. Other improvements involved an LLE step prior to SPE and the same continuous system, including postcolumn derivatization and changing the UV-chromatographic detector to a conventional fluorimetric detector (sensitivity increased about 50 times with respect to previous methods)

[37], or a laser-induced fluorescence detector (sensitivity 10 times higher than with the conventional fluorimetric detector) [38].

Almost all subsequent developments of the team in this research line during the XXI century have involved commercial automated high-pressure SPE stations (Prospekt1, Prospekt2 or Symbiosis Pharma, all from Spark-Holland) on-line connected to the chromatograph in such a way that the mobile phase acts as eluent, thus inserting in the chromatographic column the total amount of the compounds retained in the CN cartridge. The first contribution —for determination of 25(OH)D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ — involved a first manual LLE step followed by the SPE step by Prospekt1 and the final LC–DAD (diode array detector) step. A cleaner chromatogram, better reproducibility and shorter analysis time were thus achieved [40]. A subsequent development allowed total automation of the method by the use of a robotic workstation to develop the LLE step. In this way vitamins A and E, and the 25(OH)D_3, 24,25$(OH)_2D_3$ metabolites were determined (the wavelength of maximum absorption for each analyte was selected taking advantage of the DAD) [41]. A further application allowed vitamins A and E, and the 25(OH)D_3, 24,25$(OH)_2D_3$ metabolites to be determined in the normality range of these compounds in healthy individuals within the 18–80 year-old interval [42]. Avoidance of the LLE step and use of the serum sample mixed with 0.5 mL of ACN containing sodium dodecyl sulfate allowed direct insertion in the Prospekt1 system and chromatographic separation–DAD determination of fat soluble vitamins A, D_2, D_3, α- and δ-tocopherol, and 25(OH)D_3 in 20 min [43].

A key improvement of the methods for the target compounds was achieved by change the DAD to a triple quad mass spectrometry (QqQ-MS) detector. The first application of this detector, always coupled to LC equipment, involved manual sample preparation consisting of protein precipitation with methanol, LLE with hexane, evaporation of the extractant and reconstitution of the residue with methanol prior to injection in the chromatograph for separation of vitamins A, K_1, K_2, D_2, D_3 and E (as α- and δ-tocopherol), and the 25(OH)D_2, 25(OH)D_3, and 1,25$(OH)_2D_3$ metabolites [44]. The use of the commercial SPE station Prospekt2 on-line connected to the LC–QqQ-MS arrangement allowed to work with only 0.2 mL of serum sample injected (after protein precipitation) into the sample loop of the SPE station provided with Hysphere cartridges automatically conditioned. The analysis time was 20 min [45]. The features of the method made feasible its application to cross-sectional studies [47,48]. The most recent innovation in the equipment for sample preparation used by the authors' team was substitution of the Prospekt 2 by the Symbiosis Pharma model, designed for high sampling throughput with samples maintenance at 4 ºC, with subsequent in depth optimization of volumes and times for the equilibration, sorption and elution steps [49].

3.2. Derivatization

Derivatization steps based on dehydration [123–126], cycloaddition [127–130], silanization [116–118,131,132], or charge-transfer complex formation [133] have been implemented as a pre- or postcolumn step in either LC or GC.

(1) Dehydration reactions occur by exposure of vitamin D and its metabolites to high temperature. The non-specific reaction produces B-ring cyclation to yield pyro and isopyro isomers. The number of isomeric structures thus produced significantly complicates subsequent individual separation and identification. Cyclation constitutes a hard limitation for development of methods involving GC. Selective cyclation to obtain given products [122] or more thermostable products by isotachysterol formation [134–136] has been reported as a step prior to GC–MS [122].

(2) Diels–Alder cycloaddition contributes to enhancing sensitivity and selectivity in the determination of vitamin D and its metabolites, a reaction that can be developed by very different reagents and has been implemented both in pre- and postcolumn location in LC–MS methods [119,127–130].

(3) Silanization reactions have been used to minimize unspecific adsorption of vitamin D and its metabolites in GC mainly owing to the presence of hydroxyl groups on these molecules. A number of silane-derivatives have been used with this aim [93–100,137–139].

(4) Charge transfer-complex formation has also been implemented, mainly for the determination of vitamin D in pharmaceuticals [133].

Derivatization reactions have been applied by the authors' research team, always in poscolumn location, with the aim of increasing sensitivity as the necessary selectivity was supplied by LC separation. A Diels–Alder reaction using 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) formed an adduct with maximum absorption at 337 nm, which increased the sensitivity 5-times with respect to the method without derivatization [36]. A dehydration method favored by a strong-acid medium was also postcolumn developed which improved fluorimetric detection (with conventional [37] or laser-induced detectors [38]).

3.3. Sample volume

The sample volume of either serum or plasma required for determination of vitamin D metabolites has experienced a spectacular decrease thanks to the improvement of sample preparation steps, and increased sensitivity of the detectors used (mainly MS detectors). Thus, the isotope dilution-mass fragmentography assay for $1,25(OH)_2D$ published in 1979 [140] required 20 mL of serum subjected to LLE with a chloroform–methanol mixture after addition of [26-$^2H_3$]-$1,25(OH)_2D_3$, and purification by LC. Then, the metabolite in the purified material

was converted into the trimethylsilyl derivative and analyzed by GC–MS. The lower limit of quantitation (LLOQ) was 13 pmol/L (5 pg/mL), with a CV of 5%; but the large sample volume limited the general applicability of the assay. The evolution of analytical equipment and sample preparation devices led to a method published in 2010, based on LC–MS/MS [141] by which both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ could be measured in 30 μL of sample. An LLOQ for $1,25(OH)_2D_3$ of 15 ng/L (36 pmol/L), a CV of 5–15 % across physiological concentrations, and a total run time per sample of 30 min supported the excellent features of the method. Sample preparation involved a complex on-line process using a perfusion column, followed by a chain of two monolithic columns to clean and enrich the sample prior to LC–MS/MS analysis without derivatization. A recently published method uses a 20 μL serum sample volume for determination of four vitamin D metabolites —*viz.* $25(OH)D_3$, 3-epi-$25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$; with LOQ of 0.091, 0.020, 0.013, and 0.024 ng/mL, respectively. The very long sample preparation process involved SPE on wells plates, incubation, evaporation, and derivatization for 1 h prior to injection into the LC–MS/MS system [53].

In the studies developed by the authors' research team the maximum sample volume used was 2 mL [32–38,40,41], then reduced to 1 mL after exhaustive optimization [42,44,46], and to 0.5 mL by eliminating the LLE step [43], and, finally 0.2 mL when the Prospekt2 workstation was used. In our more recent research the sample volume has been 240 μL in all instances as a compromise between the required sensitivity for determination of the more diluted metabolites and the possibility of obtaining samples from patients in critical state [49,142,143].

3.4. Analysis time

A sometimes crucial analytical parameter as analysis time is very difficult to be deduced from most of the publications on this subject. A number of published methods for determination of vitamin D and its metabolites only specific the time required for the chromatographic–detection step, despite obtainment of the analytical sample [144] has involved deproteination, centrifugation, LLE, evaporation of the extractant to dryness one or several times, derivatization with enough completion requiring 1 h or even more, etc.

In the recent research developed by the authors, once the method for determination of vitamin D and its metabolites was enough validated for massive application, the time required for analysis was 15 min, achieved by overlapping of the chromatographic–detection steps of one sample with the SPE of the next [49,142,143]. The only pretreatment after sample thawing is centrifugation/filtration prior to location in the thermostated autosampler.

4. Methods for Determination of Vitamin D and its Metabolites

Methods for vitamin D metabolite measurement can be divided into two main groups: immunochemical methods and those based on liquid chromatography separation, mainly with

MS detection.

4.1. Immunoassay methods

Immunoassays dominate 25(OH)D testing (90%) in clinical laboratories and have evolved from laborious manual RIA formats to fully automated assays on random access analyzer platforms. These automated immunoassays are mainly based on a competitive principle (Ab or protein binding formats) but recently, a promising sandwich assay based on the metatype antibody principle has become available [145,146]. Automated immunoassays seem attractive for reasons of high throughput capabilities. However, they have some major shortcomings such as limited sensitivity and dynamic range, difficulties in DBP displacement, non equimolar detection of 25(OH)D_2 and 25(OH)D_3, interferences from heterophilic antibodies, and from gel and clot activator in blood collection tubes [147], lack of adequate standardization, and cross-reactivity towards other major circulating vitamin D metabolites, 24,25$(OH)_2D_3$ being the most abundant. Particularly, in specific populations with altered DBP status, as in dialysis patients, intensive care unit patients and in pregnancy, immunoassays show remarkable differences when compared to LC–MS/MS which have been attributed to inefficient release of 25(OH)D from DBP [148–150].

Immunoassay manufacturers correct for 24,25$(OH)_2D_3$ cross-reactivity in a fixed manner, which may lead to overcorrection, and to falsely low 25(OH)D as a result, in patients with lowered 24,25$(OH)_2D_3$ levels, as in chronic kidney disease [151,152].

The most commonly used methods for 1,25$(OH)_2D_3$ quantitation are competitive RIA with ^{125}I as the radio-label, or enzyme immunoassays that require extensive sample purification to minimize contribution of other vitamin D metabolites [153]. In vitamin D intoxication, elevated levels of 25(OH)D can interfere with 1,25$(OH)_2D_3$ measurement using RIA owing to 25(OH) D cross-reactivity of the 1,25$(OH)_2$D antibody [154–156]. More recently, fully automated chemiluminescence immunoassays that accurately and precisely measure 1,25$(OH)_2$D have become available. They show good correlation with well validated LC–MS/MS assays [157–162].

An area of concern in relation to immunoassays is the variability in the detection of 25(OH)D_2. Some assays claim to have 100% cross-reactivity with exogenously added 25(OH) D_2 and 25(OH)D_3 and are therefore equipotent for the measurement of the two metabolites. Other assay manufacturers admit to lower cross-reactivity with exogenous 25(OH)D_2 (75% [kit insert from IDS, Boldon, UK], 52% [product insert from Abbott, North Chicago, IL]), while some assays were specifically designed to measure only 25(OH)D_3 (product insert from Roche, Indianapolis, IN). Reports have confirmed the variability of commercial immunoassays to detect 25(OH)D_2 [163–166].

Immunoassay remains the predominant mode of measurement for 25(OH)D, although problems with equimolar recovery of the D_2 and D_3 metabolites remain an issue.

Almost all immunoassays show a high cross-reactivity with $24,25(OH)_2D$, which increases in concentration with increasing sun exposure; and as 25(OH)D increases and/or is metabolized to $1,25(OH)_2D$, this provides an increased supply of the two substrates for the 24-hydroxylase enzyme. Concentrations in the region of 10–15 nmol/L have been recorded for $24,25(OH)_2D$ in serum using GC–MS [167], with reported circulation levels of 10–15% that of 25(OH)D.

Concerns have been raised about a possible contribution to $1,25(OH)_2D$ measurement from other 1α-hydroxylated metabolites [168], and cross-reactivity for $1,25(OH)_2D_3$ 26,23-lactone, $1,24,25(OH)_3D_3$, and $1,25,26(OH)_3D_3$ has been demonstrated in both the Diasorin and IDS assays [169].

There have been major advances in semi automation and full automation of immunoassays utilizing nonradioactive tracers, which have been incorporated into both specialist-dedicated immunoassay systems.

4.2. Methods involving chromatographic separation

Liquid chromatography–tandem MS (LC–MS/MS) is regarded as the gold standard method for measurement of serum 25(OH)D concentrations due to its inherent analytical sensitivity and specificity [170–174], but the approach is equally powerful to detect other relevant vitamin D metabolites as well, either in single or multiple analyte format. The simultaneous measurement of vitamin D metabolites may provide better understanding of vitamin D metabolism in health and disease and predict which metabolite, or even combination of metabolites, may be the best indicators of vitamin D status.

Chromatography-based methods for vitamin D metabolite analysis are less susceptible to matrix effects than immunoassays. LC separations with subsequent detection, either by molecular absorption or molecular emission, have now largely been replaced by LC–MS/MS. This last approach has the advantage of selectivity, accuracy, and precision in analytical measurements as well as multiplexing capabilities, features that often greatly exceed those of immunoassays. LC–MS/MS is an approach that requires relatively expensive hardware and technical expertise operating in a batch-wise. Accurate and sensitive measurement requires optimization of many steps including sample preparation, calibration, chromatographic separation mode, choice of internal standard, ionization and transition selection for mass spectrometric detection [175]. Among the various ionization modes, ESI and APCI are the most commonly used techniques in MS vitamin D analysis, with minor use of atmospheric pressure ionization. There are reportedly over 50 vitamin D metabolites [176], apart from

numerous chemically synthesized analogs [177] and these must also be considered potential sources of assay interference. Most of the existing LC–MS/MS methods have adequate sensitivity for the measurement of 25(OH)D_3, 25(OH)D_2 and their respective C3-epimers, but the improvement in sensitivity required to include measurement of other relevant vitamin D metabolites that circulate at much lower concentrations is not always possible.This is the case with quantitation of 1,25$(OH)_2$D, which is present in serum at extremely low concentrations. The co-existence of many other higher abundant vitamin D metabolites that can interfere in 1,25$(OH)_2$D measurement constitutes an additional drawback. The LC–MS/MS platform has allowed development of methods of choice for 1,25$(OH)_2D_3$ analysis as it generates a higher selectivity in measurement when compared to immunoassays. Still, the accurate quantification of 1,25$(OH)_2D_3$ by LC–MS/MS is a challenge because of its low serum concentrations and lack of ionizable polar groups that result in poor ionization efficiency in ESI and APCI. Furthermore, specific care is needed to avoid potential interference from other dihydroxylated vitamin D metabolites, such as (3-epi) 24,25$(OH)_2D_3$, 23,25$(OH)_2D_3$, 25,26$(OH)_2D_3$ and 4β,25$(OH)_2D_3$, as they have the same molecular masses and fragmentation patterns. To enhance the detection response of the poorly ionizable compounds, derivatization strategies have been employed, mostly using Cookson-type triazoline-diones to react with the diene moiety of vitamin D, which enhances stability across the diene, and lends polar side chains to enhance ionization [9,178–182]. In some cases, mobile phase additives for adduct formation, such as ammonium, lithium or methylamine, have been used in order to further improve ionization efficiency [141, 182,183]. Recently, a new, commercially available reagent (Ampliflex™ Diene —AD—, AB Sciex, USA) was developed for derivatizing 1,25$(OH)_2$D that results in a 10-fold higher signal-to-noise ratio compared to PTAD [184]. The reaction product is optimized for MS/MS analysis due to its quaternary amine functional group and activated dienophile moiety. In contrast to PTAD, the fragmentation of the AD-1,25$(OH)_2D_3$ product is limited to several defined peaks with the quantifying SRM product containing the entire 1,25$(OH)_2$D structure, which results in different *m/z* values for the 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$ product ions. This is beneficial in preventing isobaric cross talk between the two analytes. Also, the AD-1,25$(OH)_2$D metabolite products are more polar, but remain soluble in organic solvents. This hydrophilic property of the derivatization reaction products allows for the use of more rapid LC separation techniques [184]. Improvements in sample preparation have come from inmunoaffinity extraction(IAE) allowing analyte enrichment and removal of isobaric interferences and matrix effects present in patient serum [181,182,185].

The evolution of the chromatographic methods, mainly based on the type of the coupled detector, for determination of vitamin D and its metabolites can be shown through the contributions of the authors' research team. As commented before, the first methods were based on LC–conventional molecular absorption detector with no innovation in the separation–detection step, but in sample preparation [32–36]. Keeping the same chromatographic

separation, improvement by post-column derivatization and use of a conventional fluorimetric detector [37] or a laser-induced fluorescence detector [38] was achieved. The coupling of a DAD to the LC [40–43] avoided the derivatization step and provided similar sensitivity with drastic reduction of sample volume and automation of sample preparation [43], as a prelude of the optimization and application of the LC–MS/MS platform [44–48].

All the previous contributions of the authors' group established the basis for the recent improvements on sample collection and preparation [142], on stability of the target compounds [54], the use of SIL-ISs to support quantitative analysis and DEQAS samples for external validation of the method [49], and incorporation of two-dimensional LC to MS/MS for inclusion of the C3-epimer-25(OH)D in the analysis of vitamin D metabolites [143]. Thus, the way for massive application of the final method to the determination of the target compounds in serum or plasma samples from different cross-sectional and longitudinal studies was paved [186].

4.3. The use of SIL-ISs and validation in LC–MS methods to improve the determination of vitamin D metabolites

SIL-ISs are essential for compensating for ionization suppression or enhancement effects and correction of extraction losses during sample preparation.

Isotope dilution-electrospray LC–MS/MS methods performed on ''bench top'' analyzers became popular in the mid-2000s with protein precipitation of the sample, LLE, short run times, and computer processing of chromatograms contributing to higher throughput and ease of use in the determination of vitamin D and its metabolites [187,188]. Deuterated 25(OHD_2 and D_3 internal standard material improves accuracy and verifies recovery, thereby reducing the problem of ion suppression [189].

Isotope dilution LC–MS/MS is currently considered the gold standard method for 25(OH)D measurement, being able to simultaneously quantitate 25(OH)D_2 and 25(OH)D_3, with summation of the two values resulting in total 25(OH)D. A review of the International Vitamin D External Quality Assurance Scheme (DEQAS) results for the LC–MS/MS group highlights the spread of results generated by these methods. While the majority of the methods (70–75 %) are positively biased against the all-laboratory trimmed mean (ALTM), some are close to the mean (15–20%) or negatively biased depending on the 25(OH)D concentration measured (5–10%). There has also been concern raised regarding the presence of the 3-epi-25OHD epimer of 25(OH)D, which, because of the achiral nature of LC–MS/MS, cannot be separated from 25(OH)D by the majority of current methods. The presence of an epimer may increase the total 25(OH)D concentration measured by LC–MS/MS methods compared to immunoassays.

In 2009, the National Institute of Technology (NIST) released SRM 2972, ethanolic

25(OH)D3 and 25(OH)D2 calibration solutions to improve standardization of vitamin D assays. SRM 2972 has now been replaced by SRM 2972a 25(OH)D calibration solutions, which contain two ethanolic solutions of 25(OH)D3 as well as single solutions of 25(OH)D2 and 3-epi-25(OH)D3 [190].

5. Foreseeable/Desirable Future of Vitamin D Analysis

Since vitamin D and its metabolites have been found to be related to many acute and chronic biological disorders (cardiovascular diseases, cancer, diabetes, fractures, depression and respiratory tract infections, among others) their analysis has become of great interest. Generally, the total and free concentrations of vitamin D metabolites are estimated but free circulating metabolites are technically more complex to quantitate owing to their low concentration. In addition, diseases associated with vitamin D deficiency are usually related to concentrations even lower than normal levels.

One of the major advantages of LC–MS/MS assays is the capability of measuring multiple vitamin D metabolites simultaneously. While most current LC–MS/MS methods offer good results for relatively abundant vitamin D metabolites such as 25(OH)D, they do not provide the same performance for dihydroxymetabolites such as $1,25(OH)_2D_2$ or $24,25(OH)_2D_2$. This situation should change in the future by focusing the development/improvement of the methods on achieving more sensitive and selective quantitation [174].

Another critical aspect of great interest deals with the wide range of compounds resulting from vitamin D metabolism. The present evidences of the relationship between a number of biological disorders and the concentration, or concentration ratios, of low concentrated metabolites make mandatory their accurate determination to establish the suspected influence of given metabolites on the biological state of the organism with respect to the disease [50]. Either the intake of the given metabolites or the modification of the metabolic pathways to increase their concentration in the patient could be a medium-term objective after the short-term development of the appropriate analytical methods for their accurate measurement.

6. References

1. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and preventionof vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011; 96: 1911–1930.

2. Jones G, Strugnell SA, De Luca HF. Current understanding of the molecular actions of vitamin D. Physiol Rev. 1998; 78: 1193–1231.

3. Di Rosa M, Malaguarnera M, Nicoletti F, Malaguarnera L. Vitamin D3: a helpful immuno-modulator. Immunology 2011; 134: 123–139.

4. Jones G. Metabolism and biomarkers of Vitamin D. Scand J Clin Lab Invest. 2012; 72: 7–13.

5. Kamao M, Tatematsu S, Hatakeyama S, Sakaki T, Sawada N, Inouye K, et al.C-3 epimerization of vitamin D3 metabolites and further metabolism of C-3 epimers: 25-hydroxyvitamin D3 is metabolized to 3-epi-25-hydroxyvitamin D3 and subsequently metabolized through C-1alpha or C-24 hydroxylation. J Biol Chem. 2004; 279: 15897–15907.

6.Baecher S, Leinenbach A, Wright JA, Pongratz S, Kobold U, Thiele R. Simultaneous quantification of four vitamin D metabolites in human serum using high performance liquid chromatography tandem mass spectrometryfor vitamin D profiling. Clin Biochem. 2012; 45: 1491–1496.

7. Kaufmann M, Gallagher JC, Peacock M, Schlingmann KP, Konrad M, DeLuca HF, et al. Clinical utility of simultaneous quantitation of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D by LC–MS/MS involving derivatization with DMEQ-TAD. J Clin Endocrinol Metab. 2014; 99: 2567–2574.

8. Berg AH, Powe CE, Evans MK, Wenger J, Ortiz G, Zonderman AB, et al. 24,25-dihydroxyvitamin D3 and vitamin D status of community-dwelling black and white Americans. Clin Chem. 2015; 61: 877–884.

9. Aronov PA, Hall LM, Dettmer K, Stephensen CB, Hammock BD. Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultra-performance liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem. 2008; 391: 1917–1930.

10. Hanson C, Jones G, Lyden E, Kaufmann M, Armas L, Anderson-Berry A. Vitamin D metabolism in the premature newborn: a randomized trial. Clin Nutr. 2015; 35: 835–841.

11. Wagner D, Hanwell HE, Schnabl K, Yazdanpanah M, Kimball S, Fu L, et al.The ratio of serum 24,25-dihydroxyvitamin D3 to 25-hydroxyvitamin D3 is predictive of 25 hydroxyvitamin D3 response to vitamin D3 supplementation. J Steroid BiochemMol Biol. 2011; 126: 72–77.

12. Edouard T, Husseini A, Glorieux FH, Rauch F. Serum 24,25-dihydroxyvitamin D concentrations in osteogenesis imperfecta: relationship to bone parameters. J Clin Endocrinol Metab. 2012; 97: 1243–1249.

13. Kobold U. Approaches to measurement of vitamin D concentrations – mass spectrometry. Scand J Clin Lab Invest Suppl. 2012; 243: 54–59.

14. Cashman KD, Hayes A, Galvin K, Merkel J, Jones G, Kaufmann M, et al. Significance of serum 24,25-dihydroxyvitamin D in the assessment of vitamin D status: a double-edged sword? Clin Chem. 2015; 61: 636–645.

15. Eyles D, Anderson C, Ko P, Jones A, Thomas A, Burne T, et al. A sensitive LC/MS/MS assay of 25OH vitamin D3 and 25OH vitamin D2 in dried blood spots. Clin Chim Acta2009; 43: 145–151.

16. Ogawa S, Ooki S,Morohashi M, Yamagata K, Higashi T. A novel Cookson-type reagent for enhancing sensitivity and specificity in assessment of infant vitamin D status using liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2013; 27: 2453–2460.

17. Ogawa S, Kittaka H, Shinoda K, Ooki S, Nakata A, Higashi T. Comparative evaluation of new Cookson-type reagents for LC/ESI-MS/MS assay of 25-hydroxyvitamin D3 in neonatal blood samples. Biomed Chromatogr. 2016; 30: 938–945.

18. Heath AK, Williamson EJ, Ebeling PR, Kvaskoff D, Eyles DW, English DR. Measurements of 25-hydroxyvitamin D concentrations in archived dried blood spots are reliable and accurately reflect those in plasma. J Clin Endocrinol Metab. 2014; 99: 3319–3324.

19. Larkin EK, Gebretsadik T, Koestner N, Newman MS, Liu Z, Carroll KN, et al. Agreement of blood spot card measurements of vitamin D levels with serum, whole blood specimen types and a dietary recall instrument. PLoS ONE 2011; 6: e16602.

20. Hoeller U, Baur M, Roos FF, Brennan L, Daniel H, Fallaize R, et al. Application of dried blood spots to determine vitamin D status in a large nutritional study with unsupervised sampling: The Food4Me project. Br JNutr. 2016; 115: 202–211.

21. Newman MS, Brandon TR, Groves MN, Gregory WL, Kapur S, Zava DT. A liquid chromatography/tandem mass spectrometry method for determination of 25-hydroxy vitamin D2 and 25-hydroxy vitamin D3 in dried blood spots: a potential adjunct to diabetes and cardiometabolic risk screening. J Diabetes Sci Technol. 2009; 3: 156–162.

22. Higashi T, Suzuki M, Hanai J, Inagaki S, Min JZ, Shimada K, et al. A specificLC/ESI-MS/MS method for determination of 25-hydroxyvitamin D3 in neonatal dried blood spots containing a potential interfering metabolite, 3-epi-25-hydroxyvitamin D3. J Sep Sci. 2011; 34: 725–732.

23. Ogawa S, Ooki S, Shinoda K, Higashi T. Analysis of urinary vitamin D3 metabolites by liquid chromatography/tandem mass spectrometry with ESI-enhancing and stable isotope-coded derivatization. Anal Bioanal Chem. 2014; 406: 6647–6654.

24. Higashi T, Shibayama Y, Fuji M, Shimada K. Liquid chromatography–tandem mass spectrometric method for the determination of salivary 25-hydroxyvitamin D3: a noninvasive tool for the assessment of vitamin D status. Anal Bioanal Chem. 2008; 391: 229–238.

25. Holmøy T, Moen SM, Gundersen TA, Holick MF, Fainardi E, Castellazzi M,et al. 25-hydroxyvitamin D in cerebrospinal fluid during relapse and remission of multiple sclerosis. MultScler. 2009; 15: 1280–1285.

26. Gröschl M. Current status of salivary hormone analysis. Clin Chem. 2008; 54: 1759–1769.

27. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. VitaminD and DBP: the free hormone hypothesis revisited. J Steroid Biochem Mol Biol. 2014; 144: 132–137.

28. Fairney A, Saphier PW. Studies on the measurement of 25-hydroxy vitamin D in human saliva. Br J Nutr. 1987; 57: 13–25.

29. Higashi T, Goto A, Morohashi M, Ogawa S, Komatsu K, Sugiura T, et al.Development and validation of a method for determination of plasma 25-hydroxyvitamin D3 3-sulfate using liquid chromatography/tandem mass spectrometry. J Chromatogr B AnalytTechnol Biomed Life Sci. 2014; 969: 230–234.

30. Nagubandi S, Londowski JM, Bollman S, Tietz P, Kumar R. Synthesis and biological activity of vitamin D3 3 beta-sulfate. Role of vitamin D3 sulfates in calcium homeostasis. J Biol Chem. 1981; 256: 5536–5539.

31. Luque-Córdoba D, Luque de Castro MD. Metabolomics: A potential way to know the role of vitamin D on multiple sclerosis. J Pharm Biomed Anal. 2017; 136: 22–31.

32. Ortiz-Boyer F, Fernández-Romero JM, Luque de Castro MD, Quesada JM. Improved procedure for the dual cartridge cleanup of hydroxyvitamin D3 metabolites in plasma. J LiqChrom&RelTechnol. 1998; 21: 503–518.

33. Ortiz-Boyer F, Fernández-Romero JM, Luque de Castro MD, Quesada JM. Continuous cleanup/preconcentration procedure of hydroxyvitamin D3 metabolites in plasma as an alternative to batch solid-phase extraction. J Chromatogr B 1997; 696: 43–51.

34. Ortiz-Boyer F, Fernández-Romero JM, Luque de Castro MD, Quesada JM. Quantitation of circulating hydroxyvitamin D3 in human plasma by a continuous cleanup/concentration procedure prior to HPLC–UV detection. Clin Chim Acta 1998; 274: 139–149.

35. Ortiz-Boyer F, Fernández-Romero JM, Luque de Castro MD, Quesada JM. Aminopropyl-silica as an advantageous alternative to nonpolar sorbents for continuous cleanup/preconcentration of vitamin D3 metabolites. Chromatographia 1998; 47: 367–372.

36. Ortiz-Boyer F, Fernández-Romero JM, Luque de Castro MD, Quesada JM. Determination of vitamins D2, D3, K1 and K3 and some hydroxy metabolites of vitamin D3 in plasma using a continuous clean-up–pre concentration procedure coupled on-line with liquid chromatography–UV detection. Analyst 1999; 124: 401–406.

37. Ortiz-Boyer F, Fernández-Romero JM, Luque de Castro MD, Quesada JM. Determination of vitamin D3

hydroxymetabolites in plasma at the sub-part per trillion levels using on-line cleanup/preconcentration and HPLC–fluorimetric post-column derivatisation. Talanta 1999; 50: 57–66.

38. Ortiz-Boyer F, Fernández-Romero JM, Luque de Castro MD, Quesada JM. Enhanced sensitivity by laser-induced fluorescence for the determination of calcitriol and other vitamin D3 metabolites in plasma. Chromatographia 1999; 50: 399–406.

39. Luque de Castro MD, Fernández-Romero JM, Ortiz-Boyer F, Quesada JM. Determination of vitamin D3 metabolites: state-of-the-art and trends. J Pharm Biomed Anal. 1999; 20: 1–17.

40. Mata-Granados JM, Caballo-López A, Luque de Castro MD, Quesada JM. Automated method for the determination of vitamin D3 hydroxymetabolites in serum. Anal Bio Anal Chem. 2003; 377: 287–292.

41. Mata-Granados JM, Luque de Castro MD, Quesada JM. Fully automated method for the determination of 24,25(OH)2 and 25(OH) D3 hydroxyvitamins, and vitamins A and E in human serum by HPLC. J Pharm Biomed Anal. 2004; 35: 575–582.

42. Quesada JM, Mata-Granados JM, Luque de Castro MD. Automated method for the determination of fat-soluble vitamins in serum. J Steroid Biochem Mol Biol. 2004; 89–90: 473–477.

43. Mata-Granados JM, Quesada JM, Luque de Castro MD. Fully automatic method for the determination of fat soluble vitamins and vitamin D metabolites in serum. Clin Chim Acta 2009; 403: 126–130.

44. Priego-Capote F, Jiménez JR, Mata-Granados JM, Luque de Castro MD. Identification and determination of fat-soluble vitamins and metabolites in human serum by liquid chromatography/triple quad rupole mass spectrometry with multiple reaction monitoring. Rapid Commun Mass Spectrom. 2007; 21: 1745–1754.

45. Mata-Granados JM, Ferreiro-Vera C, Luque de Castro MD, Quesada-Gómez JM. Determination of the principal metabolites of vitamin D in blood by means of on-line solid phase extraction with liquid chromatography–tandem mass spectrometry. Rev Osteoporos Metab Miner. 2010; 2: 55–61.

46. Mata-Granados JM, Luque de Castro MD, Quesada-Gómez JM. Inappropriate serum levels of retinol, α-tocopherol, 25 hydroxyvitamin D3 and 24, 25 dihydroxyvitamin D3 levels in healthy Spanish adults: Simultaneous assessment by HPLC. Clin Biochem. 2008; 41: 676–680.

47. Mata-Granados JM, Vargas-Vasserot J, Ferreiro-Vera C, Luque de Castro MD, Guerrero-Pavón R, Quesada-Gómez JM. Evaluation of vitamin D endocrine system (VDES) status and response to treatment of patients in intensive care units (ICUs) using an on-line SPE–LC–MS/MS method. J Steroid Biochem Mol Biol. 2010; 121: 452–455.

48. Mata-Granados JM, Cuenca-Acevedo R, Luque de Castro MD, Sosa M, Quesada-Gómez JM. Vitamin D deficiency and high serum levels of vitamin A increase the risk of osteoporosis evaluated by Quantitative Ultrasound Measurements (QUS) in postmenopausal Spanish women. Clin Biochem. 2010; 43: 1064–1068.

49. Mena-Bravo A, Ferreiro-Vera C, Priego-Capote F, Maestro MA, Mouriño A, Quesada-Gómez JM, Luque de Castro MD. Quantitative analytical method to evaluate the metabolism of vitamin D. Clin Chim Acta 2015; 442: 6–12.

50. Müller MJ, Volmer DA. Mass spectrometric profiling of vitamin D metabolites beyond 25-hydroxyvitamin D. Clin Chem. 2015; 61: 1033–1048.

51. Ersfeld DL, Sudhaker Rao D, Jean-Jacques B, James L, Sackrison J, Miller AB, et al. Analytical and clinical validation of the 25 OH vitamin D assay for the LIASON® automated analyzer. Clin Biochem. 2004; 37: 867–874.

52. Agborsangaya C, Toriola AT, Grankvist K, Surcel HM, Holl K, Parkkila S, et al. The effects of storage time and sampling season on the stability of serum 25-hydroxy vitamin D and and rostenedione. Nutr Cancer 2009; 62: 51–57.

53. Satoh M, Ishige T, Ogawa S, Nishimura M, Matsushita K, Higashi T, et al. Development and validation of the simultaneous measurement of four vitamin D metabolites in serum by LC–MS/MS for clinical laboratory applications.

Anal Bioanal Chem. 2016; 408: 7617¬–7627.

54. Mena-Bravo A, Calderón-Santiago M, Luque de Castro MD, Priego-Capote F. Stability study for analysis of vitamin D and metabolites in human serum by liquid chromatography coupled to tandem mass spectrometry. Sent to J Chromatogr B for publication. 2018.

55. Clemens TL, Adams JS, Nolan JM, Holick MF. Measurementof circulating vitamin D in man. Clin ChimActa 1982; 121:301–308.

56. Barragry JM, France MW, Corless D, Gupta SP, Switala S, Boucher BJ, et al. Intestinal cholecalciferol absorption in the elderly and in younger adults. Clin Sci Mol Med. 1978; 55:213–220.

57. Clemens TL, Zhou XY, Myles M, Endres D, Lindsay R. Serum vitamin D2 and vitamin D3 metabolite concentrations and absorption of vitamin D2 in elderly subjects. J Clin Endocrinol Metab. 1986; 63: 656–660.

58. Gallacher SJ, Cowan RA, Fraser WD, Logue FC, Jenkins A,Boyle IT. Acute effects of intravenous 1 alpha-hydroxycholecalciferol on parathyroid hormone, osteocalcin and calcitriolin man. Eur J Endocrinol. 1994; 130: 141–145.

59. Jones G. Pharmacokinetics of vitamin D toxicity. Am J Clin Nutr. 2008; 88:582S–586S.

60. Papapoulos SE, Clemens TL, Sandler LM, Fraher LJ, Winer J,O'Riordan JL. The effect of renal function on changes incirculating concentrations of 1,25 dihydroxycholecalciferol after an oral dose. Clin Sci (Lond) 1982; 62: 427–429.

61. Hollis BW, Assessment and interpretation of circulating 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D in the clinical environment, Rheum Dis Clin North Am. 2012; 38: 29–44.

62. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab. 2011; 96: 53–58.

63. Kushnir MM, Ray JA, Rockwood AL, Roberts WL, La'ulu SL, Whittington JE, et al. Rapid analysis of 25-hydroxyvitamin D2 and D3 by liquid chromatography–tandem mass spectrometry and association of vitamin D and parathyroid hormone concentrations in healthy adults. Am J Clin Pathol. 2010; 134: 148–156.

64. Holick MF. Vitamin D deficiency in 2010: health benefits of vitamin D and sunlight: a D-bate. Nat Rev Endocrinol. 2011; 7: 73–75.

65. Institute of Medicine. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC. 2011.

66. Poongkodi K. Causes and treatment of vitamin D deficiency. In: Vitamin D deficiency: Causes & Treatment. Unpublished manuscript 2018.

67. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. N Engl J Med. 2013; 369: 1991–2000.

68. Aloia J, Mikhail M, Dhaliwal R, Shieh A, Usera G, Stolberg A, et al. Free 25(OH)D and the vitamin D paradox in African Americans. J Clin Endocrinol Metab. 2015;100: 3356–3363.

69. Stubbs JR, Zhang S, Friedman PA, Nolin TD. Decreased conversion of 25-hydroxyvitamin D3 to 24,25-dihydroxyvitamin D3 following cholecalciferol therapy in patients with CKD. Clin J Am Soc Nephrol. 2014; 9: 1965–1973.

70. Singh RJ, Taylor RL, Reddy GS, Grebe SK. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. J Clin Endocrinol Metab. 2006; 91: 3055–3061.

71. Van den Ouweland JM, Beijers AM, Van Daal H. Fast separation of 25-hydroxyvitamin D3 from 3-epi-25-

hydroxyvitamin D3 in human serum by liquid chromatography–tandem mass spectrometry: variable prevalence of 3-epi-25-hydroxyvitamin D3 in infants, children, and adults. Clin Chem. 2011; 57: 1618–1619.

72. Bailey D, Perumal N, Yazdanpanah M, Al Mahmud A, Baqui AH, Adeli K, et al. Maternal-fetal-infant dynamics of the C3-epimer of 25-hydroxyvitamin D. Clin Biochem. 2014; 47: 816–822.

73.Yazdanpanah M, Bailey D, Walsh W, Wan B, Adeli K. Analytical measurementof serum 25-OH-vitamin D3, 25-OH-vitamin D2 and their C3-epimers by LC–MS/MS in infant and pediatric specimens. Clin Biochem. 2013; 46: 1264–1271.

74.Strathmann FG, Sadilkova K, Laha TJ, Lesourd SE, Bornhorst JA, Hoofnagle AN, et al. 3-epi-25 hydroxyvitamin D concentrations are not correlated with age in a cohort of infants and adults. Clin Chim Acta 2012; 413: 203–206.

75. Granado-Lorencio F, Muñoz García-Heras L, Blanco-Navarro I, Pérez-Sacristán B. Assessment of 3-epi-25-OH-D3 in preterm and full term infant samples and its relationship to demographic, anthropometric and biochemical determinants. Clin Biochem. 2014; 47: 853–856.

76. Goldman MM, Viec KV, Caulfield MP, Reitz RE, McPhaul MJ, Clarke NJ. The measurement of 3-epimer 25-hydroxyvitamin D by mass spectrometry in clinical specimens detects inconsequential levels in adult subjects. J Investig Med. 2014; 62: 690–695.

77. Vieth R. Vitamin D supplementation, 25-hydroxyvitamin D concentrations and safety. Am J Clin Nutr. 1999; 69: 842–856.

78.Vieth R. Vitamin D toxicity, policy, and science. J Bone Miner Res. 2007; 22: V64–V68.

79. Heaney RP, Armas LA. Quantifying the vitamin D economy. Nutr Rev. 2015; 73: 51–67.

80. Durup D, Jørgensen HL, Christensen J, Schwarz P, Heegaard AM, Lind B. A reverse J-shaped association of all-cause mortality with serum 25-hydroxyvitamin D in general practice: the CopD study. J Clin Endocrinol Metab. 2012; 97: 2644–2652.

81. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, et al. Annual high-dose oral vitamin D and falls and fractures in older women. A randomized controlled trial. JAMA 2010; 303: 815–822.

82. Suzuki M, Yoshioka M, Hashimoto M, Murakami M, Noya M, Takahashi D, et al. Randomized,double-blind, placebo-controlled trial of vitamin D supplementationin Parkinson disease. Am J Clin Nutr. 2013; 97: 1004–1013.

83. Lin AM, Fan SF, Yang DM, Hsu LL, Yang CH. Zinc-induced apoptosis in substantianigra of rat brain: Neuroprotection by vitamin D3. Free Radic Biol Med. 2003; 34: 1416–1425.

84. Dean ED, Mexas LM, Capiro NL, McKeon JE, DeLong MR, Pennell KD, et al. 25-Hydroxyvitamin D depletion does not exacerbateMPTP-induced dopamine neuron damage in mice. PLoS One. 2012; 7: e39227.

85. Wang JY, Wu JN, Cherng TL, Hoffer BJ, Chen HH, Borlongan CV, et al. Vitamin D3 attenuates 6-hydroxydopamine-induced neurotoxicity in rats. Brain Res. 2001; 904: 67–75.

86. Shinpo K, Kikuchi S, Sasaki H, Moriwaka F,Tashiro K. Effect of 1, 25-dihydroxyvitamin D3 on cultured mesencephalic dopaminergic neurons to the combined toxicity caused by L-buthioninesulfoximine and 1-methyl-4-phenylpyridine. J Neurosci Res. 2000; 62: 374–382.

87. Kosakai A, Ito D, Nihei Y, Yamashita S, Okada Y, Takahashi K, et al. Degeneration of mesencephalicdopaminergic neurons in Klotho mouse related to vitamin D exposure. Brain Res. 2011; 1382: 109–117.

88. Hypponen E, Sovio U, Wjst M, Patel S, Pekkanen J, Hartikainen AL, et al. Infantvitamin D supplementation and allergic conditions in adulthood: northern Finland birth cohort 1966. Ann N Y Acad Sci. 2004; 1037: 84–95.

89. Autier P, Boniol M, Pizot C, Mullie P. Vitamin D status and ill health: asystematic review. Lancet Diabetes Endocrinol.

2014; 2: 76–89.

90. Luque de Castro MD, Priego-Capote F. The analytical process to search for metabolomics biomarkers. J Pharm Biomed Anal. 2017; 147: 341–349.

91. Luque de Castro MD. Could metabolomics clarify the multiple sclerosis – vitamin D metabolites relationship? J Mult Scler. 2016; 3: 1710–1711.

92. Trafford DJH, Coldwell RD, Makin HLJ. Gas chromatography—mass spectrometry in the investigation of on-column dehydration of steroid hormones during gas–liquid chromatography. J Pharm Biomed Anal. 1991; 9: 1095–1105.

93. Hummer L, Nilas L, Tjellesen L, Christiansen C. A selective and simplified radioimmunoassay of 25-hydroxyvitamin D3. Scand J Clin Lab Invest. 1984; 44: 163–167.

94. Rhodes CJ, Claridge PA, Traffordand DJH, Makin HLJ. An evaluation of the use of Sep-Pak C18 cartridges for the extraction of vitamin D3 and some of its metabolites from plasma and urine. J Steroid Biochem. 1983; 19: 1349–1354.

95. Mawer EB, Hann JT, Berry JJL, Davis M. Vitamin D metabolism in patients intoxicated with ergocalciferol. Clin Sci. 1985; 68: 135–141.

96. Saggese G, Bertelloni S, Baroncelli GI. Radioreceptor 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D assay using ultrafine stationary phases in HPLC step. G Ital Chim Clin. 1986; 11: 177–182.

97. Mawer EB, Hann JT. Rapid automated HPLC assay for recalcitriol and calcidiol using trans-calcidiol as an ultra-violet-absorbing internal standard. J Chromatogr B 1987; 59: 305–316.

98. Cilliers JJL, Van-Niekerk PJ, Opperman LA, Ross FP, Sly MR. Determination of serum 25-hydroxyvitamin-D3 by HPLC using 25-hydroxyvitamin-D2 as an internal standard. J Micronutr Anal. 1987; 3: 285–293.

99. Juskova V, Wildtova V, Pacovsky V. Determination of 1,25-dihydroxyvitamin D3 in plasma using thin-layer chromatography and modified competitive protein binding assay. J Chromatogr A 1984; 290: 107–112.

100. Hollis BW, Frank NE. Solid phase extraction system for vitamin D and its major metabolites in human plasma. J Chromatogr B 1985; 343: 43–49.

101. Blanco-Gomis D, Escotet-Arias VJ, Fidalgo-Alvarez LE, Gutiérrez-Alvarez MD. Simultaneous determination of vitamins D3, E and K1 and retinylpalmitate in cattle plasma by liquid chromatography with a narrow-bore column. J Chromatogr B 1994; 660: 49–55.

102. Mawer EB, Berry JL, Cundall JP, Still PE, White A. Radioimmunoassay using a monoclonal antibody that is equipotent for ercalciriol and calcitriol (1, 25-dihydroxy vitamin D2 and D3. Clin Chim Acta 1990; 190: 199–209.

103. Johnsson H, Hessel H. High performance liquid chromatographic determination of cholecalciferol (vitamin D3) in food: a comparison with a bioassay method. Int J Vitam Nutr Res. 1987; 57: 357–365.

104. Indyk H, Woolard DC. Powders and infant formulas by HPLC. J Micronutr Anal. 1985; 1: 121–141.

105. Careri M, Lugari MT, Mangia A, Manini P, Spagnoli S. Identification of vitamins A, D and E by particle beam liquid chromatography–mass spectrometry. J Anal Chem. 1995; 351: 768–776.

106. Delgado-Zamarreño M, Sánchez-Pérez A, Gómez-Pérez MC, Hernández-Méndez JS. Directly coupled sample treatment–high-performance liquid chromatography for on-line automatic determination of liposoluble vitamins in milk. J Chromatogr. 1995; 694: 399–406.

107. Mattila P, Piironen V, Backman C, Asunmaa A, Uusi-Rauva E, Koivistinen P. Determination of vitamin D3 in egg yolk by high-performance liquid chromatography with diode array detection. J Food Compos Anal. 1992; 5: 281–290.

108. Mattila P, Piironen V, Uusi-Rauva E, Koivistoinen P. Cholecalciferol and 25-hydroxycholecalciferol contents in

fish and fish products. J Food Compos Anal. 1995; 8: 232–243.

109. Indyk H, Woollard DC. The determination of vitamin D in supplemented milk powders by HPLC. NZJ Dairy Sci Technol. 1985; 20: 19–28.

110. Meydani SN, Shapiro AC, Meydani M, Macauley JB, Blumberg JB. Effect of age and dietary fat (fish, corn and coconut oils) on tocopherol status of C57BL/6Nia mice. Lipids. 1987; 22: 345–350.

111. Johannsen FH. A generally applicable method for the determination of the D-vitamins cholecalciferol (D3) or ergocalciferol (D2). Landwirtsch Forsch. 1987; 40: 32–40.

112. Van Niekerk PJ, Smit SCC. The determination of vitamin D in margarine by high performance liquid chromatography. JAOCS. 1980; 57: 417–421.

113. Bligh EC, Dyer WJA. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37: 911–917.

114. Shimada K, Mitamura K, Mukouyama M, Okura T, Sagaya K. Separation and characterization of 25-hydroxyvitamin D3 3-sulfate in human plasma by high-performance liquid chromatography. J Chromatogr Sci. 1995; 33: 82–85.

115. Lawson DEM, Douglas J, Lean M, Sedrani S. Estimation of vitamin D3 and 25-hydroxyvitamin D3 in muscle and adipose tissue of rats and man. Clin Chim Acta 1986; 157: 175–181.

116. Coldwell RD, Trafford DJH, Makin HLJ. Improved chromatograhic determination of 25-hydroxyvitamins D2 and D3. Clin Chem. 1985; 31: 1919–1920.

117. Coldwell RD, Trafford DJH, Varley MJ, Kirk DN, Makin HLJ. Measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3, 24,25-dihydroxyvitamin D2 and 25,26-dihydroxyvitamin D2 in a single plasma sample by mass fragmentography. Clin Chim Acta 1989; 180: 157–168.

118. Shimada K, Nakatani I, Saito K, Mitamura K. Separation and characterization of monoglucuronides of vitamin D3 and 25-hydroxyvitamin D3 in rat bile by high-performance liquid chromatography. Biol Pharm Bull. 1996; 19: 491–494.

119. Vreeken RJ, Honing M, Van-Baar BLM, Ghijsen RT, De-Jong GJ, Brinkman UAT. On-line post-column Diels–Alder derivatization for the determination of vitamin D3 and its metabolites by liquid chromatography/thermospray mass spectrometry. Biol Mass Spectrom. 1993; 22: 621–632.

120. Kaune R, Harmeyer J. The quantitative determination of vitamin D3 and its metabolites in plasma. Biol Chem Hoppe-Seyler 1986; 367: 1135–1140.

121. Scharla S, Schmidt-Gayk H, Reichel H, Mayer E. A sensitive and simplified radioimmunoassay for 1,25-dihydroxyvitamin D3. Clin Chim Acta 1984; 142: 325–338.

122. Coldwell, RD, Trafford DJH, Makin HLJ. Mass fragmentographic assay for 25-hydroxyvitamin D in plasma without derivatization: enhanced sensitivity for metabolites of vitamins D2 and D3 after pre-column dehydration. J Mass Spectrom. 1995; 30: 348–356.

123. Higashi T, Awada D, Shimada K. Simultaneous determination of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in human plasma by liquid chromatography–tandem mass spectrometry employing derivatization with a Cookson-type reagent. Biol Pharm Bull. 2001; 24: 738–743.

124. Trafford DJH, Seamark DA, Turnbull H, Makin HLJ. High-performance liquid chromatography of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in human plasma: use of isotacysterols and a comparison with gas chromatography–mass spectrometry. J Chromatogr B. 1981; 226: 351–360.

125. Shimizu M, Iwasaki Y, Ishida H, Yamada S. Determination of 25-hydroxyvitamin D3 in human plasma using a non-

radioactive tetranorvitamin D analogue as an internal standard. J Chromatogr B 1995; 672: 63–71.

126. Iwata T, Yamaguchi M, HanazonoH, Imazato Y, Nakamura M, Ohkura Y. Determination of vitamin D3 and 25-hydroxyvitamin D3 in sera by column-switching high performance liquid chromatography with fluorescence detection. Anal Sci. 1990; 6: 361–366.

127. Shimizu M, Gao Y, Aso T, Nakatsu K, Yamada S. Fluorometric assay of 25-hydroxyvitamin D3 and 24R,25dihydroxyvitamin D3 in plasma. Anal Biochem. 1992; 204: 258–264.

128. Yeung B, Vouros P, Reddy GS. Characterization of vitamin D3 metabolites using continuous-flow fast atom bombardment tandem mass spectrometry and high-performance liquid chromatography. J Chromatogr A 1993; 645: 115–123.

129. Shimizu M, Kamachi S, Nishii Y, Yamada S. Synthesis of a reagent for fluorescence-labeling of vitamin D and its use in assaying vitamin D metabolites. Anal Biochem. 1991; 194: 77–81.

130. Yeung B, Vouros P, Reddy GS. Derivatization of vitamin D metabolites for analysis by capillary HPLC–tandem mass spectrometry. Am Lab. 1994; 26: 12–16.

131. Halket JM, Ganschow I, Lisboa BP. Gas chromatographic–mass spectrometric properties of boronate esters of 24R,25-dihydroxycholecalciferol. J ChromatogrA 1980; 192: 434–440.

132. Komuro S, Nakatsuka I, Yoshizake A, Iba K. Quantitative determination of F6-1,25(OH)2 vitamin D3 in human serum by gas chromatography/mass spectrometry with high-resolution selected ion monitoring. Bio Mass Spectrom. 1994; 23: 33–38.

133. Sánchez-Pérez A, Gallego-Matilla MJ, Hernández-Méndez JS. A sensitive spectrophotometric method for the determination of vitamin D3 using the charge-transfer spectra of vitamin D3-iodine complex. Anal Lett. 1993; 26: 721–731.

134. Nair PP, Bucana C, De León D, Turner DA. Gas chromatographic studies of vitamins D2 and D3. Anal Chem. 1965; 37: 631–636.

135. Seamark DA, Trafford DJH, Makin HLJ. A new procedure for the formation of isotachysterol derivatives of subnanomole quantities of ergocalciferol, cholecalciferol and its metabolites prior to gas liquid chromatography. J Steroid Biochem. 1980; 13: 1057–1063.

136. Verloop A, Koevoet AL, van Moorselaar R, Havinga E. Studies on vitamin D and related compounds IX: Remarks on the iodine-catalyzed isomerisations of vitamin D and related compounds. Recl Trav Chim Pyas-Bas. 1959; 78: 1004–1014.

137. Seamark DA, Trafford DJH, Makin HLJ. The estimation of vitamin D and some metabolites in human plasma by mass fragmentography. Clin Chim Acta 1980; 106: 51–62.

138. Holmberg I, Kristiansen T, Sturen M. Determination of 25-hydroxyvitamin D3 in serum by high performance liquid chromatography and isotope dilution–mass spectrometry. Scand J Clin Lab Invest. 1984; 44: 275–282.

139. Coldwell RD, Trafford DJH, Makin HLJ, Varley MJ, Kirk DN. Specific mass fragmentograh pic assay for 25,26-dihydroxyvitamin D in human plasma using a deuterated internal standard. J Chromatogr B. 1985; 39: 289–302.

140. Bjorkhem I, Holmberg I, Kristiansen T, Pedersen JI. Assayof 1,25-dihydroxy vitamin D3 by isotope dilution–mass fragmentography. Clin Chem.1979; 25: 584–588.

141. Casetta B, Jans I, Billen J, Vanderschueren D, Bouillon R. Development of a method for the quantification of 1-alpha, 25(OH)2-vitamin D3 in serum by liquid chromatographytandem mass spectrometry without derivatization. Eur J Mass Spectrom. 2010; 16: 81–89.

142. Mena-Bravo A, Priego-Capote F, Luque de Castro MD. Study of blood collection and sample preparation for analysis of vitamin D and its metabolites by liquid chromatography–tandem mass spectrometry. Anal Chim Acta. 2015; 879: 69–76.

143. Mena-Bravo A, Priego-Capote F, Luque de Castro MD. Two-dimensional liquid chromatography coupled to tandem mass spectrometry for vitamin D metabolite profiling including the C3-epimer-25-monohydroxyvitamin D3. J Chromatogr A. 2016; 1451: 50–57.

144. Luque de Castro MD, Priego-Capote F. Analytical Applications of Ultrasound. Elsevier: Amsterdam. 2007.

145. Saleh L, Mueller D, von Eckardstein A. Analytical and clinical performance of the new Fujirebio 25-OH vitamin D assay, a comparison with liquid chromatography–tandem mass spectrometry (LC–MS/MS) and three other automated assays. ClinChem Lab Med. 2015; 54: 617–625.

146. Cavalier E, Lukas P, Bekaert AC, Peeters S, Le Goff C, Yayo E, et al. Analyticaland clinical evaluation of the new Fujirebio Lumipulse G non-competitive assay for 25(OH)-vitamin D and three immunoassays for 25(OH)D in healthy subjects, osteoporotic patients, third trimester pregnant women, healthy African subjects, hemodialyzed and intensive care patients. Clin Chem Lab Med. 2015; 54: 1347–1455.

147. Yu S, Cheng X, Fang H, Zhang R, Han J, Qin X, et al. 25OHD analogues and vacuum blood collection tubes dramatically affect the accuracy of automated immunoassays. Sci Rep. 2015; 5: 14636.

148. Cavalier E, Lukas P, Crine Y, Peeters S, Carlisi A, Le Goff C, et al. Evaluationof automated immunoassays for 25(OH)-vitamin determination in different critical populations before and after standardization of the assays. Clin Chim Acta. 2014; 431: 60–65.

149. Heijboer AC, Blankenstein MA, Kema IP, Buijs M. Accuracy of 6 routine 25-hydroxyvitamin D assays: influence of vitamin D binding protein concentration. Clin Chem. 2012; 58: 543–548.

150.Depreter B, Heijboer AC, Langlois MR. Accuracy of three automated 25-hydroxyvitamin D assays in hemodialysis patients. Clin Chim Acta2013; 415: 255–260.

151. Bosworth CR, Levin G, Robinson-Cohen C, Hoofnagle AN, Ruzinski J, Young B, et al. The serum 24, 25-dihydroxyvitamin D concentration, a marker of vitamin D catabolism, is reduced in chronic kidney disease. Kidney Int. 2012; 82: 693–700.

152. de Boer IH, Sachs MC, Chonchol M, Himmelfarb J, Hoofnagle AN, Ix JH,et al. Estimated GFR and circulating 24, 25-dihydroxyvitamin D3 concentration: a participant-level analysis of 5 cohort studies and clinical trials. Am J Kidney Dis. 2014; 64: 187–197.

153. Seiden-Long I, Vieth R. Evaluation of a 1,25-dihydroxyvitamin D enzyme immunoassay. Clin Chem. 2007; 53: 1104–1108.

154. Hawkes CP, Schnellbacher S, Singh RJ, Levine MA. 25-Hydroxyvitamin D can interfere with a common assay for 1,25-dihydroxyvitamin D in vitamin D intoxication. J Clin Endocrinol Metab. 2015; 100: 2883–2889.

155.Ketha H, Wadams H, Lteif A, Singh RJ. Iatrogenic vitamin D toxicity in aninfant–a case report and review of literature. J Steroid Biochem Mol Biol. 2015; 148: 14–18.

156. Araki T, Holick MF, Alfonso BD, Charlap E, Romero CM, Rizk D, et al.Vitamin D intoxication with severe hypercalcemia due to manufacturing and labeling errors of two dietary supplements made in the United States. J Clin Endocrinol Metab. 2011; 96: 3603–3608.

157.van Helden J, Weiskirchen R. Experience with the first fully automated chemiluminescence immunoassay for the quantification of 1alpha, 25-dihydroxy-vitamin D. Clin Chem Lab Med. 2015; 53: 761–770.

158.Valcour A, Zierold C, Podgorski AL, Olson GT, Wall JV, DeLuca HF, et al. A novel, fully-automated,

chemiluminescent assay for the detection of 1, 25-dihydroxyvitamin D in biological samples. J Steroid Biochem Mol Biol. 2015; 164: 120–126.

159. Hussein H, Ibrahim F, Boudou P. Evaluation of a new automated assay for the measurement of circulating 1,25-dihydroxyvitamin D levels in daily practice. Clin Biochem. 2015; 48: 1160–1162

160. Miller N, Gruson D. Implementation of automated testing for 1,25-dihydroxyvitamin D: return of experience from a core-laboratory. Clin Biochem. 2015; 49: 298–301.

161. Denimal D, Lemaire-Ewing S, Duvillard L. Performance of a new 1,25-dihydroxyvitamin D fully automated assay on IDS-iSYS system. Clin Biochem. 2015; 48: 1209–1210.

162. Souberbielle JC, Cavalier E, Delanaye P, Massart C, Brailly-Tabard S, Cormier C, et al. Serum calcitriol concentrations measured with a new direct automated assay in a large population of adult healthy subjects and in various clinical situations. ClinChimActa. 2015; 451: 149–153.

163. Glendenning P, Taranto M, Noble JM, Musk AA, Hammond C,Goldswain PR, et al.Current assays overestimate 25-hydroxyvitamin D3 and underestimate 25-hydroxyvitamin D2 compared with HPLC: need for assay-specific decision limits and metabolite-specific assays. Ann Clin Biochem. 2006; 43: 23–30.

164. Binkley N, Krueger D, Lensmeyer G. 25-hydroxyvitamin D measurement, 2009: a review for clinicians. J Clin Densitom. 2009; 12: 417–427.

165. Binkley N, Krueger DC, Morgan S, Wiebe D. Current status of clinical 25-hydroxyvitamin D measurement: an assessment of between-laboratory agreement. Clin Chim Acta 2010; 411:1976–1982.

166. Carter GD, Carter R, Jones J, Berry J. How accurate are assays for 25-hydroxyvitamin D? Data from the International Vitamin D External Quality Assessment Scheme. Clin Chem. 2004; 50: 2195–2197.

167. Coldwell RD, Trafford DJ, Makin HL, Varley MJ, Kirk DN. Specific estimation of 24,25-dihydroxyvitamin D in plasma by gas chromatography–mass spectrometry. Clin Chem. 1984; 30: 1193–1198.

168. Hollis BW. 1,25-Dihydroxyvitamin D3–26,23-lactone interferes in determination of 1,25-dihydroxyvitamin D by RIA after immunoextraction. Clin Chem. 1995; 41:1313–1314.

169. Hollis BW, Kamerud JQ, Kurkowski A, Beaulieu J, Napoli JL. Quantification of circulating 1,25-dihydroxyvitamin D by radio immunoassay with 125I-labeled tracer. Clin Chem. 1996; 42: 586–592.

170. van den Ouweland JM, Vogeser M, Bächer S. Vitamin D and metabolites measurement by tandem mass spectrometry. Rev Endocr Metab Disord. 2013; 14: 159–184.

171. El-Khoury JM, Reineks EZ, Wang S. Progress of liquid chromatography–massspectrometry in measurement of vitamin D metabolites and analogues. Clin Biochem. 2011; 44: 66–76.

172.Gathungu RM, Flarakos CC, Reddy GS, Vouros P. The role of mass spectrometry in the analysis of vitamin D compounds. Mass Spectrom Rev. 2013; 32: 72–86.

173. Higashi T, Shimada K, Toyo'oka T. Advances in determination of vitamin D related compounds in biological samples using liquid chromatography–mass spectrometry: a review. J Chromatogr B 2010; 878: 1654–1661.

174.Volmer DM, Mendes LR, Stokes CS. Analysis of vitamin D metabolic markers by mass spectrometry: current techniques, limitations of the "gold standard"method, and anticipated future directions. Mass Spectrom Rev. 2015; 34: 2–23.

175. Vogeser M, Seger C. Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory. Clin Chem. 2010; 56: 1234–1244.

176. Bouillon R, Okamura WH, Norman AW. Structure-function relationships in the vitamin D endocrine system.

Endocr Rev. 1995; 16: 200–257.

177. Brown AJ, Slatopolsky E. Vitamin D analogs: therapeutic applications and mechanisms for selectivity. Mol Aspects Med. 2008; 29: 433–452.

178. Duan X, Weinstock-Guttman B, Wang H, Bang E, Li J, Ramanathan M, et al.Ultrasensitive quantification of serum vitamin D metabolites using selective solid-phase extraction coupled to microflow liquid chromatography andisotope-dilution mass spectrometry. Anal Chem. 2010; 82: 2488–2497.

179. Wang Z, Senn T, Kalhorn T, Zheng XE, Zheng S, Davis CL, et al. Simultaneous measurement of plasma vitamin D3 metabolites, including 4beta, 25-dihydroxyvitamin D3, using liquid chromatography–tandem mass spectrometry. Anal Biochem. 2011; 418: 126–133.

180. Ding S, Schoenmakers I, Jones K, Koulman A, Prentice A, Volmer DA. Quantitative determination of vitamin D metabolites in plasma using UHPLC–MS/MS. Anal Bioanal Chem. 2010; 398: 779–789.

181.Strathmann FG, Laha TJ, Hoofnagle AN. Quantification of 1alpha, 25-dihydroxy vitamin D by immunoextraction and liquid chromatography–tandemmass spectrometry. Clin Chem. 2011; 57: 1279–1285.

182. Yuan C, Kosewick J, He X, Kozak M, Wang S. Sensitive measurement of serum 1α, 25 dihydroxyvitamin D by liquid chromatography–tandem massspectrometry after removing interference with immunoaffinity extraction. Rapid Commun Mass Spectrom. 2011; 25: 1241–1249.

183. Kissmeyer AM, Sonne K. Sensitive analysis of 1α, 25-dihydroxyvitamin D3 in biological fluids by liquid chromatography–tandem mass spectrometry. J Chromatogr A. 2001; 935: 93–103.

184. Hedman CJ, Wiebe DA, Dey S, Plath J, Kemnitz JW, Ziegler TE. Developmentof a sensitive LC/MS/MS method for vitamin D metabolites: 1,25dihydroxyvitamin D2 & 3 measurement using a novel derivatization agent. J Chromatogr B. 2014; 953–954: 62–67.

185. Mahlow J, Bunch DR, Wang S. Quantification of 1,25-dihydroxyvitamin D2 and D3 in serum using liquid chromatography–tandem mass spectrometry. Methods Mol Biol. 2016; 1378: 291–300.

186. Maunsell Z, Wright DJ, Rainbow SJ. Routine isotope dilution liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D2 and D3. Clin Chem. 2005; 51:1683–1690.

187. Tsugawa N, Suhara Y, Kamao M, Okano T. Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography–tandem mass spectrometry. Anal Chem. 2005; 77: 3001–3007.

188.Vogeser M, Kyriatsoulis A, Huber E, Kobold U. Candidate reference method for the quantification of circulating 25-hydroxyvitaminD3 by liquid chromatography–tandem mass spectrometry.Clin Chem. 2004; 50: 1415–1417.

189. Annesley TM. Ion suppression in mass spectrometry. Clin Chem. 2003; 49:1041–1044.

190. Bedner M, Lippa KA. 25-Hydroxyvitamin D isomerizes to pre-25-hydroxyvitamin D in solution: considerations for calibration in clinical measurements. Anal Bioanal Chem. 2015; 407: 8079–8086.

Vitamin D deficiency: Causes & Treatment

Chapter 4

Vitamin D in Supplements and Medicines

Žane Temova Rakuša; Robert Roškar*

University of Ljubljana, Faculty of Pharmacy, Ljubljana, Slovenia

[*]***Correspondence to: Robert Roškar,*** *University of Ljubljana, Faculty of Pharmacy, Ljubljana, Slovenia.*

Email: robert.roskar@ffa.uni-lj.si.

Abstract

Vitamin D deficiency is highly prevalent condition worldwide with potential serious consequences. Vitamin D supplementation is recommended for all age groups by various guidelines. However, the use of vitamin D supplements is associated with a certain risk of lack or insufficient efficiency as well as adverse effects, mostly due to manufacturing and labelling errors. The obtained results from our own independent analysis of 23 preparations in various pharmaceutical dosage forms, available on the European market are in accordance with the published studies from all around the world and reveal high variability in the determined vitamin D content in individual preparations. The variations were more significant in supplements compared with medicines. The determined vitamin D content was higher than stated on the label in most of the tested preparations, reaching up to 156% in our study and up to 200% in the published data. Even though the use of vitamin D overages is a routine, it poses a serious danger of excessive vitamin D intakes. These can be responsible for toxic effects, mediated mostly through hypercalcaemia. This chapter hereinafter summarizes 15 reports of vitamin D intoxication due to manufacturing or labelling errors. Such results are not only concerning but also reveal the urgent need for a more strict regulation of vitamin D supplements. Their quality control is of particular importance in the supplementation of infants, which are the most sensitive population to the potential harm of vitamin D deficiency or vitamin D toxicity.

Abbreviations: Anses: French Food Safety Agency; cps: capsule; D-A-CH: Germany, Austria, Switzerland; DRV: Dietary Reference Values; DS: dietary supplements; ES: Endocrine Society; FAO: Food and Agriculture Organization of the United Nations; Gulf: United Arab Emirates and the Gulf region; HPLC: High-Performance Liquid Chromatography; ICH: International Conference on Harmonization; IOM: Institute of Medicine; m: month; NCM: Nordic Council of Ministers; NHRMC: Australian National Health and Medical Research Council; NL: Netherlands; OTC: Over the Counter medicines; SACN: UK Scientific Advisory Committee on Nutrition; SCF: Scientific Committee for Food; SE: standard error; tbl: tablet; VDD: Vitamin D deficiency; VDI: Vitamin D insufficiency; UAE: United Arab Emirates; UL: Upper tolerable limits; WHO: World Health Organization; y: years; 25(OH)D: 25-hydroxyvitamin D.

1. Vitamin D Deficiency and Prevalence

Vitamin D Deficiency (VDD) is defined as serum concentration of its major circulating form 25-hydroxyvitamin D [25(OH)D] of less than 20 ng/mL and vitamin D insufficiency (VDI) as 25(OH)D level between 21 and 29 ng/mL [1,2]. According to these definitions, 20–100% of U.S., Canadian, and European elderly men and women are estimated to be vitamin D deficient [2–4].Vitamin D deficiency and insufficiency are also highly prevalent in young and middle-aged adults worldwide, as VDD occurs in 4-55% and VDI in 25-80% of adults [5]. Pregnant and lactating women are especially at risk of vitamin D deficiency. A recent systematic review of vitamin D status revealed that the prevalence of VDD in pregnant women by WHO region is 10-54% and of VDI 50-90% [6,7], where the prevalence of VDD in breastfeeding women is 25-85% [8–10]. The main reports on vitamin D deficiency in children reveal high prevalence of VDD (1-85%) and VDI (30-90%) also in the paediatric age group [11–17]. Therefore, vitamin D deficiency is currently considered a pandemic, as it is estimated to affect around 1 billion people [3].

2. Causes of Vitamin D deficiency

The main source of vitamin D for the human kind is endogenous synthesis in the skin following exposure to sunlight. Therefore, vitamin D deficiencies are most often caused by decreased exposure of the skin to sunlight. Other factor, such as use of sunscreens, aging, skin pigmentation, season, latitude and time of the day also decrease its endogenous synthesis [18]. Furthermore, VDD is associated with some conditions (such as breastfeeding and obesity) and diseases (skin diseases, malabsorption, celiac disease, Crohn's disease, cystic fibrosis, liver or renal failure…). VDD can also be a consequence of the use of some medications, including antifungal medications, anticonvulsants, glucocorticoids and medications to treat AIDS/HIV [1].

3. Consequences of Vitamin D Deficiency

Severe vitamin D deficiency can lead to rickets in infants and small children and osteomalacia in adults. Both manifest as a consequence of impaired bone mineralisation, caused by inefficient absorption of dietary calcium and phosphorus. Typical symptoms include hypocalcemic seizures, muscle weakness, growth failure, decreased bone mass and characteristic bone changes or fractures. Less severe vitamin D deficiencies are associated with secondary

hyperparathyroidism, hypocalcaemia and hypophosphatemia. Other, nonspecific symptoms include irritability, lethargy and developmental delay. Prolonged VDI leads to low mineral bone density and an increased risk of osteoporosis, especially in post-menopausal women. Besides these well-known effects on musculoskeletal system, vitamin D deficiency is also related to several non-skeletal health problems, including many chronic conditions and diseases, which are associated with the modern way of life. Low vitamin D levels are largely described in allergies and autoimmune diseases (such as multiple sclerosis and Crohn's disease) as well as diabetes (type I and II) [19]. VDD is also associated with neurological and psychiatric diseases, including depression, schizophrenia, dementia, fibromyalgia, Alzheimer's and Parkinson's disease, as well as with the pathogenesis of different types of cancers (colorectal, prostate, breast and ovarian cancer) [20]. Moreover, several clinical trials and epidemiological studies have shown a strong association between VDD and increased risk of cardiovascular diseases and metabolic syndrome [21].

4. Vitamin D Supplementation

Food sources, naturally rich with vitamin D are quite rare and include fatty fish, red meat, eggs and wild mushrooms. Further sources of dietary vitamin D are fortified foods. In the United States and Canada, milk, infant formula, some bread products, orange juices, cereals, yogurts and cheeses are fortified with vitamin D [4]. In most European countries the fortification of foods with vitamin D is forbidden, due to an outbreak of vitamin D intoxication in young children in the 1950s. Nonetheless, some countries with high prevalence of vitamin D deficiency, such as Sweden and Finland, fortify milk products and margarine. Currently, there is growing support and development of strategies for fortifying foods with vitamin D in other European counties as well [4]. However, within the food fortification as a strategy to reduce VDD, problems associated with the stability of vitamin Darise. It is known that vitamin D is susceptible to degradation, especially when exposed to light, oxygen, elevated temperature and humidity [50]. The stability of vitamin D in foodstuffs during cooking has been shown to vary widely, resulting in vitamin D loss by 10-60% [22]. The content of vitamin D in fortified foods is also questionable, as it was found to vary from 50% to 150% of the declared to the declared value [23].

Therefore, the most effective and controlled way to prevent and treat VDD and VDI is vitamin D supplementation. Recommendations on vitamin D supplementation have been changing during the past two decades, following the latest scientific and clinical developments. Previously, vitamin D supplementation was recommended only for population groups at high risk of deficiency and for the treatment of VDD. These groups include infants and young children (<5 years), pregnant and breastfeeding women, teenagers, elderly population (≥65 years), particularly institutionalised ones, people with limited exposure to the sun (e.g. those who cover their skin for cultural reasons) and people with darker skin pigmentation. The

amount of vitamin D produced by exposure to sunlight in the summer months was presumed to be adequate for achieving sufficient serum 25(OH)D concentrations during winter for most people. However, it is now known that this is not the case. A combination of various factors: limited dietary sources of vitamin D, reduced sun exposure and increased use of sunscreens for the prevention of skin cancer, use of some medications, body fat content, age as well as several diseases has recently led to a widespread VDD in all age groups [2,4].

4.1. Vitamin D supplementation in the general population

According to the current clinical guidelines, vitamin D supplementation is recommended in all life stage groups at suggested daily amounts, depending on age and clinical circumstances. Dietary Reference Values (DRVs) are defined as the complete set of nutrient recommendations and reference values, such as population reference intakes, the average requirement, adequate intake level and the lower threshold intake [24]. Recommendations on DRVs for vitamin D by the UK Scientific Advisory Committee on Nutrition (SACN) [25], German Nutrition Society for the German-speaking countries (Germany, Austria, Switzerland, D-A-CH) [26], the French Food Safety Agency (Anses) [27], Nordic Council of Ministers (NCM) [28], Health Council of the Netherlands (NL) [29], the Scientific Committee for Food (SCF) for the European Community [30], U.S. Institute of Medicine of the National Academy of Sciences (IOM) [31], the Endocrine Society in the USA (ES) [32], Australian National Health and Medical Research Council and New Zealand Ministry of Health (NHRMC) [33], WHO/FAO [34] and in the UAE and the Gulf region [35] are summarized in **Table 1**.

Table 1: Overview of Dietary Reference Values (DRV) for vitamin D

		Life stage group						
Authority		Infants	Children		Adults	Elder	Pregnancy	Lactation
SACN	Age	0- <12 m	1-17 y		≥18 y			
	DRV (IU/day)	340-400	400		400	400	400	400
D-A-CH	Age	0- <12 m	1-18 y		≥19 y	≥65 y		
	DRV (IU/day)	400	800		800	800	800	800
Anses	Age	0- <12 m	1-3 y	4-19 y	20-74 y	≥75 y		
	DRV (IU/day)	800-1000	400	200	200	400-600	400	400
NCM	Age	0- <12 m	1-18 y		18-74 y	≥75 y		
	DRV (IU/day)	400	400		400	800	400	400
NL	Age	0- <12 m	1-18 y		18-69 y	≥70 y		
	DRV (IU/day)	400	400		400	800	400	400
SCF	Age	0-11 m	1-17 y		18-64 y	≥65 y		
	DRV (IU/day)	400-1000	0-600		0-400	400	400	400

IOM	Age	0- <12 m	1-18 y	19-70 y		≥71 y		
	DRV (IU/day)	400	600	600		800	600	600
ES	Age	0- <12 m	1-18 y	19-70 y		≥70 y		
	DRV (IU/day)	400-1000	600-1000	1500-2000		1500-2000	600-2000	600-2000
NHRMC	Age	0- <12 m	1-18 y	19-50 y	51-70 y	≥70 y		
	DRV (IU/day)	200	200	200	400	600	200	200
WHO / FAO	Age	0- <12 m	1-18 y	19-50 y	51-65 y	≥66 y		
	DRV (IU/day)	200	200	200	400	600	200	200
GULF	Age	0- <12 m	1-18 y	19-65 y		≥65 y		
	DRV (IU/day)	400-600	600-1000	800-2000		1000-2000	1500-2000	1500-2000

m-months, y-years.

As can be seen in **Table 1**, the recommended intakes for vitamin D are similar or within the same range, for most of the listed countries. The DRV for vitamin D by the Endocrine Society in the USA are considerable higher, because it recommends achieving serum 25(OH)D concentrations above 30 ng/mL, with preferred range 40-60 ng/mL on behalf of the additional health benefits in reducing the risk of common cancers, autoimmune, cardiovascular and infectious diseases and type 2 diabetes [32]. Similarly, the recommended DRV for vitamin D are noticeable higher for the UAE and the Gulf region, where the residents are exposed to various risk factors for VDD, such as diet, lack of exercise, cultural habits and avoiding sun exposure due to excessive heat [35]. The goal for this area is to achieve and maintain serum 25(OH) D concentration of 30–50 ng/mL, whereas other authorities recommend supplementation to maintain 25(OH)D concentrations above 20-30 ng/mL [36].

In general, it can be concluded that obtaining the current recommended levels of vitamin D throughout the year, through diet or endogenous synthesis is nearly impossible and that all presented authorities recommend preventive vitamin D supplementation for all age groups. Although there are a variety of guidelines on vitamin D supplementation for almost every part of the world, several factors, besides environmental, should be considered when selecting the adequate DRV. These include skin pigmentation, exposure to sun, health care system, dietary habits, clothing and cultural habits, age, body weight and potential coexisting diseases.

4.2. Vitamin D supplementation in groups at risk of vitamin D deficiency

Therapy with higher vitamin D doses than the DRV (**Table 1**) is recommended for the aforementioned groups at high risk of deficiency as well as for individuals with diagnosed conditions such as: rickets, osteomalacia, osteoporosis, bone pairs, bone deformations, disorders of calcium-phosphorus metabolism, treatment with corticosteroids, antiretroviral and antiepileptic drugs, liver and renal failure, hyper- and hypoparathyroidism, diabetes type 1 and

2, growth hormone deficiency, development delay, diseases of the nervous system, autoimmune, cardiovascular and metabolic diseases. In these cases the dosing of vitamin D should be individualized depending on the serum 25(OH)D concentration in order to maintain optimal concentration of >30 ng/mL. It is recognized that for every 100 IU of vitamin D ingested, the blood level of 25(OH)D increases by approximately 0.6 to 1 ng/mL [37,38].

4.3. Vitamin D supplementation in groups at risk of vitamin D hypersensitivity

Vitamin D hypersensitivity syndrome is a condition, responsible for vitamin D accumulation along with increased efficiency of intestinal calcium absorption and mobilization from the skeleton [39]. It is biologically characterized by a tendency towards hypercalcemia and hypercalciuria with low parathormone levels. Vitamin D hypersensitivity is associated with mutations of the CYP24A1 and SLC34A1 genes as well as with conditions such as primary hyperparathyroidism, granulomatous diseases (especially sarcoidosisor tuberculosis), chronic fungal infections and some cancers (especially lymphoma). In such cases, supplementation should be supervised by a knowledgeable medical professional and carried out in an individual manner, by monitoring the calcium and 25(OH)D levels. The latter should not exceed serum concentrations of 30 ng/mL [38–40].

4.4. Upper tolerable limits of vitamin D

Endogenous vitamin D synthesis is regulated so that prolonged sunshine exposure does not lead to vitamin D toxicity. However, considering the extensive and increasing number of vitamin D supplements, available in pharmacies and on the Internet along with the current guidelines, medical personnel recommendations and media campaigns, there is a possibility of excessive vitamin D intake. These can be responsible for toxic effects, mediated through hypercalcaemia due to bone resorption of calcium and increased intestinal calcium absorption. Symptoms of vitamin D toxicity range from mild (thirst, nausea and polyuria) to severe (calcification of soft tissues, bone demineralization, associated with increased incidence of falls and fractures, seizures, increased rates of pancreatic and prostate cancer and increased risk of mortality) [41,42]. For these reasons, regulatory agencies worldwide have established guidelines for Tolerable Upper Intake Level (UL), as a simple and effective tool to prevent vitamin D overuse by the healthy population. UL is defined as the highest average chronic daily intake of vitamin D unlikely to pose a risk to adverse health effects for almost all individuals in the population. UL values for vitamin D by European Food Safety Authority (EFSA) [43], U.S. Institute of Medicine of the National Academy of Sciences (IOM) [30], the Endocrine Society USA (ES) [31], Australian National Health and Medical Research Council and New Zealand Ministry of Health (NHRMC) [32] and in the UAE and the Gulf region [35] are presented in **Table 2**.

Table 2: Overview of Upper tolerable limits (UL) for vitamin D.

		Life stage group							
		Infants		Children		Adults	Elder	Pregnancy	Lactation
EFSA	Age	0- <12 m		1-10 y	11-17 y	≥18 y			
	UL (IU/day)	1000		2000	4000	4000	4000	4000	4000
IOM	Age	0-6 m	6-12 m	1-3 y	4-8 y	9-70 y	≥71 y		
	UL (IU/day)	1000	1500	2500	3000	4000	4000	4000	4000
ES	Age	0- <12 m		1-18 y		19-70 y	≥70 y		
	UL (IU/day)	2000		4000		10000	10000	4000-10000	4000-10000
NHRMC	Age	0- <12 m		1-18 y		19-70 y	≥70 y		
	UL (IU/day)	1000		3200		3200	3200	3200	3200
GULF*	Age	0- <12 m		1-10 y	11-18 y	≥18 y			
	UL (IU/day)	1000		2000	4000	4000	4000	4000	4000

*UL limit for obese adults and obese elderly people is 10000 IU/day; for institutionalized people it is 5000 IU/day.

5. Preparations with Vitamin D

In preparations in pharmaceutical dosage forms (dietary supplements and medicines) vitamin D is used as vitamin D2 (ergocalciferol) or vitamin D3 (cholecalciferol). The latter is the preferred form of vitamin D for supplementation, as it was proved more effective in raising and maintaining serum 25(OH)D levels [44–47]. However, these finding are disputable, as other studies have found them to be equally effective [48,49]. Preparations containing only vitamin D are available as most proper preparations for breastfed infants who have no need for supplementation with other vitamins. In addition to these, there are many available preparations with a combination of vitamin D with other vitamins (multivitamin preparations). Vitamin D is commercially available in various dosage forms including capsules (oil- and water-soluble), tablets, concentrates, chewable and gummy forms, sprays, injections and oral drops, as the only dosage form acceptable for infants [50]. Vitamin D amounts in different preparations vary greatly, reaching even 500 IU per drop in oral solutions. These preparations should be used with caution because of the ease of dispensing excessive vitamin D amounts to infants with just a few drops.

The available vitamin D preparations are registered as dietary or food supplements, non-prescription medicinal products (Over the Counter medicines - OTC) or prescription medicinal products. These differ in their definition, legislation, intended use and place of issuance.

The main difference is that supplements are used to supplement the diet by increasing the total dietary intake and unlike medicines, supplements are not intended to treat, diagnose, prevent, or cure diseases. In the EU, food supplements are defined with the Food Supplements Directive 2002/46/EC, which also establishes a list of allowable vitamins and minerals and sets labelling requirements. Other regulations are governed by individual EU Member States [51]. In the USA these preparations are referred to as dietary supplements (DS) and are regulated by Federal Food, Drug and Cosmetic Act [52] and Dietary Supplement Health and Education Act [53].The used term in Australia is complementary medicines and they are regulated with the Australian Regulatory Guidelines for Complementary Medicines [54], whereas DS in New Zealand are managed with the Dietary Supplements Regulations as part of the Food Act [55].

In general, DS are regulated as food and therefore have fewer restrictions than registered medicines. In most countries, in contrast with medicines, evidence of quality, efficacy and/or safety is not required for DS. Nonetheless, this is an upgrading field and as such, it is currently under examination and discussion by various regulatory agencies. The FDA has thus implemented a current Good Manufacturing Practice policy to ensure the quality of DS [56]. The regulation in Australia is even more stringent, as it requires pre-market approval based on quality and safety assurance [57].

One of the main aspects in the quality assurance process is content evaluation of the active ingredient in the sense of verifying the accuracy of the labelled amount. Since the European Pharmacopoeia as well as the national pharmacopoeias of the EU Member States mostly describes general test procedures, the European Medicines Agency (EMA) establishes maximum acceptable tolerance limits in the active substance content in medicines up to the end of the proposed shelf life of ± 5%. Release limits wider than ± 5% would need to be justified based on experimental results [54]. The acceptance limit for DS according to the European Commission is 80-150% of the label claim [55]. The USP acceptance limit for medicines is mostly 90-110% [58]. The acceptance limits for vitamin D in both forms, in medicines, according to their USP monographs are 100-120% (vitamin D2) and 90-120% (vitamin D3) of their labelled amount. USP provides different acceptance limits for DS with vitamin D for different dosage forms. Therefore, vitamin D tablets and capsules should contain 90-165%, whereas oral solutions should contain 90-120% of the labelled amount [56].

6. Vitamin D Content in Commercial Preparations

Production of quality commercial preparations with vitamin D can be quite a challenge, mainly due to its instability, causing losses during manufacture and storage [50]. In favour of producing commercially acceptable products, the technique that manufacturers often implement is the addition of an ‘overage’ of the stability-sensitive vitamin D. ‘Overage’ is defined as the difference between the formulated and declared levels and is usually added to ensure

the labelled content at the end of the declared shelf life [59]. However, according to the ICH guidelines on Pharmaceutical Development, any overage in the manufacture of medicines should be justified considering the safety and efficacy of the product. Information should be provided on the amount of overage, reason for the overage (e.g., to compensate for expected and documented manufacturing losses) and justification for the amount of overage [60]. The well-established use of vitamin D overage in preparations, on one hand, and its intrinsic instability and degradation during manufacture and storage, on the other hand, cause diversity in its content in commercial preparations. The re-emergence of diseases associated with VDD in the last decade, has led to a considerable rise in the usage and as well as in the number of commercially available vitamin D preparations. In relation to this, there is also an increased interest in their quality evaluation, in terms of content analysis, which is the scope of several recently published studies. In an initially published study on this topic, vitamin D content was evaluated in 4 vitamin D dietary supplements on the Polish market. The determined vitamin D content was in the range 107-138% compared with the labelled content [61]. The results obtained from a study on 16 pharmaceutical preparations with vitamin D (not specified weather they are DS, OTC or prescription medicines) from the Jordanian market revealed that only 3 preparations had vitamin D content >90%. The determined content ranged from 5 to 94% of the stated dose [62]. Comparable results were obtained in an Indian study of 14 pharmaceutical preparations (again, not specified). Vitamin D content varied from 9 to 165% of the labelled claim. Only 4 preparations were found to be within the acceptable range (90 to 125%) as defined by Indian Pharmacopeia [63]. Similar variability was determined in 15 dietary supplements from the Dutch market, specifically intended for infants. Compared to the declared values, vitamin D content ranged from 8% to 177% [23]. Similarly, a study of 15 vitamin D preparations in the USA revealed significant variations in vitamin D content in the individual preparations (9-140%), as well as between tablets from different containers with the same lot number of the same preparation (52-136% of the labelled claim) [64]. The results from a more comprehensive USA study of 54 multivitamin supplements showed similar variability in vitamin D content, ranging from 7 to 172% according to the declared amount [65]. A study on 12 dietary supplements with vitamin D from the New Zealand market revealed an even greater variability in its content, ranging from 8 to 201%. However, the content of vitamin D in the two prescription medicines included in the study was 90±4% and 97±2% of the labelled amount [66]. Difference in quality, in terms of the labelled amount accuracy between medicines and supplements was also observed in a recently published Slovenian study on 3 medicines (vitamin D content: 100-131%) and 3 supplements (vitamin D content: 100-153%) with vitamin D [67].

The actual vitamin D content in numerous preparations, registered as medicines or supplements, was also evaluated as a part of our research work. The tested pharmaceuticals in various dosage forms are available on the European market; the majority was purchased

in Slovenia. Vitamin D content was determined according to a published stability-indicating HPLC method [67]. Samples from each preparation were prepared and analysed in at least triplicate. For individual preparation, more batches with different lot numbers, were purchased and analysed. The obtained results, expressed as mean content of the analysed batches ±SE, when more batches were analysed or as mean content of the single tested batch are summarized in Table 3. More detailed results on vitamin D content, expressed as a percent of the declared amount, including the individual results of the tested batches are presented in **Figure 1**.

Table 3: Vitamin D content in supplements and medicines available on the European market, determined in our laboratory.

Mark	Dosage form	Registered as	Label claim	Number of analysed batches	Average amount found± SE (standard error)
1	Oral drops	Dietary supplement	30760 IU/mL	1	42232 IU/mL
2	Oral drops	Veterinary medicine	25000 IU/mL	1	29906 IU/mL
3	Oral drops	Prescription medicine	20000 IU/mL	4	20147 ± 209 IU/mL
4	Oral drops	Prescription medicine	14400 IU/mL	1	14043 IU/mL
5	Oral drops	Dietary supplement	12320 IU/mL	1	14774 IU/mL
6	Oral drops	Dietary supplement	12000 IU/mL	1	7755 IU/mL
7	Oral drops	Veterinary medicine	5000 IU/mL	1	6830 IU/mL
8	Oral drops	Prescription medicine	4000 IU/mL	4	4223 ± 17 IU/mL
9	Oral drops	Dietary supplement	4000 IU/mL	3	3999 ± 34 IU/mL
10	Oral drops	Dietary supplement	2400 IU/mL	1	2682 IU/mL
11	Oral drops	Prescription medicine	2000 IU/mL	5	2598 ± 47 IU/mL
12	Oral drops	Dietary supplement	320 IU/mL	1	345 IU/mL
13	Oral drops	Dietary supplement	200 IU/mL	1	240 IU/mL
14	Oral spray	Dietary supplement	1000 IU/mL	2	998 ± 22 IU/mL
15	Syrup	Dietary supplement	200 IU/mL	3	204 ± 3 IU/mL
16	Syrup	Dietary supplement	40 IU/mL	1	42 IU/mL
17	Tablets	Non-prescription medicine	500 IU/tbl	1	648 IU/tbl
18	Tablets	Non-prescription medicine	400 IU/tbl	1	488 IU/tbl
19	Tablets	Dietary supplement	400 IU/tbl	3	623 ± 7 IU/tbl
20	Tablets	Dietary supplement	400 IU/tbl	3	483 ± 13 IU/tbl
21	Tablets	Dietary supplement	200 IU/tbl	1	293 IU/tbl
22	Tablets	Non-prescription medicine	80 IU/tbl	1	97 IU/tbl
23	Capsules	Dietary supplement	600 IU/cps	1	728 IU/cps

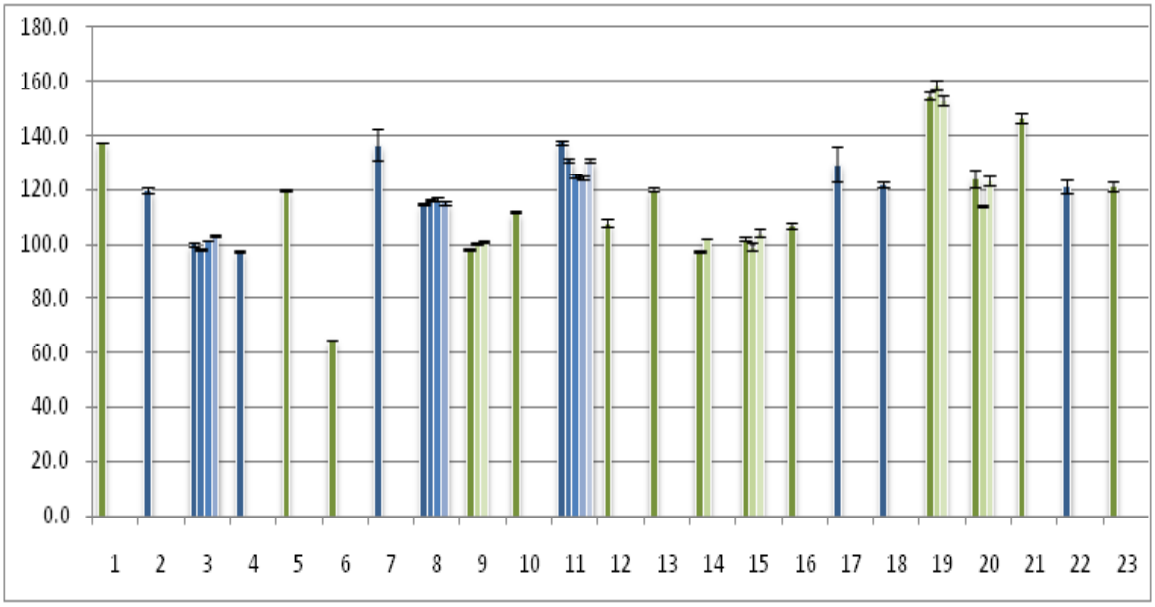

Figure 1: Vitamin D content (expressed as a percent of the declared amount with the belonging SE, n ≥ 3) in medicines (blue) and supplements (green), available on the European market and determined in our laboratory.

The obtained results are additional confirmation that in general, medicines, which are more strictly regulated, are also superior to supplements in the sense of lower variability between different batches and disparity in the actual and declared content (**Figure 1** and **Table 3**). The determined vitamin D content in the tested medicines ranged 98-130% and was similar in veterinary medicines (between 120 and 137%), whereas supplements showed substantial variability: 65-156% of the labelled amount (**Figure 1**). The determined vitamin D content was found consistent with the label claim (100 ± 5%) in approximately 20% of the tested preparations (**Figure 1**). It is also evident, that the use of vitamin D overage is a common practice among manufacturers, as only 1 out of 23 tested preparations has significantly lower vitamin D content, whereas 15 preparations had vitamin D contents higher than 110% of the declared amount (**Figure 1**). Vitamin D overages of 20% were most frequently used, which were observed in roughly one third of the tested preparations. Special attention should also be drawn at the declared vitamin D content, particularly in the tested oral drops and syrups, ranging from 40 IU/mL up to 30760 IU/mL (**Table 3**). The dose of these high-dose preparations, recommended by the manufacturer, itself exceeds most of the generally accepted Dietary Reference Values (**Table 1**). Such high concentrations in combination with inaccurate or inappropriate dosing, pose a high risk of excess vitamin D intake, which can lead to unwanted or ever toxic effects, especially if used for infants or small children. The used vitamin D overage is an additional risk factor and cause for concern. These high-dose preparations should therefore be used with extreme caution and avoided for the supplementation of infants and small children.

The main cause of the frequently used vitamin D overages lays in its instability. It is generally accepted that many environmental factors, including light, oxygen, elevated temperature and humidity affect its stability [50]. During the manufacture process and storage in closed original containers it is protected from these external influences. However, once the original containers are opened, vitamin D is exposed to these factors, resulting in its degrada-

tion and decreased content. Nonetheless, shelf life after opening is generally not defined by the manufacturers and is a common reason for disagreement between medical professionals. Despite the fact that the evaluation of vitamin D stability in commercial preparations is an important aspect of their quality, especially in the case of considerably lower initial content (around 60% in preparation 7, **Figure 1**), this research area still remains quite unexplored. To our knowledge, there is currently only one published study evaluating the effect of storage conditions on the shelf life of commercial preparations with vitamin D. The results reveal that its content generally decreases after opening the preparations to an extent dependent on the formulation and storage temperature. Considerable decrease of around 10% in vitamin D content was observed in one liquid vitamin D preparation, registered as medicine in the first 10 days of storage at ambient temperature (25 °C). The decrease was even more extensive with increases in the storage temperature, which may occur in real life [50].

Stability issues are generally more evident in solutions compared with solid dosage forms. Considering that solutions are the only dosage form acceptable for infants, they should be verified in terms of vitamin D content and shelf life after opening in order to avoid insufficient supplementation and possible consequent complications. Therefore, disparity between the actual and declared vitamin D content in preparations in both directions can be associated with serious consequences due to either excessive or insufficient vitamin D intake, especially in the most sensitive, paediatric population. The importance of appropriate regulation, via stringent quality control, emerges in such cases. The regulatory framework should ensure that vitamin D preparations (both medicines and supplements) are appropriate for use, based on benefit risk profile and that they are of adequate quality.

7. Quality Control Assessment and Toxicity

Vitamin D supplementation has gained increased acceptance due to the current guidelines and recommendations by the healthcare professionals as well as the high prevalence of vitamin D deficiency. However, it is associated with a certain risk of potential adverse toxic effect, particularly when using errantly manufactured or wrongly labelled preparations. A review of the literature revealed an increased number of publications on vitamin D toxicity after 2001 and especially after 2009. Vitamin D supplementation is generally considered safe, supported by studies revealing that even doses of 10000 IU/day, administered through longer time periods are not associated with vitamin D toxicity [4]. A recent review article reports that the most common causes of vitamin D toxicity are errors in formulation or fortification, followed by inappropriate prescribing or dispensing and errors in administration [68]. Examples of vitamin D toxicity as a result of errors in preparations are summarized in **Table 4**.

Table 4: Reports of vitamin D intoxication as a result of inaccurately manufactured or labelled supplements.

Country, year, reference	Affected patients	Symptoms	Vitamin D content (fold)*
USA, 2001, [69]	1 adult	Hypercalcemia symptoms	26 to 434
USA, 2004, [70]	1 adult, 3 children	Fatigue, constipation, back pain, forgetfulness, nausea, vomiting	470
Netherlands, 2010, [71]	2 adults	Life-threatening hypercalcemia	100 to 1000
Dominican Republic, 2011, [72]	9 adults	Hypercalcemia	400
USA, 2011, [73]	2 adults	Fatigue, excessive thirst, polyuria, muscle aches, poor mentation	117**
Ecuador, 2012, [74]	1 adult	Submandibular pain and hypercalcemia	Unknown
Australia, 2013, [75]	1 adult	Nausea, vomiting, abdominal discomfort	1000
Turkey, 2013, [76]	3 children	Abdominal pain, vomiting, poor appetite, failure to gain weight, irritation, constipation	Unknown
Italy, 2013, [77]	3 adults	Hypercalcaemia and renal insufficiency	880
Turkey, 2013, [78]	7 children	Symptoms of hypercalcemia	4000
Italy, 2014, [79]	2 children	Abdominal pain, constipation and vomiting	Higher than declared
Brazil, 2014, [80]	1 adult	Worsening of renal function, pruritus, muscle weakness, lack of appetite, weight lost	2000
USA, 2015, [81]	1 child	Emesis, diarrhoea, lethargy, dehydration	3
Netherlands, 2016, [82]	1 adult	Headaches, nausea, reduced appetite, weight loss	78
Brazil, 2016, [83]	1 adult	Hypercalcemia, acute renal function impairment	Unknown

*Fold of the determined vitamin D content according to the label claim;
**Along with the manufacturing error, there was a labelling error, recommending 10 cps instead of one cps per day.

As can be seen in **Table 4**, typical symptoms of vitamin D intoxication are a consequence of hypercalcemia and include fatigue, constipation, nausea, vomiting, polyuria and muscle weakness; more severe symptoms are associated with renal failure, which can be life-treating. Vitamin D intoxication, as a result of errors in pharmaceutical preparations, has been reported in both adult and paediatric population. As demonstrated in **Table 4** inaccurately manufactured and labelled vitamin D supplements are reported worldwide and pose a serious health problem, as the determined vitamin D amounts diverge significantly from the declared contents. The determined vitamin D amounts in these particular preparations were 3-4000 folds higher that stated on the label. Such case reports on vitamin D intoxication in conjunc-

tion with errant manufacture and labelling has not been published for vitamin D preparations, registered as medicines. Even though vitamin D intoxication is a rare condition, it is nearly always linked to supplement use and can be life-threatening. Therefore, greater awareness and caution is needed when using vitamin D supplements. Evidently, there is a substantial need for improvement in the quality of vitamin D supplements, in conjunction with more specific, strict and effective legislation to ensure their safety and also appropriate supervision of preparations, registered as supplements.

8. Conclusion

Vitamin D sufficiency is crucial for health maintenance due to its numerous functions, including calcium homeostasis and bone mineralization along with many non-skeletal effects, especially in autoimmune, cardiovascular diseases and cancer. Vitamin D deficiency is highly prevalent condition worldwide. Consequently, vitamin D supplementation is strongly recommended by numerous agencies and scientific organizations and has become widely accepted by the general population. Despite the wide therapeutic index, vitamin D supplementation can lead to toxicity and life-threatening hypercalcemia, as a result of excess vitamin D intake, especially in individuals with vitamin D hypersensitivity syndrome as well as in infants and children. In spite of that, the quality and safety of vitamin D preparations cannot be taken for granted. Due to vitamin D instability, the manufacture of vitamin D preparations is challenging and requires certain pharmaceutical expertise. The shortcut approach, which is used by most manufacturers, is the addition of vitamin D overage. The literature review along with the presented results from the currently most extensive study on vitamin D preparations available in the European market reveal that variations in the determined content compared to the stated amount were higher in dietary supplements compared to medicines. The presented cases of vitamin D intoxication, as a result of inaccurately manufactured or labelled supplements, emphasize the urgent need for a more strict regulation in the field of supplements, in general, and especially in the case of vitamin D supplements. Another, equally important aspect in the need for appropriate supervision by routine quality control and content determination in vitamin D preparations is the possible insufficient supplementation. Lower vitamin D intakes, than expected may be a result of either low initial content or vitamin D instability and its degradation during storage. As can be concluded from the obtained results, lower initial vitamin D contents are seldom in commercial preparations, but the differences between the actual and declared content can be very broad. Such deviations should be particularly taken into consideration when supplementing infants, as preparations are often their main source of the indispensable vitamin D.

9. References

1. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr 2008; 87: 1080S–1086S.

2. Palacios C, Gonzalez L. Is vitamin D deficiency a major global public health problem? J Steroid Biochem Mol Biol. 2014; 144 Pt A: 138–145.

3. Holick MF. High prevalence of vitamin D inadequacy and implications for health. Mayo Clin Proc 2006;81:353–73.

4. Holick MF. Vitamin D deficiency. N Engl J Med 2007;357:266–81.

5. Ben-Shoshan M. Vitamin D Deficiency/Insufficiency and Challenges in Developing Global Vitamin D Fortification and Supplementation Policy in Adults. Int J Vitam Nutr Res 2012;82:237–59.

6. Kiely M, Hemmingway A, O'Callaghan KM. Vitamin D in pregnancy: current perspectives and future directions. Ther Adv Musculoskelet Dis 2017;9:145–54.

7. Saraf R, Morton SMB, Camargo CA, Grant CC. Global summary of maternal and newborn vitamin D status - a systematic review. Matern Child Nutr 2016;12:647–68.

8. Zhao Y, Yu Y, Li H, Chang Z, Li Y, Duan Y, et al. Vitamin D status and the prevalence of deficiency in lactating women from eight provinces and municipalities in China. PLoS One 2017;12:e0174378.

9. Sharif S, Farasat T, Shoaib H, Saqib M, Fazal S. Vitamin D Levels Among Pregnant and Lactating Women. J Coll Physicians Surg Pak. 2013;23(12):862-5.

10. Gellert S, Ströhle A, Hahn A. Breastfeeding woman are at higher risk of vitamin D deficiency than non-breastfeeding women - insights from the German VitaMinFemin study. Int Breastfeed J 2016;12:19.

11. Oren Y, Shapira Y, Agmon-Levin N, Kivity S, Zafrir Y, Altman A, et al. Vitamin D insufficiency in a sunny environment: a demographic and seasonal analysis. Isr Med Assoc J 2010;12:751–6.

12. Larkin A, Lassetter J. Vitamin D deficiency and acute lower respiratory infections in children younger than 5 years: identification and treatment. J Pediatr Health Care 2014;28:572-82; quiz 583-4.

13. Zhao X, Xiao J, Liao X, Cai L, Xu F, Chen D, et al. Vitamin D Status among Young Children Aged 1–3 Years: A Cross-Sectional Study in Wuxi, China. PLoS One 2015;10:e0141595.

14. Mellati AA, Sharifi F, Faghihzade S, Mousaviviri SA, Chiti H, Kazemi SAN. Vitamin D status and its associations with components of metabolic syndrome in healthy children. J Pediatr Endocrinol Metab 2015;28:641–8.

15. Voortman T, van den Hooven EH, Heijboer AC, Hofman A, Jaddoe VW, Franco OH. Vitamin D Deficiency in School-Age Children Is Associated with Sociodemographic and Lifestyle Factors. J Nutr 2015;145:791–8.

16. Zhu Z, Zhan J, Shao J, Chen W, Chen L, Li W, et al. High prevalence of vitamin D deficiency among children aged 1 month to 16 years in Hangzhou, China. BMC Public Health 2012;12:126.

17. Pludowski P, Grant WB, Bhattoa HP, Bayer M, Povoroznyuk V, Rudenka E, et al. Vitamin D Status in Central Europe. Int J Endocrinol 2014;2014:1–12.

18. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am J Clin Nutr 2004;80:1678S–88S.

19. Muszkat P, Camargo MBR, Griz LHM, Lazaretti-Castro M. Evidence-based non-skeletal actions of vitamin D. Arq Bras Endocrinol Metabol 2010;54:110–7.

20. Pludowski P, Holick MF, Pilz S, Wagner CL, Hollis BW, Grant WB, et al. Vitamin D effects on musculoskeletal

health, immunity, autoimmunity, cardiovascular disease, cancer, fertility, pregnancy, dementia and mortality-a review of recent evidence. Autoimmun Rev 2013;12:976–89.

21. Kheiri B, Abdalla A, Osman M, Ahmed S, Hassan M, Bachuwa G. Vitamin D deficiency and risk of cardiovascular diseases: a narrative review. Clin Hypertens 2018;24:9.

22. Jakobsen J, Knuthsen P. Stability of vitamin D in foodstuffs during cooking. Food Chem 2014;148:170–5.

23. Verkaik-Kloosterman J, Seves SM, Ocké MC. Vitamin D concentrations in fortified foods and dietary supplements intended for infants: Implications for vitamin D intake. Food Chem 2017;221:629–35.

24. EFSA (European Food Safety Authority). Technical Report: Dietary Reference Values for nutrients 2017.

25. SACN (Scientific Advisory Committee on Nutrition). Vitamin D and Health. 2016.

26. D-A-CH (Deutsche Gesellschaft für Ernährung; Osterreichische Gesellschaft für Ernährung; Schweizerische Gesellschaft für Ernährung). Referenzwerte für die Nährstoffzufuhr. Bonn, Germany: Neuer Umschau Buchverlag; 2015.

27. Anses/CIQUAL (Agence nationale de sécurité sanitaire de l'alimentation de l'environnement et du travail/Centre d'information sur la qualité des aliments. French food composition table. 2012.

28. Nordic Council of Ministers. Nordic Nutrition Recommendations. Integrating nutrition and physical activity. 2012.

29. Health Council of the Netherlands. Dietary reference intakes: calcium, vitamin D, thiamin, riboflavin, niacin, pantothenic acid, and biotin. Publication no. 2000/12; 2000.

30. SCF (Scientific Committe for Food). Nutrient and energy intakes for the European Community. Reports of the Scientific Committee for Food. Luxembourg: Commission of the European Communities; 1993.

31. IOM (Institute of Medicine). U.S. Department of Health & Human Services. Dietary Reference Intakes for Calcium and Vitamin D. Washington, D.C.: National Academies Press; 2011.

32. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 2011;96:1911–30.

33. NHMRC (Australian National Health and Medical Research Council and (MoH) New Zealand Ministry of Health. Nutrient Reference Values for Australia and New Zealand. 2006.

34. WHO/FAO (World Health Organization and Food and Agriculture Organization of the United Nations). Vitamin and mineral requirements in human nutrition Second edition. 2004.

35. Wimalawansa SJ, Pludowski P, Anouti F Al. Clinical practice guidelines for vitamin D in the United Arab Emirates. J Steroid Biochem Mol Biol 2018;175:4–11.

36. Pludowski P, Holick MF, Grant WB, Konstantynowicz J, Mascarenhas MR, Haq A, et al. Vitamin D supplementation guidelines. J Steroid Biochem Mol Biol 2018;175:125–35.

37. Hossein-nezhad A, Holick MF. Vitamin D for health: a global perspective. Mayo Clin Proc 2013;88:720–55.

38. Grant WB, Joan Boucher B, Kimball SM, Rusin A, Rusin S, Płudowski P, et al. Vitamin D Supplementation Guidelines for General Population and Groups at Risk of vitamin D Deficiency in Poland-Recommendations of the Polish Society of Pediatric endocrinology and Diabetes and the expert Panel with Participation of National Specialist C 2018;9:1.

39. Baudart P, Molin A, Cesini J, Jones G, Kaufmann M, Kottler ML, et al. Calcium pyrophosphate deposition disease revealing a hypersensitivity to vitamin D. Jt Bone Spine 2017;84:349–51.

40. Pronicka E, Ciara E, Halat P, Janiec A, Wójcik M, Rowińska E, et al. Biallelic mutations in CYP24A1 or SLC34A1 as a cause of infantile idiopathic hypercalcemia (IIH) with vitamin D hypersensitivity: molecular study of 11 historical IIH cases. J Appl Genet 2017;58:349–53.

41. Alshahrani F, Aljohani N. Vitamin D: Deficiency, Sufficiency and Toxicity. Nutrients 2013;5:3605–16.

42. Hathcock JN, Shao A, Vieth R, Heaney R. Risk assessment for vitamin D. Am J Clin Nutr 2007;85:6–18.

43. EFSA (European Food Safety Authority). Scientific opinion on the tolerable upper intake level of vitamin D. EFSA J 2012;10:2813.

44. Armas LAG, Hollis BW, Heaney RP. Vitamin D 2 Is Much Less Effective than Vitamin D 3 in Humans. J Clin Endocrinol Metab 2004;89:5387–91.

45. Hossein-nezhad A, Holick MF. Optimize dietary intake of vitamin D. Curr Opin Clin Nutr Metab Care 2012;15:567–79.

46. Björkman M, Sorva A, Tilvis R. Responses of parathyroid hormone to vitamin D supplementation: A systematic review of clinical trials. Arch Gerontol Geriatr 2009;48:160–6.

47. Heaney RP. Vitamin D in health and disease. Clin J Am Soc Nephrol 2008;3:1535–41.

48. Holick MF, Biancuzzo RM, Chen TC, Klein EK, Young A, Bibuld D, et al. Vitamin D 2 Is as Effective as Vitamin D 3 in Maintaining Circulating Concentrations of 25-Hydroxyvitamin D. J Clin Endocrinol Metab 2008;93:677–81.

49. Biancuzzo RM, Clarke N, Reitz RE, Travison TG, Holick MF. Serum Concentrations of 1,25-Dihydroxyvitamin D 2 and 1,25-Dihydroxyvitamin D3 in Response to Vitamin D2 and Vitamin D3 Supplementation. J Clin Endocrinol Metab 2013;98:973–9.

50. Temova Ž, Roškar R. Shelf life after opening of prescription medicines and supplements with vitamin D3 for paediatric use. Eur J Hosp Pharm 2017;24:115–9.

51. DIRECTIVE 2002/46/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements (Text with EEA relevance). n.d.

52. U.S. Food and Drug Administration. Federal Food, Drug, and Cosmetic Act (FD&C Act). U.S. Government Publishing Office; 2011.

53. Office of Dietary Supplements, National Institutes of Health USD of H& HS. Dietary Supplement Health and Education Act of 1994. 1994.

54. Australian Government; Department of Health. Australian regulatory guidelines for complementary medicines. 2018.

55. Ministry of Health.Parliamentary Counsel office. Dietary Supplements Regulations 1985 (SR 1985/208) – New Zealand Legislation. Wellington: New Zealand: 1985.

56. Nutrition C for FS and A. Current Good Manufacturing Practice in Manufacturing, Packaging, Labeling, or Holding Operations for Dietary Supplements; Small Entity Compliance Guide n.d.

57. Australian Government; Department of Health and Ageing. Australian Regulatory Guidelines for Complementary Medicines (ARGCM) Part III: Evaluation of Complementary Medicine Substances. 2011.

58. United States Pharmacopeial Convention. USP29 General Chapters: Pharmaceutical Compounding - Nonsterile Preparations. 2005.

59. Food Supplements Europe. Good Manufacturing Practice for Manufacturers of Food Supplements. Brussels, Belgium: 2014.

60. International Conference on Harmonization (ICH). Pharmaceutical Development Q8(R2). vol. 8. 2009.

61. Kłackow G. AEL. Simultaneous determination of vitamins A, D3 and E in multiple pharmaceutical preparations by HPLC method. Acta Pol Pharm 2000;57:167–70.

62. Al-Qadi E, Battah A, Hadidi K. Development of High-Performance Liquid Chromatographic Method for Vitamin D3 Analysis in Pharmaceutical Preparation. Jordan J Pharm Sci 2010;3.

63. Khadgawat R, Ramot R, Chacko KM, Marwaha RK. Disparity in cholecalciferol content of commercial preparations available in India. Indian J Endocrinol Metab 2013;17:1100–3.

64. LeBlanc ES, Perrin N, Johnson JD, Ballatore A, Hillier T. Over-the-Counter and Compounded Vitamin D: Is Potency What We Expect? JAMA Intern Med 2013;173:585.

65. Andrews KW, Pehrsson PR, Betz JM. Variability in Vitamin D Content Among Products for Multivitamin and Mineral Supplements. JAMA Intern Med 2013;173:1752.

66. Garg S, Sabri D, Kanji J, Rakkar PS, Lee Y, Naidoo N, et al. Evaluation of vitamin D medicines and dietary supplements and the physicochemical analysis of selected formulations. J Nutr Health Aging 2013;17:158–61.

67. Temova Ž, Roškar R. Stability-Indicating HPLC-UV Method for Vitamin D3 Determination in Solutions, Nutritional Supplements and Pharmaceuticals. J Chromatogr Sci 2016;54:1180–6.

68. Taylor PN, Davies JS. A review of the growing risk of vitamin D toxicity from inappropriate practice. Br J Clin Pharmacol 2018;84:1121–7.

69. Koutkia P, Chen TC, Holick MF. Vitamin D Intoxication Associated with an Over-the-Counter Supplement. N Engl J Med 2001;345:66–7.

70. Klontz KC, Acheson DW. Dietary Supplement–Induced Vitamin D Intoxication. N Engl J Med 2007;357:308–9.

71. Kaptein S, Risselada AJ, Boerma EC, Egbers PHM, Nieboer P. Life-threatening complications of vitamin D intoxication due to over-the-counter supplements. Clin Toxicol 2010;48:460–2.

72. Lowe H, Cusano NE, Binkley N, Blaner WS, Bilezikian JP. Vitamin D Toxicity due to a Commonly Available "Over the Counter" Remedy from the Dominican Republic. J Clin Endocrinol Metab 2011;96:291–5.

73. Araki T, Holick MF, Alfonso BD, Charlap E, Romero CM, Rizk D, et al. Vitamin D Intoxication with Severe Hypercalcemia due to Manufacturing and Labeling Errors of Two Dietary Supplements Made in the United States. J Clin Endocrinol Metab 2011;96:3603–8.

74. Granado-Lorencio F, Rubio E, Blanco-Navarro I, Pérez-Sacristán B, Rodríguez-Pena R, García López FJ. Hypercalcemia, hypervitaminosis A and 3-epi-25-OH-D3 levels after consumption of an "over the counter" vitamin D remedy. A case report. Food Chem Toxicol 2012;50:2106–8.

75. Bell DA, Crooke MJ, Hay N, Glendenning P. Prolonged vitamin D intoxication: presentation, pathogenesis and progress. Intern Med J 2013;43:1148–50.

76. Ahmet A, Gönül Ç, Ayhan A, Ceyhun D, Ece B. Acute Vitamin D Intoxication Possibly Due to Faulty Production of a Multivitamin Preparation. J Clin Res Pediatr Endocrinol 2013;5:136–9.

77. Benemei S, Gallo E, Giocaliere E, Bartolucci G, Menniti-Ippolito F, Firenzuoli F, et al. It's time for new rules on vitamin D food supplements. Br J Clin Pharmacol 2013;76:825–6.

78. Kara C, Gunindi F, Ustyol A, Aydin M. Vitamin D Intoxication Due to an Erroneously Manufactured Dietary Supplement in Seven Childrene244. Pediatrics 2014;133:240.

79. Conti G, Chirico V, Lacquaniti A, Silipigni L, Fede C, Vitale A, et al. Vitamin D intoxication in two brothers: be

careful with dietary supplements. J Pediatr Endocrinol Metab 2014;27:763–7.

80. Marins TA, Galvão T de FG, Korkes F, Malerbi DAC, Ganc AJ, Korn D, et al. Vitamin D intoxication: case report. Einstein (São Paulo) 2014;12:242–4.

81. Ketha H, Wadams H, Lteif A, Singh RJ. Iatrogenic vitamin D toxicity in an infant – a case report and review of literature. J Steroid Biochem Mol Biol 2015;148:14–8.

82. Zigenhorn M, Westerman EM, Rietveld AP. Hypervitaminosis D due to a dietary supplement. Ned Tijdschr Geneeskd 2016;160:A9360.

83. Guerra V, Vieira Neto OM, Laurindo AF, Paula FJA de, Moysés Neto M. Hypercalcemia and renal function impairment associated with vitamin D toxicity: case report. J Bras Nefrol 2016;38:466–9.

Vitamin D deficiency: Causes & Treatment

Chapter 5

Vitamin D Deficiency in Children with Chronic Kidney Disease

Nirupama Gupta MD

Division of Nephrology, Department of Pediatrics, College of Medicine, University of Florida, Gainesville, FL, 32610 USA

Phone: 352-273-9182; Fax: 352-294-8072, Email: peacock7@ufl.edu

Abbreviations: 1,25(OH)2D: calcitriol; 7-DHC: 7-dehydrocholesterol; 25(OH)D: 25-hydroxyvitamin D; BMD: bone mineral density; CKD: chronic kidney disease; CKD-MBD- CKD: Mineral and Bone Disorder; *CYP2R1* gene: encodes the 25-hydroxylase enzyme; *CYP24A1* gene: encodes the 24-hydroxylase enzyme; *CYP27B1* gene: encodes the 1α-hydroxylase enzyme; FGF23: fibroblast growth factor 23; GFR: glomerular filtration rate; IOM: Institute of Medicine; KDIGO: Kidney Disease Improving Global Outcomes; NKF/KDOQI: National Kidney Foundation/Kidney Disease Outcomes Quality Initiative; PD: peritoneal dialysis; PTH: parathyroid hormone; RCT: randomized controlled trial; RDA: recommended dietary allowance; SHPT: secondary hyperparathyroidism; UV: ultraviolet; Vitamin D2 (ergocalciferol); Vitamin D3 (cholecalciferol); VDBP: Vitamin D binding protein; VDR -Vitamin D receptor

1. Introduction

Chronic kidney disease (CKD) is associated with disturbances of mineral and bone metabolism, which includes abnormalities of hypocalcemia, hyperphosphatemia, and Vitamin D metabolism. The alteration of mineral bone metabolism can lead to secondary hyperparathyroidism, metabolic bone disease, and extra-skeletal calcifications. The primary focus of the current chapter is to review vitamin D deficiency among children with CKD and to discuss the available therapeutic options available in the treatment and prevention of vitamin D deficiency and its related effects.

2. Vitamin D: Normal Metabolism

Vitamin D is a fat-soluble pro-hormone that is produced endogenously in the skin and can be found in certain foods. Humans acquire approximately 80% of their vitamin D from sunlight-induced cutaneous synthesis, while the rest comes from diet and supplement [1]. The two major forms of vitamin D are ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Vitamin D2 is commonly found in vegetable sources and “fortified” foods whereas vitamin D3 is found in animal-based foods and is synthesized in the skin. Dietary sources of vita-

min D are important during the winter months, when there is insignificant synthesis of vitamin D3 in the skin; the only significant sources of vitamin D (D2 or D3) are animal liver, fatty fish, egg yolks, fish oils, and commercially irradiated mushrooms [2].

Vitamin D2 is manufactured through the ultraviolet irradiation of ergosterol from yeast; whereas, vitamin D3 is from the conversion of 7-dehydrocholesterol (7-DHC) to previtamin D in the epidermal layer of the skin by solar ultraviolet (UV) B radiation (wavelength 290-315 nm) [3]. Previtamin D from the skin is then rapidly converted to vitamin D3 by a temperature-dependent process. Excess previtamin D3 can be destroyed by sunlight; thus, excessive exposure to sunlight does not cause vitamin D3 intoxication [3].

Both vitamin D2 and D3 have similar metabolism; they both are incorporated into chylomicrons after ingestion and absorbed into the lymphatic system. From the circulation, they either enter the liver to undergo further processing or are taken up by the adipose tissue for storage. For Vitamin D to become biologically active, it must undergo two steps of activation.

In the first step, after vitamin D is transported by the vitamin D-binding protein (VDBP) into the liver, it undergoes hydroxylation at the carbon 25 position by the 25-hydroxylase enzyme (coded by the *CYP2R1* gene) to become 25-hydroxyvitamin D [25(OH)D]. 25(OH) D3 is the main circulating form of vitamin D and has a relatively long half life of 2-3 weeks [4]. 25(OH)D circulates bound to VDBP (85–90 %) or albumin (10–15 %), with <1 % in its free forms (Denburg 2013). VDBP is a 58 kDa protein that has a higher binding affinity than albumin for the vitamin D metabolites [5,6]. The binding of vitamin D to VDBP affects their half-life, rate of uptake by target cells, and clearance through hepatic metabolism and biliary excretion.

In the second step, 25(OH)D is converted to 1,25(OH)2D (calcitriol) in the kidney. The VDBP- 25(OH)D complexes are filtered by the glomerulus and are reabsorbed via megalin/cubilin receptor-mediated endocytosis at the level of the proximal tubular brush border [5,6]. Once inside the tubular cell, 25(OH)D is released and converted in the mitochondria by 25-hydroxyvitamin D-1α-hydroxylase enzyme (coded by the *CYP27B1* gene), to form 1,25(OH)2D. This final product is the biologically active form of vitamin D responsible for maintaining calcium and phosphorus homeostasis.

CYP24A1 encodes the key enzyme (24-hydroxylase) involved in the catabolism of both 25(OH)D and 1,25$(OH)_2$D to form 24R,25$(OH)_2$D3 and 1α,24,25(OH)3D3, respectively. In vitro and animal studies have shown that renal CYP24A1 is suppressed by the parathyroid hormone (PTH) and induced by fibroblast growth factor 23 (FGF23) [5]. The 24-hydroxylation is the first of a five step process in the C-24 oxidation pathway to form the biologically inactive, water soluble calcitroic acid [2,3,5,7]. *CYP24A1* is expressed in most tissues and is regulated

by 1,25$(OH)_2$D. In normal settings, the inactive metabolites of 25(OH)D circulate at less than 10–15% of the total concentration of 25(OH)D [8].

3. Vitamin D: Physiologic Functions of Vitamin D

Vitamin D hormone functions through a single vitamin D receptor (VDR), a superfamily of nuclear receptors, to initiate or suppress gene transcription of a wide variety of vitamin D dependent genes in vitamin D target cells [2,9]. VDR is located in the target cells of enterocytes, osteoblasts, distal renal tubular cells and parathyroid gland cells [10]. Active vitamin D is tightly regulated by PTH, calcium, phosphorus, and FGF23 levels.

When calcitriol binds to the VDR in the small intestinal cells, a cascade of events leads to the increase of calcium channel expression and entry. Active vitamin D enhances phosphorus absorption in the small intestine as well. Without vitamin D, only 10-15% of dietary calcium and about 60% of phosphorus are absorbed. With active Vitamin D, intestinal calcium absorption increases to 30-40% and phosphorus absorption to approximately 80% [3]. In general the dietary calcium is favored to support serum calcium concentrations under normal conditions; however, if this system fails, then calcium mobilization from bones and reabsorption in the kidneys is required to maintain calcium and phosphorus homeostasis.

PTH regulates calcium metabolism by increasing tubular reabsorption of calcium in the kidney, increasing mobilization of calcium from the skeleton by the activation of osteoblasts, and by increasing the renal production of 1,25$(OH)_2$D [8]. PTH also causes phosphaturia, resulting in low to normal serum phosphorus level. The presence of active vitamin D can also suppress the parathyroid gene and proliferation of the parathyroid gland cells [7].

FGF23, a bone derived hormone, down-regulates calcitriol synthesis by decreasing the expression of *CYP27B1* and upregulates calcitriol catabolism via *CYP24A1*. It also increases phosphaturia by internalizing the sodium-phosphate transporter (NaPi2a) channels from the luminal proximal tubular cells via a Klotho-FGF23 signal transduction. FGF23 is stimulated by both phosphate intake and vitamin D treatment.

4. Vitamin D: Extra-Renal Calcitriol Synthesis and Effects

Extra-renal 1α-hydroxylase is also expressed in nonrenal tissues to locally produce 1,25$(OH)_2$D, where it is believed to act in an autocrine or paracrine manner to promote additional functions of vitamin D outside the classical endocrine functions in calcium or phosphate homeostasis [2]. The VDR appears also in the placenta, macrophages, skin keratinocytes, promyelocytes, lymphocytes, colon cells, pituitary gland cells, breast cells, prostate and ovarian cells (2,3,7]. Studies shows that 1α-hydroxylase activity in extra-renal cells may contribute to tissue function, cell proliferation, and immunoregulation [6]. It is probably regulated by cy-

tokines and growth factors as part of an inflammatory response [2]. Although it is beyond the scope of this article to discuss every organ system or conditions associated with extra-renal calcitriol synthesis [3], we will discuss the effect of vitamin D in the immune and cardiovascular systems here.

5. Immune System

Calcitriol is a potent immunomodulator, whose biological effects are mediated through VDR. VDR is present in most immune cells, including T lymphocytes, neutrophils and antigen presenting cells (APCs) such as macrophages and dendritic cells [11]. $1,25(OH)_2D$ can be synthesized by monocytes and macrophages via the upregulation of extra-renal 1α-hydroxylase [12]. $1,25(OH)_2D3$ primarily affects dendritic cell maturation and macrophage differentiation, and reduces cytokine release [11]. In monocytes, vitamin D acts as a transcription factor for antibacterial peptides such as cathelicidin and beta-defensin 4A [3]. Cathelcidins are a family of antimicrobial peptides found in lysosomes, macrophages and polymorphonuclear leukocytes that serve a role to defend against bacterial infections.

Resting T-cells express almost undetectable levels of VDR, but the receptor levels increase as T-cells proliferate following an antigenic activation [13]. $1,25(OH)_2D$ can inhibit T-cell activation and proliferation by modifying the capacity of APCs to express co-stimulatory molecules, such as CD40, CD80 and CD86; it can inhibit the release of IL-12, a cytokine responsible for stimulating T-helper 1 cell development; and, it can inhibit T-helper 17 cell development and increase the production of T-helper 2 and T regulatory cells [11]. There is also evidence that $1,25(OH)_2D$ may inhibit the differentiation of B cells into plasma cells, thus modulating the production of antibodies [13]. Hence, the suppression of the adaptive immune system could be useful in treating a variety of autoimmune disorders, whereas, the stimulation of the innate immune response could be a useful first line of defense against microbial pathogens [11].

6. Cardiovascular System

VDR is also found on endothelial cells, smooth muscle cells and myocytes [9]. One of the earliest stages of the atherosclerosis process is the impairment of endothelial function [14]. Endothelial dysfunction involves a complex mechanism that includes overproduction of reactive oxidative species, inflammatory cytokines and pro-atherogenic lipoproteins, along with an imbalance between molecules for vasodilation (e.g., nitric oxide (NO), produced by endothelial cells) and vasoconstriction (e.g., endothelin-1) [15,16]. In addition, calcitriol directly regulates endothelial NO synthase [17] and suppresses the expression of renal renin production [18]. Flow mediated dilation, release of NO in response to sheer stress, is the gold standard in measuring endothelial function [16].

While vitamin D may improve endothelial function in some studies, a large meta-analysis of 16 adult studies showed no significant improvement of endothelial function with vitamin D supplementation, regardless of the type of vitamin D, method of administration and baseline 25(OH)D levels [15]. Another meta-analysis summarized of 46 prospective trials suggested no effects of 25(OH)D supplementation on systolic or diastolic blood pressure reduction [19]. However, this does not completely exclude a role for vitamin D in modulation of the renin-angiotensin-aldosterone system but suggests that the effect may be small and possibly subclinical.

7. Definition of Vitamin D Deficiency

The serum concentration of 25(OH)D is the best marker for vitamin D status because: a) there is no negative feedback to limit the conversion of pre-vitamin D metabolite from cutaneous synthesis or diet to 25(OH)D; b) has no significant storage in the liver; c) has a half life in vivo of approximately 2-3 weeks; d) and serum/plasma 25(OH)D is stable and resistant to repeated freeze-thaw cycles [20]. The serum 1,25 (OH)D level does not accurately reflect the vitamin D status because its conversion depends on the availability of its substrate 25(OH)D; b) its conversion is tightly regulated by circulating PTH, FGF23, calcium and phosphate; and, c) the half-life in vivo is approximately 4-6 hours [20].

The recommendations for the current vitamin D guidelines comes from prior studies that showed a direct relationship between PTH and vitamin D levels. For example, it was shown that PTH levels began to plateau at their nadir when 25(OH)D levels were between 30 and 40 ng/mL (70-100 nmol/L) [21]. Furthermore, healthy adults that were given 50,000 IU of vitamin D once a week for 8 weeks for a 25(OH)D level of between 11 and 25 ng/mL, had an increase in their 25(OH)D levels by more than 100% at the end of 8 weeks, and that the mean decrease on PTH levels declined by 55% in subjects who had 25(OH)D between 11 and 15ng/mL and decreased by 35% for those with 25(OH)D levels of between 16 and 19 ng/mL [22]. Those subjects who had 25(OH)D > 20 ng/mL had no significant change in their PTH level. Thus, it was suggested that vitamin D deficiency should be defined as 25(OH)D <20 ng/mL. It is also observed that intestinal calcium transport increased by 45 to 65% in women when 25(OH)D levels increased from 20 to 32ng/ml (50-80 nmol/L) [23]. It also appears that vitamin D intoxication does not occur until blood levels are 150–200 ng/mL [8]. Vitamin D intoxication is defined as a 25(OH)D level >150 ng/mL that is associated with hypercalcemia, hypercalciuria and, often, hyperphosphatemia [3].

Based on published guidelines [24-29] (Table 1), the critical lower limit of serum vitamin D concentration is not well defined. The Pediatric Endocrine Society [25] and Endocrine Society Clinical Guideline [27] base their recommendations on the effects of vitamin D on the prevention of nutritional rickets, PTH suppression and optimal gut calcium absorption. Other

experts recommend that serum 25(OH)D should be greater than 30 ng/ml [8, 29, 30] as concentrations below this are associated with hyperparathyroidism, lower bone mineral density (BMD) and hip fractures [20]. Institute of Medicine [27] recommends their definition based solely on the effects of vitamin D on bone health outcomes.

Table 1: Definitions for Vitamin D Levels (ng/ml)*

Vitamin D status	AAP [24]	Pediatric Endocrine Society [25]	National Osteoporosis Society [26]	Institute of Medicine [27]	Endocrine Society [28]	K/DOQI 2009 [29]
Vitamin D Toxicity	-	>150	>150	-	>150	-
Vitamin D Risk of Toxicity/Excess	-	>100	-	> 50	> 100	-
Vitamin D Sufficiency/ Adequacy	≥20	≥20	≥ 20	≥20	≥ 30	≥ 30
Vitamin D Insufficiency	16-20	15-20	10-19	12-20	21-29	16-30
Vitamin D Deficiency	<15	≤15	<10	<12	<20	<5-15
Severe Vitamin D Deficiency	-	≤ 5	-	<5	-	<5

*to convert from ng/ml to nmol/L multiply by 2.496 AAP - American Academy of Pediatrics

8. Impact of Ckd on Vitamin D Metabolism

8.1. Definition of Ckd

CKD is defined as any abnormality of the kidney structure, such as having markers of kidney damage and/or decreased glomerular filtration rate (GFR) < 60ml/min/1.73m2, present for > 3 months, with implications for health [31]. The markers of kidney damage include albuminuria (ACR >30mg/g; AER > 30mg/24 hours), abnormal renal histology, renal imaging, urinary sediment, electrolyte and/or tubular disorders, and history of kidney transplantation. Furthermore, CKD is further divided into stages based on the glomerular filtration rate (see Table 2).

Table 2: GFR categories in CKD [31]

CKD category	GFR (ml/min/1.73m2)	Terms
Stage 1	>90	Normal or high
Stage 2	60-89	Mildly decreased
Stage 3a	45-59	Mildly to moderately decreased
Stage 3b	30-44	Moderately to severely decreased
Stage 4	15-29	Severely decreased
Stage 5	<15	Kidney failure

9. Prevalence of Vitamin D Deficiency in Ckd

It is estimated that more than one billion people have vitamin D insufficiency or deficiency worldwide [3]. It is recognized that 30–50% of both the European and US population are vitamin D insufficient or deficient [8]. According to the NHANES 2001-2004 report, about 9% of the USA healthy pediatric population aged 1 to 21 years is vitamin D deficient (vitamin D level < 15ng/ml) and 61% are vitamin D insufficient (vitamin D level 16-29ng/ml) [32]. The NHANES 2001-2006 report states that 18% of children aged 1 to 11 years old are vitamin D deficient. Younger age (< 6 years old), girls and non-his panic blacks had the highest prevalence of vitamin D deficiency [33]. In healthy infants and toddlers (8-24 months of age), the prevalence of vitamin D deficiency (vitamin D level < 20ng/ml) is 12.1% [34].

The prevalence of pediatric CKD ranges from 15 to 74.7 cases per million children [35]. About half of the children with CKD have congenital abnormalities of the kidney and urinary tract (CAKUT), such as renal hypodysplasia/aplasia, obstructive uropathy and reflux nephropathy [36, 37]. The prevalence of vitamin D insufficiency and deficiency in children with CKD ranges between 16% and 82% [37].

In a cohort of European children with CKD stages 3-5, 94.2% had vitamin D levels below 30 ng/ml, with 68.2% having vitamin D deficiency (< 16ng/ml) [37]. In a cohort of 78 children with CKD stages 2-4 in India, vitamin D deficiency was present in 92%: 34% had insufficiency, 50% had mild deficiency and 6.7% had severe deficiency [35]. In a cohort of 167 children, vitamin D deficiency (<20 ng/ml) was present in 12.5% of healthy controls compared to 32% with CKD [38]. Among the different subgroups of children with CKD, the severity of vitamin D deficiency increases with advanced stages [38].

10. Risk Factors for Vitamin D Deficiency in Ckd

● Limited sun exposure - Children with CKD are at a greater risk for vitamin D deficiency because they are less active and have less sunlight exposure that would otherwise promote cutaneous generation of endogenous vitamin D3 [39]. It is observed that as skin pigmentation increases from type III to types V and VI, the exposure time necessary to maximize previtamin D3 formation increases from 30 minutes to 1 hour and 3 hours, respectively, likely due to melanin competition with 7-DHC [40]. However, regardless of the skin type, previtamin D3 reaches a maximum and plateaus at about 15% of the original 7-DHC and further UV-B radiation increases only in lumisterol-3 (biologically inactive photoisomer of vitamin D3) [40]. As the latitude changes, with a decrease in UV radiation from the equator to the North, the exposure time required to maximize previtamin D3 formation also increases [40].

● Chronic kidney disease - When the GFR falls < 50ml/min/1.73m2, the kidney cannot convert 25(OH)D to 1,25$(OH)_2$D. The proposed reasons are the following ([39]:

a) reduced renal mass is accompanied by reduced availability of 1a-hydroxylase

b) raised phosphate and FGF23 downregulate 1**α**-hydroxylase

c) 1**α**-hydroxylase is suppressed in an acidic and uremic milieu

d) reduced renal megalin expression

e) reduced availability of the substrate 25(OH)D

f) and secondary hyperparathyroidism depletes body stores of vitamin D by promoting *CYP24A1*

PTH levels are usually in the normal range in a majority of patients with CKD stage 2, but it rises in a significant percentage of patients with progressive kidney failure [41]. In CKD stages 2-3, hyperphosphatemia stimulates FGF23 synthesis to decrease phosphate retention [42,43]. FGF23 also suppresses renal 1**α**-hydroxylase enzyme, resulting in a reduction of active vitamin D level [42] and induces 24-hydroxylase expression, which is responsible for the degradation of $1,25(OH)_2D$ [44]. As CKD progresses, maximal FGF23 effects on renal phosphate excretion are reached and phosphate retention ensues. Moreover, the rising PTH levels with advanced CKD causes release of calcium and phosphorus from the bone [45]. Early increases in FGF23 may be the first sign of altered osteocyte function in pediatric CKD patients [42]. In late CKD, hypocalcemia, hyperphosphatemia, and low 1,25 (OH2)D levels combine to stimulate PTH secretion and development of secondary hyperparathyroidism (SHPT).

● Nutrition and Uremia - Nutritional factors also contribute to suboptimal vitamin D status in CKD. Many patients with CKD have decreased food intake of natural sources of vitamin D due to poor appetite and dietary restrictions. Uremia may also be associated with impaired GI absorption of vitamin D [44]. It is also observed that rats with uremic toxins have decreased 25(OH)D synthesis secondary to PTH-mediated reduction in liver CYP450 isoforms (namely, CYP2C11, 2J3, 3A2, 27A1), which impedes the C-25 hydroxylation of vitamin D3 [46]. In this study, rats with CKD and parathyroidectomy had improvement in their CYP450 expression and calcidiol levels when compared to those with CKD and no parathyroidectomy [46].

● Reduced megalin- It is observed that 25(OH)D tubular reabsorption is impaired due to decreased renal megalin in rats [47]. As a compensatory mechanism, downregulation of *CYP24A1* may represent an appropriate response to calcitriol deficiency as a result of GFR loss [5,48], as seen *in vivo* rat studies [46,47]. These findings may explain why some CKD patients have poor efficacy of vitamin D3 therapy on serum calcidiol levels.

● Proteinuria - Proteinuria may be a contributing factor due to increased urinary excretion of VDBP [6, 39]. In a recent multicenter study of 61 children with idiopathic nephrotic syn-

drome, the prevalence of vitamin D deficiency was 100% at diagnosis and 53% at 2–4 months of follow-up after vitamin D supplementation [49]. Furthermore, among 182 children and adolescents with CKD and ESRD, glomerular disease, particularly FSGS, was an independent risk factor for lower 25(OH)D concentrations, adjusted for age, race, season, CKD severity, and hypoalbuminemia [38, 50]. Among non-dialysis participants, FSGS was also associated with significantly lower $1,25(OH)_2D$ concentrations, adjusted for the concentration of 25(OH) D, intact PTH, and FGF23 [5].

● Dialysis - Serum vitamin D level is lower in peritoneal dialysis (PD) and hemodialysis patients compared with CKD and renal transplant patients [51]. Patients on PD have greater loss of VDBP and its bound vitamin D metabolites through the peritoneal membrane compared to hemodialysis and non-dialysis patients [52]. VDBP losses in the urine and dialysate closely mirrors a linear relationship to albumin losses in these fluids [53]. However, total 25(OH)D was not associated with serum VDBP, suggesting that impaired vitamin D metabolism likely involves mechanisms extending beyond urinary losses of binding proteins. This hypothesis is supported by recent evidence that antiproteinuric therapy (i.e, angiotensin converting enzyme inhibitor) in CKD patients reduced urinary VDBP loss, but did not impact serum VDBP or vitamin D concentrations [54]. Also, chronic hemodialysis patients exhibit defective photoproduction of cholecalciferol, despite normal epidermal content of 7-DHC substrate [55].

11. Consequences of Vitamin D Deficiency in Ckd

As the vitamin D level decreases in the body, a decrease in intestinal calcium absorption lowers the ionized calcium transiently. Hypocalcemia is recognized by calcium sensors in the parathyroid glands to increase the production and secretion of PTH, which increases calcium reabsorption in renal tubules and increases 1α-hydroxylase activity, which increases calcitriol synthesis [25]. However, with sustained vitamin D deficiency, the prolonged stimulation of the parathyroid glands leads to SPTH.

The activation of osteoblasts by PTH and vitamin D stimulates the transformation of preosteoclasts into mature osteoclasts [10]. Osteoclasts dissolve the mineralized collagen matrix in the bone, which can result in rickets (failure of mineralization of growing bone and cartilage) among children and osteomalacia in in adults. The peak incidence of rickets in normal children is between 3 and 18 months of age [24]. The symptoms of rickets can range from none to varying degrees of irritability, delay in gross motor development, and bone pain. Signs include widening of the wrists and ankles, genu varum or valgum, rachitic rosary, delayed closure of fontanelles, craniotabes and frontal bossing; tooth eruption may be delayed; and may be associated with poor growth [25]. One third of healthy infants and toddlers with a serum 25(OH)D level of 20ng/ml were noted to have some evidence of bone demineralization by standard radiograph, while only one child had signs of rickets on physical exam [56]. Thus,

subclinical vitamin D deficiency could make detection in a routine clinical practice difficult. In addition, mild rickets has been found in North American infants with serum 25(OH)D levels close to 20 ng/ml [57].

12. Bone Health

The consensus guidelines for the management of bone health in children with CKD are largely opinion based, and there is lack of agreement in particular on the target range for PTH [58]. The term used to describe the mineral, skeletal and vascular disease associated with progressive kidney failure is "CKD Mineral and Bone Disorder" (CKD-MBD).

CKD-MBD is manifested by either one or a combination of the following [59]:

1) abnormalities of calcium, phosphorus, PTH, or vitamin D metabolism,

2) abnormalities in bone histology, linear growth, or strength, and

3) vascular or other soft tissue calcification.

"Renal osteodystrophy" is the specific term used to describe the bone pathology that occurs as a complication of CKD and is therefore one aspect of the CKD-MBD. While the definitive evaluation of renal osteodystrophy requires a bone biopsy, this procedure is not routinely performed in the clinical setting. However, bone histomorphometry continues to be the gold standard for the assessment of three essential aspects of bone histology: turnover, mineralization, and volume [42].

With 90% of peak bone mass accrued by 18 years of age and cortical bone comprising 80% of skeletal mass, the growing skeleton is vulnerable to chronic disease, and an abnormal bone density impacts fracture risk [58]. Bone mineralization defects develop even in CKD stage 2, which may precede increases in PTH [41] and have been associated with increased fracture rates observed in CKD [60]. In Chronic Kidney Disease in Children (CKiD) study, the incident fracture burden in pediatric CKD was 12.5%. This fracture incidence was 2-3 times higher than sex-specific rates from the general population, with higher rates in adolescent males compared to females [60].

Defective skeletal mineralization, defined as an increase in osteoid volume/bone volume in combination with a prolongation in osteoid maturation time, is present in 29% and 79% of stage 2 CKD and 4/5 CKD, respectively [41]. Defective mineralization was associated with lower serum calcium levels and increased PTH concentrations. Bone turnover was normal in all patients with stage 2 CKD and increased in only 18% of patients with stage 3 and stage 4/5 CKD, despite the lack of therapy with active vitamin D in the majority of patients [41]. They also noted that the ability of any biochemical parameters (PTH, calcium, phosphorus, vitamin

D, FGF23) to predict bone turnover was very poor [41]. With the use of DEXA (dual energy X-ray absorptiometry) or peripheral quantitative computed tomography (pQCT) to measure BMD in childhood CKD, it is noted that higher PTH levels were associated with lower cortical BMD, lower cortical area, and greater trabecular BMD [58]. This would be anticipated given the high turnover state of hyperparathyroidism. In the only randomized control trial (RCT) of native vitamin D therapy in 40 children with CKD stage 2-3, it was shown that children on ergocalciferol who achieved 25(OH)D levels >30 ng/mL had a delayed development of secondary hyperparathyroidism compared to the placebo group [61].

However, there are few studies in children or adults with CKD that examine the effects of 25(OH)D concentrations on bone, and the optimal target level of 25(OH)D is unclear and may need to be higher than that in the general population. Moreover, there is little evidence to define PTH target levels in children with CKD Stages 2–5D and international guideline committees have suggested different recommendations, with PTH targets ranging from normal in CKD stages 2–4 to 2- to 9-fold above the upper limit of normal in children on dialysis [29, 62] (Table 3).

Table 3: Optimal PTH

CKD category	GFR (ml/ min/1.73m2)	Desired PTH level [29]	KDIGO 2017 [62]
Stage 1	>90	35-70 pg/ml	Maintain PTH at approximately 2 to 9 times upper normal limit for assay.
Stage 2	60-89		
Stage 3a	45-59		
Stage 3b	30-44		
Stage 4	15-29	71-110 pg/mL	
Stage 5/D	<15	200-300 pg/mL	

13. Therapeutic Use of Vitamin D in Ckd

Food fortification with synthetic vitamin D2 was pioneered and patented in the United States in the 1930 by Harry Steenbock at the University of Wisconsin. With increased fortification of certain foods with vitamin D, rickets was virtually eradicated in North America [2]. Currently, only 1 of 3 children is taking a vitamin D–containing supplement [33]. And although vitamin D supplementation raises 25(OH)D levels, 1 in 10 children taking vitamin D–containing supplements at current doses (100 –400 IU) had a 25(OH)D level of 20 ng/mL and over half of children had a level of 30 ng/mL [33].

Both the Kidney Disease Outcomes Quality Initiative (NKF/KDOQI) 2009 [29] and Kidney Disease Improving Global Outcomes (KDIGO) 2017 [62] experts recommend checking the serum 25(OH)D concentration to assess vitamin D status of children with and without CKD stages 2-5D at least once a year. It is also recommended to supplement with vitamin D2

or D3 when the serum 25(OH)D level falls < 30ng/ml in CKD and dialysis patients. When vitamin D level is replete, then maintenance dose of vitamin D should be continued and levels monitored at least yearly. Vitamin D deficiency and insufficiency in patients with CKD stage 1–5 should be corrected using treatment strategies recommended for the general population [29, 62].

With respect to the recommended dietary allowance (RDA) of vitamin D in the general population, the Institute of Medicine (IOM) from the US and Canada recommend that without adequate sun exposure, children and adults require 800 to 1000 IU of vitamin D per day [3]. These recommendations cover the needs of >97.5% of the population and assume minimal or no sun exposure; thus, providing further safety for individuals with lower endogenous synthesis of vitamin D [27]. More importantly, the dietary reference intake is developed for "normal healthy persons" and not intended for individuals with specific disease states.

14. Vitamin D2 or D3?

Despite their structural differences, both vitamin D2 and D3 are are believed to be equipotent and exhibit identical sets of biological responses around the body through the same VDR mediated regulation of genes as 1α,25$(OH)_2$D3 [2]. A meta-analysis including seven heterogeneous studies indicated that regardless of the dosage, frequency or administration (oral or intramuscular), vitamin D3 was more effective at raising serum 25(OH)D concentrations compared to vitamin D2 [63]. Therapy with cholecalciferol, when compared with ergocalciferol, is more effective at raising serum 25(OH)D in non-dialysis-dependent CKD patients using the same dosage (50,000 IU weekly) [64]. It has also been reported that vitamin D3 increases the total 25(OH)D concentration more than vitamin D2 and that vitamin D2 supplementation was associated with a decrease in 25(OH)D3 [65]. One explanation for this difference could be a faster and more selective catabolism of vitamin D2 by nonspecific cytochrome P450s in the liver and intestine that may limit vitamin D2 action preferentially in target cells where it is expressed [2]. Three randomized trials in healthy children and those with nutritional rickets have examined the effects of vitamin D2 and D3 supplementation [20]. Although the patient cohorts, dosage of vitamin D, frequency of administration and duration of treatment varied widely, there was no difference in 25(OH)D levels between vitamin D2 and D3 supplementation. It must also be noted that the available pharmaceutical vitamin D3 dosages greatly differ from one country to another. Moreover, ergocalciferol (vitamin D2) is the form available by prescription in the United States.

15. Treatment With Native Vitamin D Supplementation In Children With Ckd

Currently, there is no clear consensus between guideline committees on the type of native vitamin D supplement, its dosage, frequency of administration or duration of treatment in healthy children or children with CKD. Currently, pediatric nephrologists recommend nu-

tritional vitamin D in about 73% of cases with CKD 2-5D, and that about 35% of cases had supplemental vitamin D even with levels > 30ng/ml [66].

All guidelines [24-29] (Table 4) recommend a loading regimen or intensive replacement period for a variable duration of 4–12 weeks followed by a maintenance regimen. Unlike the dosage recommendations for vitamin D treatment in healthy children that are based on age, NKF/KDOQI 2009 recommends escalating doses for intensive replacement depending on the baseline 25(OH)D level. In children with CKD, one RCT showed that normal 25(OH)D levels were more difficult to achieve and maintain in CKD stages 3–4 compared with stage 2 [60], suggesting that higher doses of ergocalciferol may be required in children with CKD stages 3-4. It is not recommended to administer vitamin D analogs to treat vitamin D deficiency. Until further studies in children with CKD and on dialysis are available, there is suggestion of using a treatment schedule guided by age and vitamin D level for native vitamin D supplementation in children with CKD Stages 2–5D [20].

Table 4: Vitamin D Supplementation Varies with Society Recommendations

Vitamin D Supplementation in Healthy Children				**Therapy for Vitamin D Deficiency in Healthy Children**
Society	Age (years)	RDA or Maintenance (IU/day)	Maintenance Upper Limit (IU/day)	Treatment
AAP [24]	Birth-18 yo	400	N/A	N/A
Pediatric Endocrine Society [25]	< 1 mo 1-12 mo > 12mo	400 400 400	N/A	1000 IU/day x 2-3 months 1000-5000 IU/day x 2-3 mo 5000 IU/day x 2-3 mo Stoss therapy: 50,000 IU of vitamin D2 weekly for 8 weeks (teenagers and adults)
National Osteoporesis Society [26]	1-6 mo 6mo-12yo 12-18 yo	400-600	N/A	3000 IU daily x 8-12 weeks 6000 IU x 8-12 weeks 10,000 IU x 8-12 weeks
Institute of Medicine [27]	0-6 mo 6-12 mo 1-3 4-8 9-18	400 400 600 600 600	1000 1500 2500 3000 4000	N/A
Endocrine Society [28]	< 1 1-18 > 18	400-1000 600-1000 1500-2000	2000 4000 4000	2000 IU/day (or 50,000 IU/wk) for 6 weeks 2000 IU/day (or 50,000 IU/wk) for 6-8 weeks 6000 IU/day (or 50,000 IU/wk) for 8 weeks
Vitamin D Supplementation in children with CKD				**Therapy for Vitamin D Deficiency in Healthy Children**

NKF/KDOQI [29]	1-18	200-1000	N/A	Vitamin D < 5 ng/ml: Initial Dose: 8000 IU/day (or 50,000 IU/ week) x 4 weeks Maintenance Dose: 4000 IU/day (or 50,000 IU twice monthly) x 2 months Vitamin D 5-15 ng/ml: Initial Dose: 4000 IU/day (or 50,000 IU every other week) x 3 months Vitamin D 16-30 ng/ml: Initial Dose: 2000 IU/day (or 50,000 IU every 4 weeks) x 3 months

RDA – Recommended Dietary Allowances; covering requirement of > 97.5% of the population N/A - not available

16. Treatment With Active Vitamin D Analogs in Children With Ckd

Vitamin D analogs are chemically synthesized, preactivated versions of active vitamin D2 that mimic the actions of the endogenously synthesized active form in any or all of its calcemic or noncalcemic actions [2]. Vitamin D analogs have been shown to up-regulate VDR and *CYP24A1* while down-regulating renal *CYP27B1* expression at the mRNA and protein levels, thereby, turning off the synthesis of endogenous 1**α**,25$(OH)_2$D3 [2].

Vitamin D analogs are recommended in patients with CKD stages 3-5 in whom serum PTH levels are progressively rising and remain persistently above the upper normal limit for assay despite the correction of modifiable factors. Even though serum concentrations of PTH and alkaline phosphatase are poor markers of bone status, at present, they are the only tools available in clinical practice to guide active vitamin D therapy. Bone biopsies are highly invasive and rarely performed in clinical practice.

Vitamin D analogs [67] that are available for use in paediatric CKD patients include 1-alfacalcidol, calcitriol, paricalcitol and doxercalciferol (Table 5). There are no head-to head trials of all the vitamin D analogs and only limited data comes from a single RCT comparing calcitriol and doxercalciferol in peritoneal dialysis patients [68]. Moreover, there are no RCTs in children with CKD Stages 2–3 primarily assessing the effect of vitamin D analogs versus placebo or ergocalciferol/cholecalciferol on secondary hyperparathyroidism. In children on haemodialysis, two RCTs showed a significant reduction in PTH with thrice-weekly intravenous calcitriol [69] and thrice-weekly intravenous paricalcitol [70] versus placebo control. A significantly increased risk of hypercalcaemia was reported with intravenous calcitriol [69], but not with paricalcitol [70]. A recent meta-analysis concludes that the overall quality of the evidence available is poor and there are no data to indicate any superiority of paricalcitol over other vitamin D analogs in lowering PTH or reducing the burden of mineral loading [71]. Thus, there are no data supporting the clinical superiority of any vitamin D analogues available in the U.S. compared with calcitriol or placebo.

Vitamin D analogs may also be started prior to repletion of 25(OH)D stores provided the child is normocalcaemic. Vitamin D analogs should be started in the lowest dose to achieve target PTH concentrations and maintain normocalcaemia. Calcitriol is not preferred for stoss therapy (high doses of Vitamin D; "stoss therapy", from the German word *stossen* meaning "to push") because it is expensive, has a short half-life and does not build up vitamin D stores [25]. Subsequent titration of vitamin D therapy may be performed based on trends in serum calcium, phosphate and PTH levels.

Table 5: Available Formulations of Vitamin D [24,25,67]

Dosage Form	Strength	Trade Names
Vitamin D2 (ergocalciferol)		
Oral Soultion	8000 IU/ml	Calcidiol, Calciferol, Drisdol
Capsule	50,000 IU	Drisdol
Tablet	400 IU	various
Vitamin D3 (cholecalciferol)		Baby drops
Oral drops	400-, 1000-, 2000 IU/drop	D-Vi-Sol, Just D
Oral solution	400 IU/ml	Dialyvite, Decara
Capsule	400-, 1000-, 2000-, 5000-, 25,000- IU	Thera-D, others
Tablet	400-, 1000-, 2000-, 5000-IU	
1,25(OH)2D (calcitriol)		
Oral solution	1 mcg/ml	Rocaltrol
Capsule	0.25-,0.5-mcg	Rocaltrol
Intravenous	1 mcg/ml	Calcijex, Zemplar

1.0 mcg of vitamin D = 40 IU, 1.0 mg of vitamin D = 40,000 IU

17. References

1. Holick MF. Resurrection of vitamin D deficiency and rickets. J Clin Invest. 2006; 116: 2062-72.

2. Jones G. Extrarenal vitamin D activation and interactions between vitamin D2, vitamin D3 and vitamin D analogs. Annu. Rev. Nutr. 2013; 33:23–44.

3. Holick MF. Vitamin D deficiency. N. Engl. J. Med. 2007; 357: 266–281.

4. Melamed ML and Thadhani RI. Vitamin D therapy in chronic kidney disease and end stage renal disease. Clin J Am Soc Nephrol. 2012; 7: 358–365.

5. Denberg MR, Kalkwarf HJ, de Boer IH, Hewison M, Shults J, Zemel BS, et al. Pediatr Nephrol. 2013; 28:1843–1853.

6. Gois PHF, Wolley M, Ranganathan D and Seguro AC. Vitamin D deficiency in chronic kidney disease: Recent evidence and controversies. Int. J. Environ. Res. Public Health. 2018; 15: 1773-89.

7. Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. Physiol Rev. 1998; 78:1193–231.

8. Holick, M.F. Vitamin D Status: Measurement, Interpretation, and Clinical Application. Ann. Epidemiol. 2009; 19: 73–78.

9. Brandenburg VM, Vervloet MG, and Marx N. The role of vitamin D in cardiovascular disease: from present evidence to future perspectives. Atherosclerosis. 2012; 225: 253–263.

10. DeLuca HF. Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr. 2004; 80: Suppl:1689S-1696S.

11. Sterling KA, Eftekhari P, Girndt M, Kimmer PL, and Raj DS. The immunoregulatory function of vitamin D: implications in chronic kidney disease. Nat Rev Nephrol. 2012; 8: 403–412.

12. Townsend K, Evans KN, Campbell MJ, Colston KW, Adams JS, Hewison M. Biological actions of extra-renal 25-hydroxyvitamin D-1a-hydroxylase and implications for chemoprevention and treatment. J Steroid Biochem Mol Biol. 2005; 97: 103–109.

13. Hewison, M. Vitamin D and the Immune System: New Perspectives on an Old Theme. Endocrinol. Metab. Clin. N. Am. 2010; 39: 365–379.

14. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol. 2004; 15: 1983–1992.

15. Hussin AM, Ashor AW, Schoenmakers I, Hill T, Mathers JC, Siervo M. Eur J Nutr. 2017; 56:1095-1104.

16. Alyami A, Soares MJ, Sherriff JL, Mamo JC. Vitamin D and endothelial function. Indian J Med Res 2014; 140: 483-490

17. Andrukhova O, Slavic S, Zeitz U, Riesen SC, Heppelmann MS, Ambrisko TD et al. Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice. Mol Endocrinol. 2014; 28(1):53–64

18. de Borst MH, Vervloet MG, Wee PM, Navis G. Cross talk between the renin-angiotensin-aldosterone system and vitamin D-FGF23-klotho in chronic kidney disease. J Am Soc Nephrol. 2011; 22: 1603-1609.

19. Beveridge LA, Struthers AD, Khan F, Jorde R, Scragg R, Macdonald HM et al. (Eds.) Effect of vitamin D supplementation on blood pressure a systematic review and meta-analysis incorporating individual patient data. JAMA Intern Med. 2015; 175: 745–754.

20. Shroff R, Wan M, Nagler EV, Bakkaloglu S, Fischer DC, Bishop N et al. Clinical practice recommendations for native vitamin D therapy in children with chronic kidney disease Stages 2-5 and on dialysis. Nephrol Dial Transplant. 2017; 32: 1098–1113.

21. Chapuy MC, Preziosi P, Maaner M, Arnaud S, Galan P, Hercberg S, et al. Prevalence of vitamin D insufficiency in an adult normal population. Osteoporosis Int. 1997;7:439–443.

22. Malabanan AO, Veronikis IE, Holick MF. Redefining vitamin D insufficiency. Lancet. 1998; 351:805-6.

23. Malabanan AO, Holick MF. Vitamin D and bone health in postmenopausal women. J Womens Health (Larchmt). 2003; 12(2): 151-6.

24. Wagner CL, and Greer FR. Prevention of rickets and vitamin D deficiency in infants, children and adolescents. Pediatrics. 2008; 122:1142-1152.

25. Misra M, Pacaud D, Petryk A, Collett-Solberg, Kappy M. Pediatrics. 2008; 122:398-417

26. Arundel P, Shaw N. Vitamin D and Bone Health: A practical clinical guideline for the management in children and young people. National Osteoporosis Society. 2015; 1-14.

27. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the institute of medicine: what clinicians need to know. J Clin Endocrinol Metab. 2011; 96: 53–58

28. Holick MF, Binkley NC, Bischoff-Ferrari HA et al. Evaluation, treatment, and prevention of vitamin D deficiency: an endocrine society clinical practice guideline. J Clin Endocrinol Metab. 2011; 96: 1911–1930

29. K/DOQI clinical practice guidelines for nutrition in children with CKD. Am J Kidney Dis 2009; 53: S11–S104

30. Henry HL, Bouillon R, Norman AW, Gallagher JC, Lips P, Heaney RP et al. 14th Vitamin D Workshop consensus on vitamin D nutritional guidelines. J. Steroid Biochem. Mol. Biol. 2010; 121: 4–6.

31. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney Int Suppl. 2013; 3:5-133.

32. Kumar, J.; Muntner, P.; Kaskel, F.J.; Hailpern, S.M.; Melamed, M.L. Prevalence and associations of 25-hydroxyvitamin D deficiency in US children: NHANES 2001–2004. Pediatrics 2009; 124: e362–e370.

33. Mansbach JM, Ginde AA, Camargo CA Jr. Serum 25-hydroxyvitamin D levels among US children aged 1 to 11 years: do children need more vitamin D? Pediatrics 2009; 124(5):1404-10.

34. Gordon CM, Feldman HA, Sinclair L, et al. Prevalence of vitamin D deficiency among healthy infants and toddlers. Arch Pediatr Adolesc Med. 2008; 162(6):505–512

35. Kamath N and Iyengar AA. Chronic kidney disease (CKD): An observational study of etiology, severity and burden of comorbidities. Indian J Pediatr. 2017: 84(11):822–825

36. Copelovitch L, Warady BA, Furth SL. Insights from the chronic kidney disease in children (CKiD) study. Clin J Am Soc Nephrol. 2011; 6:2047–53.

37. Doyon A, Schmiedchen B, Sander A, Bayazit A, Duzova A, Canpolat N et al. Genetic, environmental, and disease-associated correlated of vitamin D status in children with CKD. Clin J Am Soc Nephrol. 2016; 11: 1145–1153.

38. Coccia P, Blazquez J, Contreras M, Ferraris V, Raddavero, Ghezzi L et al. High prevalence of vitamin D deficiency among children with chronic kidney disease and kidney transplant. Arch Argent Pediatr. 2017; 115(3):220-226

39. Shroff R, Knott C, Rees L. The virtues of vitamin D–but how much is too much? Pediatr Nephrol 2010; 25: 1607–1620.

40. Holick MF, MacLaughlin JA, Doppelt SH. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. Science. 1981; 211 (4482):590 –593.

41. Wesseling-Perry K, Pereira RC, Tseng CH et al. Early skeletal and biochemical alterations in pediatric chronic kidney disease. Clin J Am Soc Nephrol 2012; 7: 146–152.

42. Wesseling-Perry K, Salusky IB. Chronic kidney disease: mineral and bone disorder in children. Semin Nephrol. 2013; 33(2):169-79.

43. Jean G, Souberbielle JC, and Chazot C. Vitamin D in chronic kidney disease and dialysis patients. Nutrients 2017; 9: 328-343.

44. Shroff R, Wan M, Nagler EV, Bakkaloglu S, Cozzolino M, Bacchetta J et al. Clinical practice recommendations for treatment with active vitamin D analogues in children with chronic kidney disease Stages 2-5 and on dialysis. Nephrol Dial Transplant. 2017; 32: 1114-1127.

45. Portale AA, Booth BE, Halloran BP, Morris RC Jr. Effect of dietary phosphorus on circulating concentrations of 1,25-dihydroxyvitamin D and immunoreactive parathyroid hormone in children with moderate renal insufficiency. J Clin Invest 1984; 73: 1580-1589.

46. Michaud J, Naud J, Ouimet D, Demers C, Petit JL, Leblond FA et al. Reduced hepatic synthesis of calcidiol in uremia. J Am Soc Nephrol. 2010; 21: 1488–1497.

47. Takemoto, F.; Shinki, T.; Yokoyama, K.; Inokami, T.; Hara, S.; Yamada, A.; Kurokawa, K.; Uchida, S. Gene expression of vitamin D hydroxylase and megalin in the remnant kidney of nephrectomized rats. Kidney Int. 2003; 64: 414–420

48. De Boer, I.H.; Sachs, M.C.; Chonchol, M.; Himmelfarb, J.; Hoofnagle, A.N.; Ix, J.H.; Kremsdorf, R.A.; Lin, Y.S.;

Mehrotra, R.; Robinson-Cohen, C.; et al. Estimated GFR and circulating 24,25-dihydroxyvitamin D3 concentration: A participant-level analysis of 5 cohort studies and clinical trials. Am. J. Kidney Dis. 2014; 64: 187–197.

49. Selewski DT, Chen A, Shatat IF, et al. Vitamin D in incident nephroticsyndrome: a Midwest Pediatric Nephrology Consortium study. Pediatr Nephrol 2016; 31:465–472.

50. Kalkwarf HJ, Denburg MR, Strife CF, et al. Vitamin D deficiency is common in children and adolescents with chronic kidney disease. Kidney Int 2012; 81:690–697.

51. Cankaya, E.; Bilen, Y.; Keles, M.; Uyanik, A.; Akbas, M.; Gungor, A.; Arslan, S.; Aydinli, B. Comparison of Serum Vitamin D Levels Among Patients With Chronic Kidney Disease, Patients in Dialysis, and Renal Transplant Patients. Transpl. Proc. 2015; 47: 1405–1407

52. Koenig KG, Lindberg JS, Zerwekh JE et al. Free and total 1,25-dihydroxyvitamin D levels in subjects with renal disease. Kidney Int 1992; 41: 161–165

53. Prytula A,Wells D,McLean T et al. Urinary and dialysate losses of vitamin D-binding protein in children on chronic peritoneal dialysis. Pediatr Nephrol 2012; 27: 643–649

54. Doorenbos CR, de Cuba MM, Vogt L, Kema IP, van den Born J, Gans RO et al. Antiproteinuric treatment reduces urinary loss of vitamin D-binding protein but does not affect vitamin D status in patients with chronic kidney disease. J Steroid Biochem Mol Biol. 2012; 128:56–61

55. Jacob, AI, Sallman A, Santiz Z, Hollis BW. Defective photoproduction of cholecalciferol in normal and uremic humans. J. Nutr. 1984; 114: 1313–1319.

56. Gordon CM, Feldman HA, Sinclair L, Williams AL, Kleinman FK, Perez-Rossello J, et al. Prevalence of vitamin D deficiency among healthy infants and toddlers. Arch Pediatr Adolesc Med. 2008; 162(6):505-512

57. Arnaud SB, Stickler GB, Haworth JC. Serum 25-hydroxyvitamin D in infantile rickets. Pediatrics. 1976; 57(2):221–225.

58. Denberg MR. Skeletal manifestations of renal disease in childhood. Curr Opin Nephrol Hypertens. 2016; 25: 292-300

59. Kidney Disease: Improving Global Outcomes CKD-MBD Work Group. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease-mineral and bone disorder (CKD-MBD). Kidney Int Suppl. 2009; 113: S1-S130.

60. Denburg MR, Kumar J, Jemielita T, et al. Fracture burden and risk factors in childhood CKD: results from the CKiD cohort study. J Am Soc Nephrol 2016; 27:543–550

61. Shroff R, Wan M, Gullett A et al. Ergocalciferol supplementation in children with CKD delays the onset of secondary hyperparathyroidism: a randomized trial. Clin J Am Soc Nephrol 2012; 7: 216–223.

62. Kidney Disease: Improving Global Outcomes CKD-MBD Work Group. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease-mineral and bone disorder (CKD-MBD). Kidney Int Suppl 2017; 7:1-59

63. Tripkovic L, Lambert H, Hart K, Smith CP, Bucca G, et al. Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. Am J Clin Nutr. 2012; 95:1357–64

64. Wetmore, JB, Kimber C, Mahnken JD, Stubbs JR. Cholecalciferol v. ergocalciferol for 25-hydroxyvitamin D (25(OH) D) repletion in chronic kidney disease: A randomised clinical trial. Br J Nutr. 2016; 116: 2074–2081.

65. Lehmann U, Hirche F, Stangl GI, Hinz K, Westphal S, Dierkes J. Bioavailability of vitamin D(2) and D(3) in healthy

volunteers, a randomized placebo-controlled trial. J Clini Endocrinol. Metab. 2013; 98: 4339–4345.

66. Griffin LM, Denburg MR, Shults J, Furth SL, Salusky IB, et al. 2013. Nutritional vitamin D use in chronic kidney disease: a survey of pediatric nephrologists. Pediatr. Nephrol. 28:265–75

67. Lee JY, So T, Thackray J. A review on vitamin D deficiency treatment in pediatric patients. J Pediatr Pharmacol Ther 2013; 18(4):277–291

68. Wesseling-Perry K, Pereira RC, Sahney S et al. Calcitriol and doxercalciferol are equivalent in controlling bone turnover, suppressing parathyroid hormone, and increasing fibroblast growth factor-23 in secondary hyperparathyroidism. Kidney Int 2011; 79: 112–119

69. Greenbaum LA, Grenda R, Qiu P et al. Intravenous calcitriol for treatment of hyperparathyroidism in children on hemodialysis. Pediatr Nephrol 2005; 20: 622–630

70. Greenbaum LA, Benador N, Goldstein SL et al. Intravenous paricalcitol for treatment of secondary hyperparathyroidism in children on hemodialysis. Am J Kidney Dis 2007; 49: 814–823

71. Cai P, Tang X, Qin W et al. Comparison between paricalcitol and active non-selective vitamin D receptor activator for secondary hyperparathyroidism in chronic kidney disease: a systematic review and meta-analysis of randomized controlled trials. Int Urol Nephrol 2016; 48: 571–584