



The measurement of vitamin D metabolites: part I—metabolism of vitamin D and the measurement of 25-hydroxyvitamin D

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Abstract

It has been more than 80 years since the discovery of vitamin D and its ability to cure rickets in children. Vitamin D is a secosteroid and comes in two distinct forms, vitamin D₂ and vitamin D₃. During the last 40 years, the synthesis and metabolism of vitamin D were elucidated and more than 50 metabolites of vitamin D have been discovered, though commercial measurement procedures have been developed for only a few of them. The clinical significance of vitamin D in calcium and phosphorus homeostasis is well appreciated. However, recent epidemiological data have indicated that it has several extra-skeletal physiological actions which are still a matter of scientific debate. Both research findings and the debate around the interpretation of the research results have created increased interest in more measurements of vitamin D. With the ever growing family of measurable vitamin D metabolites and the measuring techniques comes a question: What metabolic product will provide the right answers and which is the best way to measure it. The right choice of analytical technique is connected with the question of which metabolite we aim to measure, what is its serum concentration, and the purpose of the measurement. The aim of the first part of this review is to provide a brief overview of vitamin D metabolism and a more detailed analysis of the existing methods and the status of standardization for the measurement of 25-hydroxyvitamin D.

Keywords Vitamin D · 25-hydroxyvitamin D · Liquid chromatography · Mass spectrometry · Immunoassays · Standardization · Vitamin D standardization program

Abbreviations

7-DHC	dehydrocholesterol or provitamin D ₃	VDBP	vitamin D binding protein
24,25(OH) ₂ D ₃	24,25-dihydroxyvitamin D ₃	VDDR	vitamin D-dependent rickets
25(OH)D ₃	calcidiol or 25-hydroxyvitamin D ₃	VDR	vitamin D receptor
1 α ,25(OH) ₂ D ₃	calcitriol or 1,25-dihydroxyvitamin D ₃	VDSP	Vitamin D Standardization Program
C3-epi-25(OH)D	C3-epimer of the 25(OH)D	CLD	chronic liver disease
CYP27B1	25(OH)D-1 α -hydroxylase	CKD	chronic kidney disease
CYP24A1	24-hydroxylase	SHPT	secondary hyperparathyroidism
PTH	parathyroid hormone	UV	ultraviolet
FGF-23	fibroblast growth factor-23	HPLC	high-performance liquid chromatography
		LC-MS/MS	liquid chromatography coupled with mass spectrometry
		CPBA	competitive protein binding assays
		RIA	radioimmunoassays
		ELISA	enzyme-linked immunosorbent assays
		CLIA	chemiluminescent assays
		RMP	reference measurement procedure
		PRM	primary reference material
		NIST	National Institute for Standards and Technology

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IFCC	International Federation of Clinical Chemistry
JCTLM	Joint Committee for Traceability in Laboratory Medicine
AACC	American Association for Clinical Chemistry
DEQAS	Vitamin D External Quality Assessment Scheme
CAP	College of American Pathologists
CDC	Center for Disease Control
EQA	External Quality Assessment

Introduction

Vitamin D metabolism

Vitamin D and parathyroid hormone (PTH) are the major regulators of calcium homeostasis and play an important role in bone metabolism [1]. Vitamin D is a fat-soluble steroid hormone and exists in two major forms, vitamin D₂ (or ergocalciferol) and vitamin D₃ (or cholecalciferol). Both are formed by UV irradiation of either ergosterol, which is found naturally in plants, phytoplankton, and fungi and is also commercially produced by UV irradiation of milk and yeast (mushrooms), or 7-dehydrocholesterol (7-DHC or provitamin D₃), which is found in all vertebrates with the exception of fish (Figs. 1 and 2) [2].

In humans, vitamin D (D here represents D₂, D₃, or both) is either produced endogenously in the skin from sun exposure or obtained from foods that naturally contain vitamin D and supplements. Foods rich in vitamin D include cod liver oil, fatty fish, UV-irradiated mushrooms, and foods fortified with vitamin D. Moreover, during exposure to sunlight, 7-DHC in the skin is converted to previtamin D₃ (Fig. 1).

These two forms of vitamin D differ in chemical structure and in pharmacokinetic properties. Chemically, they differ in one methyl group at carbon C₂₄ position and a double bond between the carbons at positions C₂₂-C₂₃ on their side chain (Fig. 3). As a consequence, they also have different molecular weights (396.65 g/mol and 384.64 g/mol, respectively).

Vitamin D synthesized in the skin diffuses into the blood, where it is transported by vitamin D binding protein (VDBP) to the liver. Vitamin D from the diet is absorbed in the small intestine and incorporated into chylomicrons, which are released into the lymphatic system, and enters the venous blood where it binds to VDBP and lipoproteins and is transported to the liver [3]. The hepatic ingress of vitamin D is modulated by its plasma carriers and liver membrane receptors [4].

Vitamin D has no biological activity and its activation involves two hydroxylation steps (Fig. 4). With the first step that takes place in the liver, vitamin D is hydroxylated at the C₂₅

position by the cytochrome p450 enzyme CYP2R1 (also called 25-hydroxylase, located in the endoplasmic reticulum) to yield 25-hydroxyvitamin D [25(OH)D] (Fig. 5). This enzyme hydroxylates both vitamin D₂ and D₃ and seems to be the main enzyme involved in vitamin D 25-hydroxylation [5]. Experimental evidence has shown that additional enzymes (mitochondrial CYP27A1 as well as microsomal CYP2D11, CYP2D25, CYP2J2/3, and CYP3A4) also contribute to 25(OH)D production by exerting 25-hydroxylase activity [6, 7]. The activity of 25-hydroxylase is poorly regulated by any feedback mechanism in the context of the vitamin D endocrine system and it seems to be dependent primarily on the concentration of vitamin D [8]. Severe liver failure affects the function of the enzyme CYP2R1. Moreover, loss-of-function mutations of the same enzyme are responsible for vitamin D-dependent rickets [VDDR], type 1B [VDDR-1B] [9].

After hydroxylation, 25(OH)D is transported by VDBP to the kidney, where it is filtered by the glomerulus and reabsorbed by the proximal tubular cells. Entry of 25(OH)D into the proximal tubular cells requires receptor-mediated uptake of the VDBP-25(OH)D complex at the brush border, degradation of VDBP, and endocytic internalization and translocation of 25(OH)D to mitochondria [10]. This is achieved via the megalin/cubulin complex [11]. Both megalin and cubulin are large, multiligand, endocytic-membrane glycoproteins expressed in epithelial cells of several tissues and involved in the endocytic uptake of many ligands, including lipoproteins, vitamin-binding proteins, other carrier proteins, hormones, enzymes, and drugs in several epithelia. The receptors might work independently, but have also been observed to interact to facilitate the uptake of several ligands [11]. It is in the mitochondria of the proximal renal tubule that 25(OH)D is hydroxylated at the carbon to position C₁, resulting in the formation of the secosteroid hormone calcitriol or 1,25-dihydroxy-vitamin D [1,25(OH)₂D], the hormonally active form of vitamin D which is responsible for most, if not all, of the biologic actions of vitamin D. Again, a cytochrome p450 enzyme, renal CYP27B1 (also called 25(OH)D-1 α -hydroxylase), is responsible for this hydroxylation. This enzyme is predominately present in the kidney (proximal straight tubules) and contributes to the majority of the circulating levels of 1,25(OH)₂D. Inactivating mutations of these enzymes are responsible for vitamin D-dependent rickets (VDDR) type 1A [VDDR-1A] [6–8, 12].

Vitamin D catabolism

Catabolism is an essential component of vitamin D metabolism (Fig. 6). Both 25(OH)D and 1,25(OH)₂D undergo catabolism via multiple side chain hydroxylations to become more polar metabolites, which are subsequently excreted in both urine and feces. The enzyme CYP24A1, a mitochondrial P450 enzyme encoded by the *CYP24A1* gene, catalyzes the

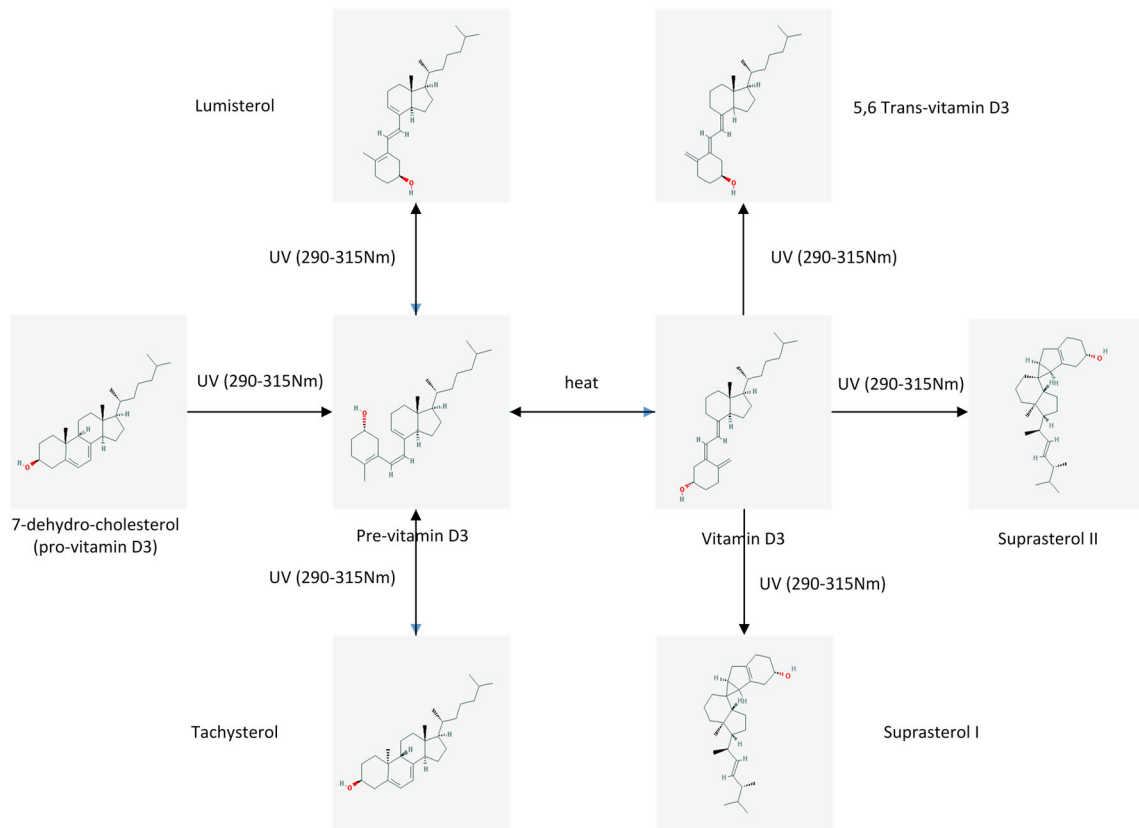


Fig. 1 When the skin is exposed to UV radiation in the wavelength range 290–315 nm, 7-dehydrocholesterol absorbs this energy, which causes chemical bonds within its molecule to break and re-arrange, resulting in the formation of pre-vitamin D. In the skin, pre-vitamin D undergoes rapid, thermally induced isomerization and vitamin D is produced. Once formed, pre-vitamin D and vitamin D continue to absorb UV. Prolonged exposure to UV radiation results in breakdown of these

molecules into biologically inactive photoproducts. For this reason, during prolonged irradiation, a steady state is reached when only 10–15% of 7-dehydrocholesterol is cutaneously converted to pre-vitamin D3. This ensures that no toxic levels of vitamin D are synthesized under excessive sun exposure conditions (the chemical formulas are taken from pubchem <https://pubchem.ncbi.nlm.nih.gov>)

first step, along with several subsequent steps of the vitamin D catabolic pathway in humans and is capable of catalyzing all of the hydroxylation steps in the catabolism of both 25(OH)D and 1 α ,25(OH) $_2$ D [7]. The 24-hydroxylase pathway

generates 24R,25(OH) $_2$ D from the hydroxylation of 25(OH)D and 1,24,25(OH) $_3$ D from the hydroxylation of 1,25(OH) $_2$ D. These metabolites are further oxidized to generate a variety of excretory products [6, 13, 14]. These 24-

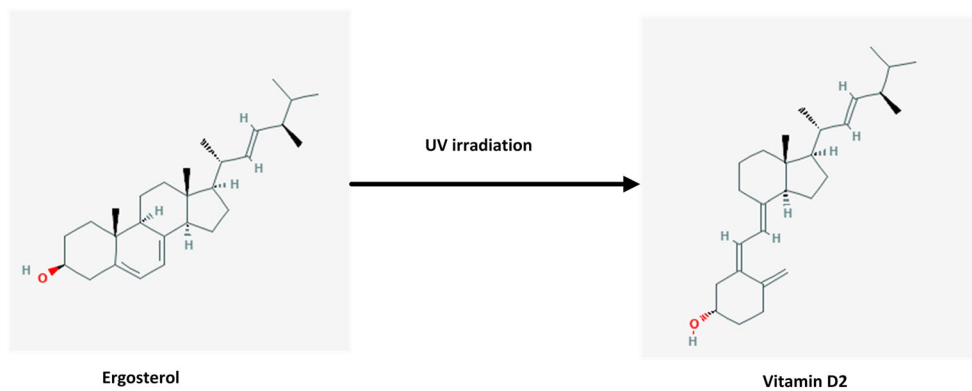
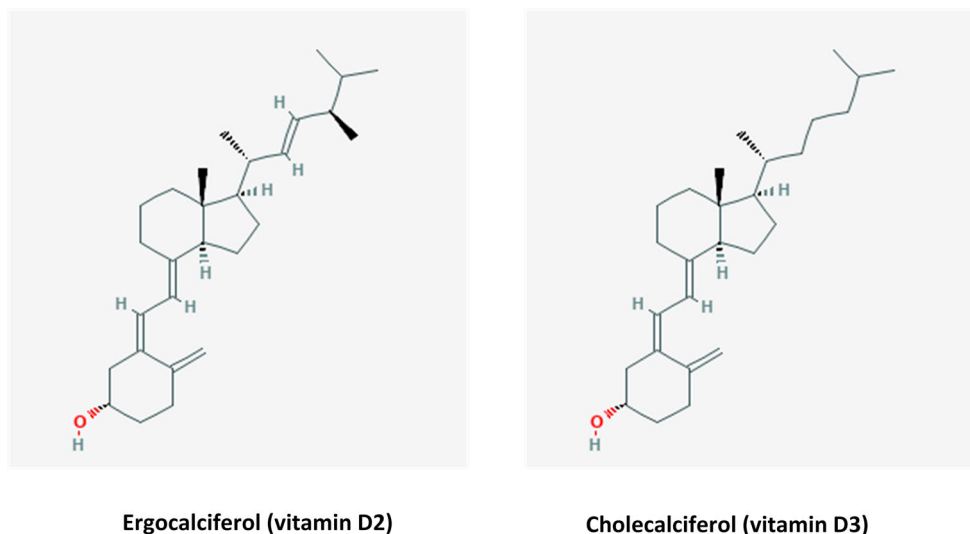


Fig. 2 Production of vitamin D from ergosterol. Ergosterol differs from 7-dehydrocholesterol in its side chain, which contains an extra methyl group at carbon 4 at position C-24. Ultraviolet (UV) radiation in the wavelength range 290–315 nm cleaves the B ring of both ergosterol and 7-dehydrocholesterol, yielding ergocalciferol and cholecalciferol,

respectively. Irradiation of milk and yeast is a commercial means of producing D2 from ergosterol, and dihydrotachysterol (DHT) is a synthetic analog of vitamin D2 (the chemical formulas are taken from pubchem <https://pubchem.ncbi.nlm.nih.gov>)

Fig. 3 The two forms of vitamin D: ergocalciferol (left) and cholecalciferol (right) (the chemical formulas are taken from pubchem <https://pubchem.ncbi.nlm.nih.gov>)



hydroxylation reactions represent the first steps in the vitamin's inactivation process, that leads to the biologically inactive product calcitriolic acid (Fig. 6) [13]. On the other hand, the 23-hydroxylase pathway ends up creating the biologically active $1\alpha,25\text{-}26,23$ lactone. Calcitriol and FGF23 can cause up-regulation of expression of 24-hydroxylase, while PTH and low calcium down-regulate its expression. The expression of the enzyme CYP24A1 (or 24-hydroxylase) in the kidney provides another means of control in vitamin D homeostasis.

CYP24A1 has been found in many tissues that express the vitamin D receptor. In the kidney, it is found in the proximal and distal tubules [14, 15]. The *CYP24A1* gene is highly inducible by $1\alpha,25(\text{OH})_2\text{D}$ in all tissues in which it is found and it acts as a control mechanism to prevent intoxication from $1\alpha,25(\text{OH})_2\text{D}$ [16]. The importance of this feedback mechanism was demonstrated when inactivating mutations of CYP24A1 were reported in children and adults with hypercalcemia [9, 17]. Another enzyme, CYP3A4, also plays a role in vitamin D catabolism. This is a microsomal cytochrome P450 enzyme which is found in numerous tissues and catabolizes both $25(\text{OH})\text{D}$ and $1,24\text{R},25(\text{OH})_3\text{D}$ in a manner similar to CYP24A1. The quantitative contribution of CYP3A4 to vitamin D catabolism compared to CYP24A1 is not well known [18]. Recently, a gain-of-function mutation in CYP3A4 was described, which leads to vitamin D deficiency through accelerated vitamin D metabolite inactivation (named type 3 vitamin D-dependent rickets or VDDR3) [19].

Other vitamin D metabolites

In addition to the primary pathway of vitamin D metabolism, there are also a number of minor metabolic pathways. It has recently been discovered that vitamin D can alternatively be metabolized through the C3-epimerization pathway that parallels the standard metabolic pathway [20]. This pathway

creates the vitamin D epimers, a group of metabolites that has attracted much attention recently. Epimers are molecules with identical structure but different stereochemical configuration. The C3-epimers of vitamin D differ from the primary molecules only in the configuration of the hydroxyl group at the 3 carbon position. Of them, the C3-epimer of the $25(\text{OH})\text{D}$ [C3-epi- $25(\text{OH})\text{D}$] is the most abundant epimer that has been found in the systemic circulation (Fig. 7) [21]. The C3-epi- $25(\text{OH})\text{D}$ can also undergo 1α hydroxylation to give C3-epi- $1\alpha,25(\text{OH})_2\text{D}$ and 24 hydroxylation to give C3-epi- $24,25(\text{OH})_2\text{D}$ and $1\alpha,24,25(\text{OH})_3\text{D}$ (Fig. 7) [22].

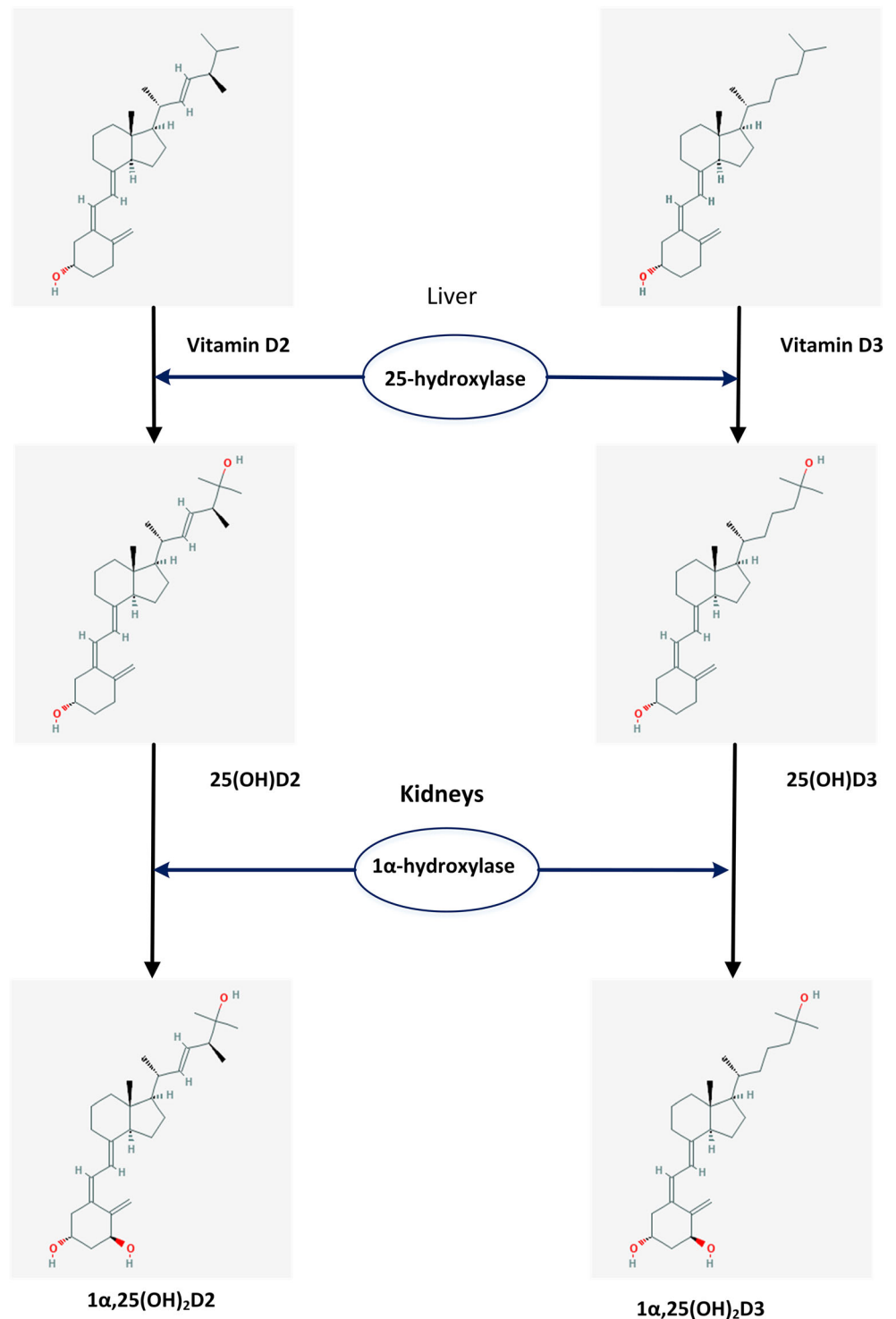
The physiological importance of the vitamin D3 epimer is not as yet very well known. The C3-epi- $25(\text{OH})\text{D}$ was initially found in neonates, in a 2006 publication by Singh et al., where it was reported that the C3-epimer was detectable in a significantly high percentage (up to 60%) of neonates and children up to 1 year of age [23]. Recently, it has been reported in adults [20, 24]. Detectable levels of the epimer range from 0 to 100% of the adults tested and it is estimated that, on average, adults have a median concentration (range) epimer of 1.72 (0–9.01) ng/ml and that the epimer makes up to 6.1% (0–47.0%) of the total $25(\text{OH})\text{D}$ [the sum of epimer + $25(\text{OH})\text{D}$] [21].

Measurement of vitamin D metabolites

Which metabolites can be measured

At present, more than 50 vitamin D metabolites have been described and characterized, with some of them exhibiting biological activity [25]. However, methods for adequate measurement been developed for only five of them (vitamin D, $25(\text{OH})\text{D}_2$ and $25(\text{OH})\text{D}_3$, $1\alpha,25(\text{OH})_2\text{D}$, $24\text{R},25(\text{OH})_2\text{D}$, and C3-epi- $25(\text{OH})\text{D}$) (Table 1), these being metabolites

Fig. 4 The two steps of activation of vitamin D (the chemical formulas are taken from pubchem <https://pubchem.ncbi.nlm.nih.gov>)



encountered in serum in concentrations that allow for their measurement with these methods [26].

The above metabolites differ significantly in their biological activity. For example, 1 α ,25(OH)₂D is five times more potent than vitamin D in its ability to absorb calcium from the intestines and to mobilize it in the bones [27]. A significant factor that determines the biological activity of a metabolite is

its affinity to VDR. Experimental studies have shown that 1,25(OH)₂D exhibits the highest affinity to VDR among all vitamin D metabolites [28], while the affinity of the rest of the metabolites is significantly lower. For instance, 25OHD and 24,25(OH)₂D exhibit approximately 900 and 5000 times lower affinity to VDR, respectively, compared to that of 1,25(OH)₂D [29].

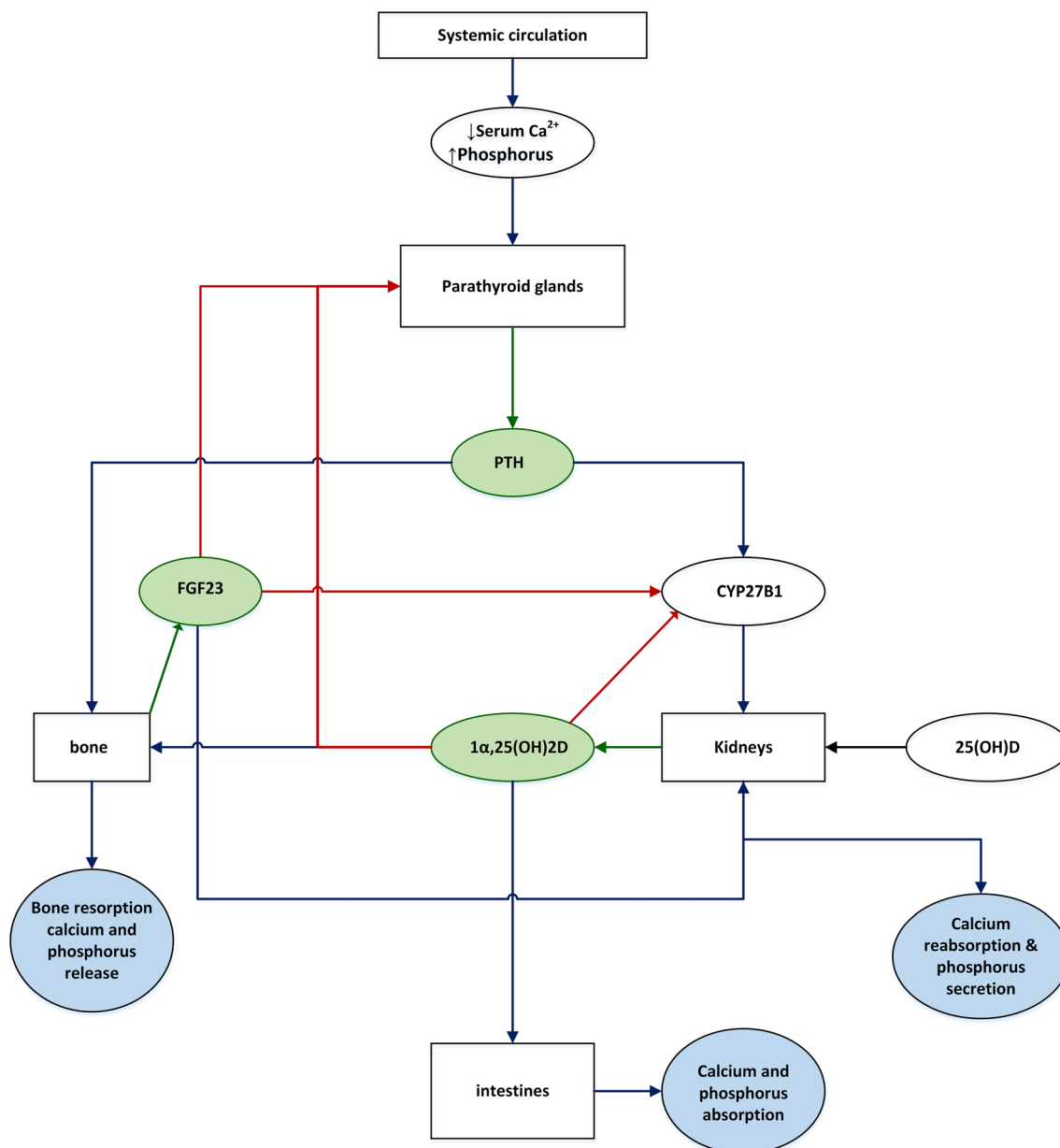


Fig. 5 Hormonal regulation of calcium and phosphorus. Their serum levels are maintained by a tight interplay between PTH, $1\alpha,25(\text{OH})_2\text{D}$ and FGF23. Green lines and shapes indicate products, blue lines indicate stimulatory effects and red lines indicate inhibitory effects

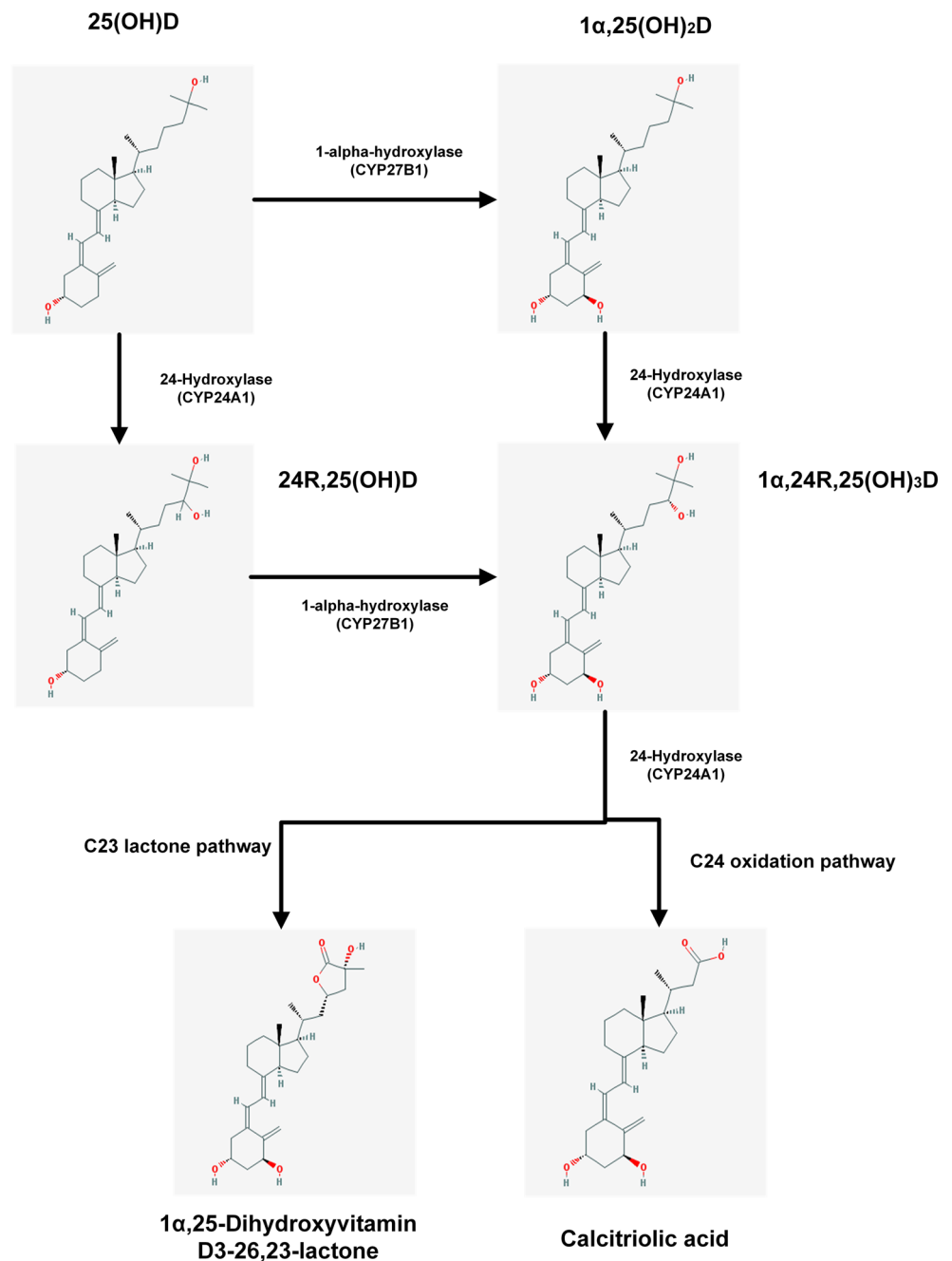
Pre-analytical considerations

Sample types and collection tubes Both serum and plasma (EDTA) can be used for the measurement of vitamin D metabolites. However, serum is the preferred matrix, since it has the advantage of being free of anticoagulants used for plasma collection, such as EDTA, heparin, or citrate, which may interfere with their measurement. However, even when serum is the medium, significant interferences are observed with certain commercial assays when Serum Clot Activator Tubes with Gel are used [30]. Saliva has also been explored in several studies and with assays based on different principles, however, often with inconsistent results. More recently, the

technological advances achieved in LC-MS/MS sensitivity and novel extraction approaches have made feasible the measurement of $25(\text{OH})\text{D}$, requiring reduced sample volume and using dried blood spots (DBSs) for sample collection [31].

Sample stability Vitamin D metabolites are stable in plasma and serum when samples are stored at room temperature ($24\text{ }^\circ\text{C}$), or at $4\text{ }^\circ\text{C}$, or frozen, as long as the metabolites are not separated from their binding protein. Therefore, no special precautions are necessary during the transport of samples to the laboratory. In stored samples, repeated cycles of freeze-thaw do not seem to have any significant effect on $25(\text{OH})\text{D}$ levels. Attention is needed only when the samples have

Fig. 6 Catabolism of vitamin D (the chemical formulas are taken from pubchem <https://pubchem.ncbi.nlm.nih.gov>)



already been pretreated and vitamin D has been separated from its binding protein. Then, samples should be kept in dark vials to avoid exposure to light and stored at < -70 °C [32].

Effect of season of the year on 25(OH)D levels Due to its biosynthesis, levels of vitamin D are directly dependent upon exposure to UV irradiation from the sun. This results in a seasonal variation and in the fact that 25(OH)D levels depend on the geographical location in which a person lives (distance from equator and altitude) [33]. Generally speaking, people living in the northern hemisphere present the highest levels of

25(OH)D during the summer and autumn and the lowest during winter and spring [34].

Single-nucleotide polymorphisms Genetic studies have revealed that single-nucleotide polymorphisms (SNPs) of genes that are involved in vitamin D metabolism pathways (i.e., DHCR7, CYP2R1, CYP3A4, CYP27A1, DBP, LRP2, CUB, CYP27B1, CYP24A1, VDR, and RXRA) have an effect on vitamin D levels. However, the effect exerted by these SNPs on the levels of circulating 25(OH)D can explain only 5% of its variability and can be considered small compared to

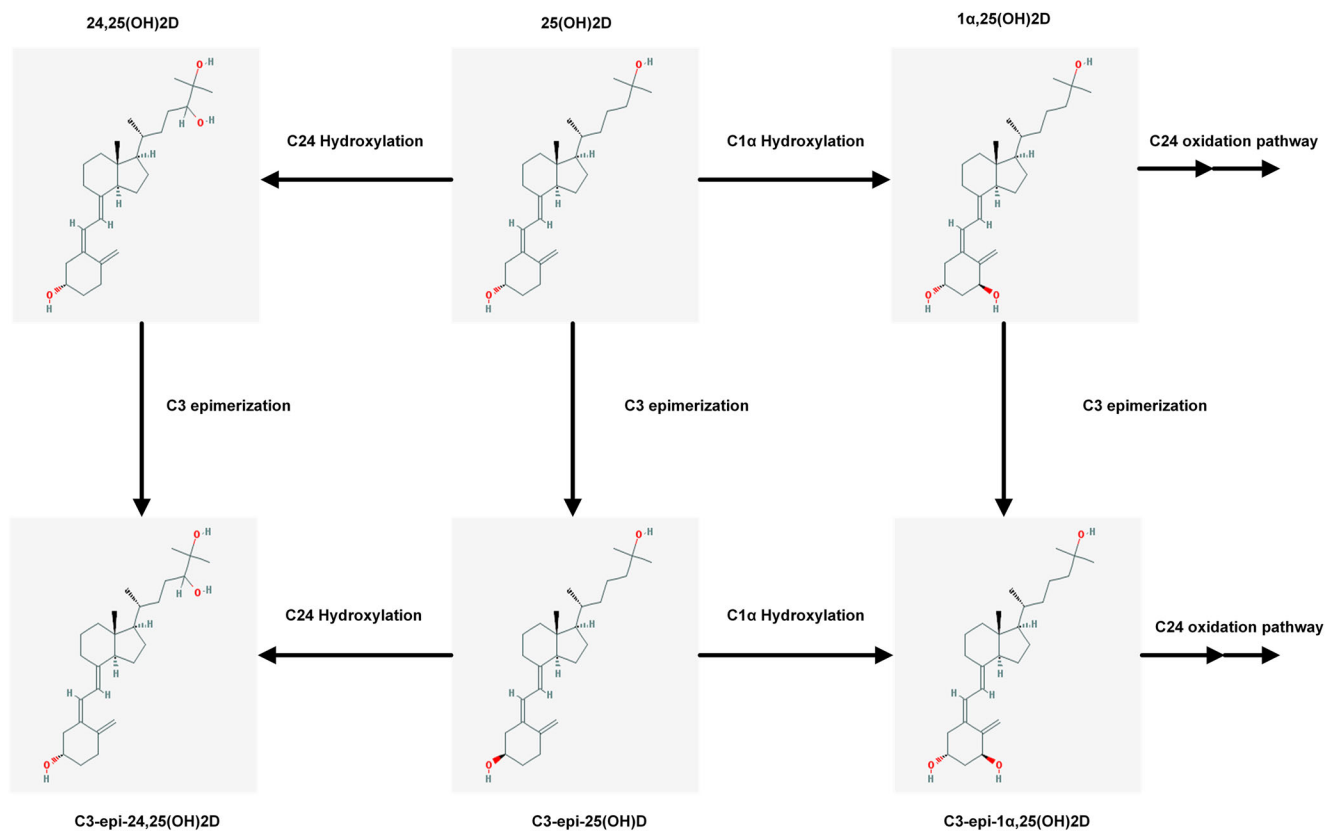


Fig. 7 Epimerization pathways of vitamin D metabolites (the chemical formulas are taken from pubchem <https://pubchem.ncbi.nlm.nih.gov>)

other environmental factors that have a more significant effect on its levels [7, 35]. Therefore, their presence does not seem to have significant clinical value in everyday practice, also taking into account the fact that most laboratory assays present an analytical variability of approximately 10%.

Age, sex, body mass index, and lifestyle Age, sex, and body fat seem to have a small effect on the seasonal variation of 25(OH)D levels, although recent studies have shown that the effect of age is small [36]. On the other hand, the lack of association of 25(OH)D levels with age can be explained in these studies, since most of the participants were < 75 years

Table 1 The major metabolites of vitamin D in humans and their concentration naturally present in the systemic circulation

Metabolite ^a	Levels in human serum
Vitamin D2/vitamin D3	18–29 nmol/L
25(OH)D3	8–165 nmol/L
25(OH)D2	< 7 nmol/L
1,25(OH) ₂ D3/1,25(OH) ₂ D2	48–168 pmol/L [0.1% of 25(OH)D]
24R,25(OH)2D	< 2.8–30 nmol/L [11% of 25(OH)D]
3-epi-25(OH)D	< 2–22 nmol/L [4% of 25(OH)D]

^a Concentrations of vitamin D2 are directly dependent upon intake either as supplement or with the food

old. It is known that in older adults, (1) calcium absorption is reduced; (2) there is intestinal resistance of calcium absorption to circulating 1,25(OH)₂D; (3) the ability of the skin to produce vitamin D is decreased; (4) VDR expression is reduced; (5) the kidneys are not able to produce 1α,25(OH)₂D compared to younger kidneys; and finally, (6) there is substrate deficiency of vitamin D [37]. Lastly, older people are more housebound and therefore less exposed to sunshine and to outdoor activities compared to younger people [38].

Body mass index (BMI) is usually increased with age, and there are several mechanisms which can explain its inverse association with low vitamin D levels, namely, physical inactivity, inappropriate diet, and incorporation of vitamin D in body fat, which makes it unavailable in the systemic circulation. Vitamin D deficiency is prevalent among people with obesity, suggesting that adipose tissue might play a role in the low vitamin D levels [39]. However, a causal relationship between obesity and low 25(OH)D levels has not to date been completely elucidated. Different mechanisms have been proposed to explain this inverse association, including such behavioral factors as reduced exposure to sunlight due to less outdoor physical activity and low dietary intake of vitamin D-enriched food. Moreover, decreased intestinal absorption, impaired hydroxylation in adipose tissue, and 25(OH)D accumulation in fat have been proposed to explain hypovitaminosis in obesity. The fact that vitamin D is a fat-soluble molecule may

lead to the hypothesis that vitamin D is sequestered in body fat depots, resulting in lower bioavailability in the obese state [40]. On the other hand, some studies have speculated that vitamin D deficiency itself could cause obesity or even prevent weight loss [40].

Several studies have demonstrated that men have higher levels of 25(OH)D, this finding being independent of age, season, and race. Finally, outdoor activities, exposure to sunlight (not only the duration of exposure but also the time of the day), dressing habits, and, most importantly, the coverage of the body (and even the type of clothes) and the use or not of sun protection all significantly affect 25(OH)D levels.

Ethnicity Although most of the studies were conducted with subjects of European descent, there are studies that have shown that levels of 25(OH)D differ according to ethnicity and skin color. This seems logical, since darker skin color protects from exposure to UV irradiation, thereby increasing the risk of vitamin D deficiency [41]. Vitamin D synthesis is highly dependent on melanin concentration in the skin, as melanin absorbs ultraviolet radiation (UVR), resulting in less efficient conversion of 7-dehydrocholesterol to provitamin D3 [42]. Therefore, dark-skinned individuals will experience slower vitamin D synthesis than light-skinned persons. This is more obvious and important at higher latitudes where the intensity and duration of sunlight are less. Metabolic differences due to race/ethnicity may provide an additional explanation.

Effect of liver and kidney disease The liver and kidneys are the two most important organs that participate in the metabolism of vitamin D.

The liver is the organ where 25-hydroxylation of vitamin D primarily takes place and the majority of VDBP is synthesized. In patients with liver disease, the prevalence of insufficiency and deficiency is much higher than in the general population, ranging between 64 and 92%, while serum 25(OH)D is inversely related to the severity of liver disease [43]. The high prevalence of vitamin D deficiency in this populations occurs regardless of the etiology of liver disease. Low vitamin D levels in chronic liver disease (CLD) may be the result of a variety of causes and mechanisms, including limited sun exposure and dietary intake, intestinal fat malabsorption due to cholestasis, and decreased hepatic hydroxylation [44]. Decreased production of VDBP and albumin might also be critical in CLD. In addition, hepatic hydroxylation is impaired, leading to low production of 25(OH)D. Hence, there will be less 25(OH)D available for production of the active hormone, whereas catabolism is increased. As VDBP has a single sterol-binding site and only 5% of total circulating DBP actually carries a vitamin D metabolite at any time, liver function would have to be severely impaired for low VDBP to have a significant role in 25(OH)D deficiency in CLD [45].

Total 25(OH)D levels decrease as severity of CLD increases, and there is a lack of correlation between total 25(OH)D levels and PTH and calcium. However, although patients with end-stage liver disease exhibit a high prevalence of low total 25(OH)D, they maintain normal serum corrected calcium levels and do not develop secondary hyperparathyroidism [46]. These observations have led to the hypothesis that total 25(OH)D levels may not be the most accurate biomarker to estimate vitamin D adequacy in patients with advanced CLD and that free 25(OH)D may be a more accurate marker, especially in patients with diminished liver synthetic ability [47].

The kidneys are essential not only for the conversion of 25(OH)D to $1\alpha,25(\text{OH})_2\text{D}$ but also for the reabsorption of 25(OH)D from renal ultrafiltrate for its recycling to the circulation to maintain serum 25(OH)D levels. Normal renal function is also essential to maintain the endocrine actions of calcitriol, while normal calcitriol, in turn, contributes to maintaining VDR in target tissues, since, through binding to VDR, it protects the receptor from degradation [48].

In chronic kidney disease (CKD), progressive loss of capacity to produce $1\alpha,25(\text{OH})_2\text{D}$ is observed. The mechanisms involved in the decrease of calcitriol production during the course of CKD are reviewed in references [14, 49]. Impaired uptake of 25(OH)D by the kidneys seems to be the main cause of $1\alpha,25(\text{OH})_2\text{D}$ deficiency, although CYP abundance and activity are also responsible [14, 50].

Decreased kidney function and calcitriol deficiency lead to hypocalcemia and are the key contributors to secondary hyperparathyroidism [14, 50]. This is more obvious among patients with end-stage renal disease, where $1\alpha,25(\text{OH})_2\text{D}$ is almost undetectable. Therefore, calcitriol replacement with the appropriate analog seems to be the therapy of choice to prevent or slow down the progression of SHPT and its adverse effects [51]. CKD is also characterized by low serum 25(OH)D levels. The main causes and risk factors for vitamin D deficiency among CKD patients are also reviewed in [50]. These levels also decrease progressively as renal function deteriorates; however, not all studies show that 25(OH)D insufficiency or deficiency are greater in these patients than in the general population [52]. Maintenance of sufficient levels of 25(OH)D could be a possible objective, since the kidney is not the only site of calcitriol production, and in CKD patients, vitamin D deficiency is also a strong predictor of accelerated renal disease and death [53]. However, the debate over what is the best treatment approach and what is the best biomarker to follow for CKD patients is still ongoing [54, 55].

Kidney disease also disrupts vitamin D catabolism. In the kidneys, 1 α -hydroxylase and 24-hydroxylase are under the competing hormonal regulation of FGF23 and PTH. However, the net effect of these two hormones on vitamin D catabolism in CKD is disputed. CKD is also characterized by high levels of FGF23 and increased serum phosphorus levels. Studies have

shown that FGF23 is responsible for the reduced expression of 1 α -hydroxylase in renal tubular cells and induces the expression of 24-hydroxylase, which is responsible for the catabolism of vitamin D. This may result in reduced production of 1 α ,25(OH)₂D. On the other hand, 24R,25(OH)₂D also seems to be lower in patients with CKD compared to healthy subjects [56]. Notably, moreover, PTH appears to increase the expression of CYP27B1 mRNA in renal tubular cells [14]. The increased phosphate levels have been correlated with low concentrations of 1 α ,25(OH)₂D, but it is not clear whether this correlation is direct or is induced by FGF23 or is a confounder along with other factors [14]. Furthermore, metabolic disturbances that are observed in patients with CKD, such as diabetes, metabolic acidosis, and uremia, it is possible to reduce the expression of CYP27B1 [57, 58].

Pregnancy Special attention must be given to pregnancy, since several studies report low levels of 25(OH)D in pregnant women. A recent meta-analysis reported that 54% of pregnant women have levels of vitamin D below 50 nmol/L [59]. Moreover, several studies have suggested that low levels of 25(OH)D during pregnancy are associated with increased risk of pre-eclampsia and gestational diabetes, as well as other complications of pregnancy [60]. However, the results from these studies associating low levels of 25(OH)D during pregnancy with adverse outcomes are conflicting. This has not only to do with possible methodological problems related to the design of the studies but also with the method of 25(OH)D quantitation. In pregnancy, VDBP is known to be increased; thus, when the measurement of 25(OH)D is performed with an immunoassay, the levels may well be underestimated due to incomplete dissociation of 25(OH)D from its binding protein. This causes significant assay variation, so that results from meta-analyses may be subject to error, especially when results are included from studies where measurements were based on immunoassays.

By contrast, when a HPLC or a LC-MS/MS method is used, dissociation of 25(OH)D from its binding protein is enhanced due to the use of strong chemical solvents in the pretreatment step of the samples [61].

Assays for the measurement of 25-hydroxyvitamin D

The measurement of 25(OH)D is performed for two major reasons: (1) to determine the nutritional status of vitamin D and (2) to monitor its therapeutic level. There are two different forms of vitamin D, as mentioned above, and, in order to adequately monitor therapy, both types of vitamin D need to be equally accurately quantitated. In fact, accurate measurement of 25(OH)D for the assessment of vitamin D status has always been a major goal of all clinical laboratories involved in measuring vitamin D metabolites.

25(OH)D is the metabolite of choice for several reasons:

- Its levels in the blood are higher than those of any other vitamin D metabolite: its serum concentration is in the range of 25–200 nmol/L, which is 1000 times higher than that of 1 α ,25(OH)₂D, whose concentration is in the range of 50 to 150 pmol/L. Furthermore, most of it is found in the systemic circulation, with limited distribution in less accessible tissues (e.g., fat) [62].
- Several clinical studies have demonstrated that there is an association between serum levels of 25(OH)D and several clinical outcomes, such as bone mineralization, fracture risk, fall risk, cancer, diabetes, and cardiovascular events.
- It has a relatively long half-life (2–3 weeks), as compared with 1 α ,25(OH)₂D (approximately 4–6 h), and, therefore, serum levels vary little within short periods of time [16, 63].
- The hydroxylase enzymes that metabolize vitamin D to 25(OH)D *in vivo* behave according to first-order reaction kinetics. This means that its rate of production is dependent on vitamin D levels and, therefore, its level in the systemic circulation is the best indicator of vitamin D nutritional status [64].
- Furthermore, 25(OH)D represents the sum of vitamin D intake and dermal production [65].
- Serum levels are relatively stable and not affected by diet (i.e., calcium intake) and lifestyle (i.e., sedative life or regular physical exercise), whereas 1 α ,25(OH)₂D levels are affected by all the latter [62, 63].
- Serum levels can determine if there is enough 25(OH)D for the extrarenal tissues to produce 1 α ,25(OH)₂D that has autocrine or paracrine action, since recent data have revealed that many of these tissues also contain the enzyme CYP27B1, responsible for converting 25(OH)D to 1,25(OH)₂D. Regulation of CYP27B1 in these non-renal tissues is generally different from that in the kidney and may be more substrate-dependent. This has led to the concept that maintenance of adequate 25OHD levels in the blood is required for vitamin D regulation of a large number of physiologic functions beyond those of the classic actions involved in bone mineral metabolism. Measurement of 1 α ,25(OH)₂D does not provide this information, since its extrarenal production does not contribute much to the systemic load [29, 66].

The assays that have been developed for the measurement of 25(OH)D can be classified into three types: (1) competitive protein binding assays (CPBA), (2) immunoassays, and (3) chemical assays.

CPBA This was the first method developed for 25(OH)D measurements and was published in 1971 [67]. In its first edition, this method used VDBP as the primary binding agent and ³H-25(OH)D as tracer. Radiolabeled ³H-25(OH)D “competed” with 25(OH)D in the sample for the available VDBP. Therefore, the

concentration of 25(OH)D was estimated by the reduction of radioactivity in ^3H -25(OH)D. However, since several vitamin D metabolites, such as 24R,25(OH) $_2$ D and even some rare ones like 25,26(OH) $_2$ D, have high affinity and bind to VDBP, this method has suffered from interferences. Moreover, it necessitated laborious preparation of the sample prior to analysis with organic solvents, with a chromatographic method, in order to release 25(OH)D in the sample from its binding proteins, which process required silicon columns. This method was supposed to detect with equal sensitivity and specificity both forms of 25(OH)D. Its analytical sensitivity was 10 nmol/L, later modification improving the sensitivity down to 5 nmol/L. Nevertheless, it remained a very laborious method and difficult to apply when large volumes of samples were needed for analysis. The method was largely abandoned by 2001, while an automated method based on this principle was available, but is now out of production. The only method that is still available today based on this principle is a manual ELISA [63].

Immunoassays These fall into three categories: (1) radioimmunoassays (RIA), (2) enzyme-linked immunosorbent assays (ELISA), and (3) chemiluminescent assays (CLIA). The development of polyclonal antibodies against 25(OH)D enabled the development of the first RIA in the early 1980s [68]. These were also competitive assays, and 25(OH)D in the sample competed with a radiolabeled 25(OH)D [^{125}I -25(OH)D]. Sample preparation was simplified by the use of acetonitrile to separate 25(OH)D from its binding protein. This method too was not free of interferences. The polyclonal antibody also reacted with 24R,25(OH) $_2$ D, 25,26(OH) $_2$ D, and 25(OH)D-26,23-lactone. Other disadvantages were the short life of reagents, difficulties and risks due to the use of radiolabeled reagents, and the manual character of the method, which excluded automated use. ELISAs were first introduced in the mid-1990s. They are also competitive in their design; however, the radiolabeled tracer was replaced by an enzyme (horse radish peroxidase). These too were manual methods and are still in production. Starting from the early 2000s, the first automated methods were developed and are now the mainstream methods for the quantitation of 25(OH)D. They are again based on the competitive principle, only the tracer is different: it is a chemiluminescent label. These CLIA assays claim better sensitivity and reproducibility, and, of course, automation allowed clinical labs to measure in a small period of time large volumes of samples. Analyzers were easy to handle and they did not require specially trained personnel. Major disadvantages included interferences from other metabolites and the huge variabilities observed between different assays from different manufacturers. This resulted in a lack of comparability, not only of measurements between clinical labs that used assays from different manufactures, but also between research studies [29, 69]. Moreover, immunoassays are affected by various other interferences [70, 71]. Nonetheless, all immunoassays continued to be designed as competitive methods. The reason is that the small

size of 25(OH)D impedes the simultaneous binding of two antibodies, and, therefore, the development of non-competitive sandwich immunoassays, which are characterized by superior analytical sensitivity and specificity [72]. Recently, Omi et al. described the development of an automated non-competitive method designed for the quantitation of 25(OH)D based on antimetatype monoclonal antibodies [73]. This non-conventional immunoassay has recently been validated and is commercially available [74].

High-performance liquid chromatography The use of HPLC for the separation and quantitation of vitamin D metabolites dates back to the early 1970s [75]. HPLC uses the difference of affinity of the molecules towards the solid phase (a resin contained in the column), or towards the liquid phase (a mixture of organic solvents and/or water based solutions known as the eluent). Both the polarity and the size and conformation of the molecule play an important role in the process. The use of columns is of major importance, and their production has evolved over the years to provide better separation between different metabolites. Detection is done primarily using UV detection at 265 nm, although electrochemical detection has been employed in several applications [29, 64, 69]. Though the above method can separate D2 from D3, the procedure, while being accurate for the measurement of 25(OH)D, its performance is wholly inadequate for the measurement of other metabolites that circulate at concentrations well below those of the 25(OH)D sample volume [64]. It has been used mainly as a research method since it requires a time-consuming manual sample preparation and an extraction phase before the sample is introduced to HPLC. The non-automation of this method is the main reason that it has not gained general acceptance by clinical labs. Each lab has to develop its own in-house application and the performance depends greatly on the selection of the instruments, the column used, and the experience of the users. The selection of an appropriate column is essential in order to be able to separate and quantitate different metabolites adequately. Nevertheless, the separation of vitamin D epimers is not done successfully, this being one of the negative sides of this method.

Liquid chromatography coupled with mass spectrometry

While HPLC used UV for the detection of various metabolites, LC-MS/MS uses a mass spectrometer for this task and relies on this to quantify different metabolites. LC-MS/MS relies on MS to differentiate between different molecular entities of vitamin D. As mentioned, in stand-alone HPLC methods, detection is achieved through a UV detector. Although UV detection is a powerful detection method for vitamin D metabolites due to their strong absorption at 264 nm, most metabolites exhibit similar UV patterns and need to be fully separated in the LC step in order to be detected and quantified separately. Therefore, metabolites that cannot be fully resolved by the LC part of the method

cannot be independently quantified and will instead be reported as a sum. This is where MS presents a big advantage. In this technique, molecules are detected by their mass-to-charge ratio (m/z), and most vitamin D metabolites have different masses. Therefore, they can be separated, detected, and quantified even if the LC part of the method does not fully separate them. However, a problem arises when some metabolites, called isobars, are present in the sample. These are molecules with the same molecular weight that form product ion pairs with the same m/z ratio. This is a problem that appears particularly with LC/MS/MS methods that typically use short chromatographic run times. For those metabolites, several techniques have been devised, and more advanced MS systems have been developed to overcome these obstacles.

Currently, LC-MS/MS methods present analytical sensitivity < 10 nmol/L well below the 25 nmol/L that signifies the severe deficiency of vitamin D. These methods are also very versatile, since they can measure multiple metabolites in a single sample in a single run (lately, methods have been developed that can quantify up to six different metabolites in a single sample), and they exhibit fewer matrix effects than immunoassays. Currently, the problems with separation of epimers seem to have been solved, as well as problems that can be created from the isobaric compounds with comparable m/z ratios. Finally, there has been significant progress in the automation of sample preparation [76].

To conclude, some drawbacks must be mentioned that are involved in both HPLC and LC-MS/MS methods that are mainly technical in nature. They require high quality water and chemical solvents; they are mostly in-house applications and this may present a problem with regulatory authorities, thus demanding very detailed validation prior to use for measurement of clinical samples; they necessitate very experienced technicians; and the initial instrumentation is expensive and requires large investment by the lab. Moreover, pre-analytical and analytical aspects in these methods need careful consideration, including sample type, protein precipitation, analyte extraction, derivatization, chromatographic separation ionization, and capabilities of the mass spectrometer. Calibration, standardization, and the use of internal standards are other important issues that impact on the accuracy of results. Only well-designed methods that are under continuous internal and external quality control allow accurate and stable measurement of 25(OH)D and other metabolites, such as 1,25(OH)₂D 24R, 25(OH)₂D and the epimer [77].

Analytical variability and the standardization of the 25(OH)D assays

Serum total 25(OH)D, as expressed by the sum of the serum concentrations of 25(OH)D₂ and 25(OH)D₃, is considered to be the best biological marker of an individual's vitamin D status. Several medical societies and government agencies

have issued vitamin D guidelines; however, differences exist in these guidelines as regards defining deficiency, insufficiency, and sufficiency, this creating a great deal of controversy [78, 79]. However, a fundamental factor that confounds efforts to develop consensus clinical and nutritional public health guidelines for interpreting serum 25(OH)D concentration is the substantial variability that existed (and still exists) in many assays that have been used over the years to measure 25(OH)D in clinical research studies [80]. The lack of assay standardization is the main source of bias, making it impossible to pool research results in order to develop consensus cut-points [81].

What is the VDSP? To overcome these problems, the Vitamin D Standardization Program (VDSP) was established in 2010 by the Office of Dietary Supplements, National Institutes of Health, as an international collaborative effort with the National Institute for Standards and Technology (NIST), Centers for Disease Control and Prevention (CDC) and Ghent University in Belgium, the AACC, the IFCC, and national health and nutrition surveys from Australia, Canada, Germany, Ireland, Mexico, South Korea, United Kingdom and the USA [82].

What is standardization? In terms of 25(OH)D, a standardized laboratory measurement is one that is “comparable across measurement system, location and time” [83]. This means that every laboratory, regardless of the type of assay it uses, the location, or the time (now or in the future), would report the same 25(OH)D result for the same sample—within certain predetermined statistical limits—as would be obtained using one of the internationally recognized gold standard RMPs developed by the NIST, Ghent University, and the CDC [84]. In other words, each “standardized laboratory” would report the true concentration of serum total 25(OH)D. It is also important to note here that standardization is different from harmonization. Harmonization is a process by which all laboratories report the same value for a given specimen, though the value is not necessarily the true one [85]. Standardization allows for the development and consistent application of evidenced-based guidelines. It is the first crucial step in comparing and contrasting results from different studies, whether they are clinical trials or observational epidemiological studies. With standardization, it is possible to achieve long-term stability of measurement results both in research and patient care.

Development of validated analytical methods In order to standardize an assay and to develop primary reference materials (PRM), critically evaluated analytical methods are required. The highest level of a validated analytical method is a Reference Measurement Procedure (RMP). A RMP is a measurement procedure that has been validated to measure what it

is intended to measure, has been thoroughly assessed for potential sources of bias, and is certified by the Joint Committee for Traceability in Laboratory Medicine (JCTLM). For well-defined, small-molecule clinical markers, RMPs are typically based on isotope dilution Mass Spectrometry (IDMS). To assist in the standardization of measurements of total 25(OH)D, the NIST first developed a RMP based on ID LC-MS/MS for the determination of 25(OH)D₂ and 25(OH)D₃ in human serum [86]. This method is now recognized by the JCTLM as a RMP. Later, Stepman et al. (at Ghent University, Belgium) also described an ID LC-MS/MS method for the determination of 25(OH)D₂ and 25(OH)D₃, which was also recognized as a RMP for 25(OH)D by the JCTLM [84]. Finally, the CDC also developed an ID LC-MS/MS method that was subsequently recognized by the JCTLM as a RMP for 25(OH)D [87]. These three methods are currently the only JCTLM-recognized RMPs for the determination of 25(OH)D₂ and 25(OH)D₃, and these RMPs play a critical role in the VDSP.

What has the VDSP achieved? The VDSP has developed a reference measurement system that is the backbone for standardizing 25(OH)D measurement in current and future assay systems [88, 89]. The components of this reference measurement system include: [1] the gold standard RMPs, (2) the NIST Standard Reference Materials (SRMs), (3) the VDSP's Standardization Certification Program which was developed, conducted, and maintained by the CDC, (4) the accuracy-based performance testing or external quality assessment schemes (PT/EQA) conducted by the College of American Pathologists and the Vitamin D External Quality Assessment Scheme or DEQAS [86, 90], (5) methods for retrospective standardization of studies completed in the past, and (6) a set of laboratory performance guidelines for both reference laboratories (those that run the RMP) and for routine laboratories developed by the VDSP [90].

The practical application of the VDSP consists in allowing the results of a clinical laboratory to be traceable to the gold standard RMP. If the lab uses a commercially available method, then the producer of the assay is responsible to ensure the traceability of its commercial method to the RMP. On the other hand, if it is an in-house developed method, then it is the lab's responsibility to ensure traceability [91]. Health care providers should understand the importance of the knowledge that their clinical assay is traceable to a RMP, since this provides information on both assay mean bias and variability (CV). This has enabled the VDSP to adopt 25(OH)D assay performance criteria developed by Stockl et al. for different types of labs and assays [90]. Specifically, for the RMPs, the limits for total CV and mean bias should be less than or equal to 5% and less than or equal to 1.7%, respectively. For routine laboratories, the limits for total CV and mean bias should be less than or equal to 10% and less than or equal to 5%, respectively. Thus, for a clinical laboratory 25(OH)D assay to be

defined as traceable to the NIST-Ghent-CDC RMPs, it must demonstrate a CV less than or equal to 10% and mean bias of less than or equal to $\pm 5\%$.

How is the performance of assays assessed? Participation of laboratories in proficiency testing or external quality assessment schemes is necessary to evaluate and improve analytical performance. The accuracy-based surveys are a very special type of EQA [92]. While there are several Vitamin D EQA programs around the world, to our knowledge, there are only two accuracy-based programs, namely, the CAP program in the USA and DEQAS for the rest of the world. Specifically, "accuracy-based" means that each laboratory's results, for each EQA sample, is compared to the "true value", as this was determined by one of the three recognized RMPs. The CDC assigns target values to the CAP materials using their RMP. The NIST did the same for DEQAS from October 2012 through July 2018; since then, the CDC has assigned target values to DEQAS serum materials or samples. The VDSP suggests that all laboratories should participate in an accuracy-based EQA scheme simply because a laboratory can determine the bias in their assay versus the true concentration, thereby helping to promote assay standardization [83].

Conclusions—key points

Vitamin D produced in the skin or ingested via the diet is biologically inert and requires two successive hydroxylations to form its biologically active form, calcitriol.

Serum total 25(OH)D, the sum of 25(OH)D₂ and 25(OH)D₃, is the best reflection of vitamin D status. It is the main circulating metabolite of vitamin D, with a long half-life, and its high concentration facilitates its measurement.

Over the years, several methods have been developed for its measurement, with automated immunoassays with liquid chromatography coupled with tandem mass spectroscopy (LC-MS/MS) being the latest methods developed. They are now considered as the preferred methods for the quantitation of 25(OH)D, since they can measure both 25(OH)D₂ and 25(OH)D₃.

Standardization was a major achievement of the VDSP, as it succeeded in minimizing the substantial variability that was associated with laboratory measurement of 25(OH)D.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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