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Review

Vitamin D: Analytical Advances, Clinical Impact, and Ongoing Debates on Health Perspectives

Etienne Cavalier [a,*] Konstantinos Makris [b] Annemieke C. Heijboer [c] Markus Herrmann [d] and Jean-Claude Souberbielle [e]

BACKGROUND: Vitamin D, acknowledged since the 1930s for its role in preventing rickets, gained additional prominence in relation to fragility fracture prevention in the late 1980s. From the early 2000s, connections between vitamin D deficiency and extra-skeletal pathologies emerged, alongside increased awareness of widespread deficits. This prompted crucial debates on optimal serum concentrations, expected to conclude when the outcomes of high-dose supplementation randomized controlled trials were available. Skepticism arose with inconclusive results from these trials.

CONTENT: This review begins with an exploration of vitamin D metabolism, followed by a detailed description of the measurement of vitamin D metabolites and the crucial role of standardization. Subsequent sections focus on the association of vitamin D with bone health and explore the extra-skeletal effects. The review concludes with a comprehensive discussion on the definition of vitamin D status and its implications for supplementation.

SUMMARY: Despite standardization efforts, assay variations and challenges still exist, especially in specific patient groups. Vitamin D supplementation has a significant impact on bone metabolism and optimal vitamin D status improves the efficacy of antiresorptive drugs such as bisphosphonates. The extra-skeletal effects of vitamin D remain debated, but may include potential benefits in conditions such as respiratory infections and cancer mortality, particularly in deficient individuals. The definition of vitamin D sufficiency is nuanced, especially when variations in population groups and analytical methods are taken into account. Despite ongoing debates and recent mega-trials tempering enthusiasm, vitamin D remains a complex and essential element in human health. Further research is needed to clarify its role in various health outcomes and guide supplementation strategies.

Introduction

Very few medications or vitamins can boast a history like that of vitamin D. Known since the 1930s for its role in prevention of rickets, its role in fragility fracture prevention only became apparent during the late 1980s. Subsequently, from the early 2000s, it became evident that a deficiency in vitamin D could also be associated with extra-skeletal pathologies and literature extensively discussed these "pleiotropic" effects of vitamin D. In parallel, awareness of a relatively common deficiency in vitamin D in the general population also quickly emerged.

Analytically, the measurement of vitamin D and its metabolites has also seen significant evolution. However, it is generally agreed that the best metabolite to reflect vitamin D status is 25-hydroxyvitamin D [25(OH)D] (1). The measurement of 25(OH)D quickly evolved from isotopic to automated chemiluminescent methods to cope with the logarithmic growth in demand from laboratories.

The ideal serum 25(OH)D concentration has been a longstanding point of contention among scientific societies and experts for numerous years (2, 3). However, it is worth noting that the discussion surrounding "clinical" cutoffs, which led to the abandonment of the "traditional" population-based reference intervals for 25(OH)D in the early 2000s, was grounded on the assumption that the measurements of 25(OH)D were standardized. This assumption implied that each analytical method in use would yield consistent concentrations. Unfortunately, this was not possible until the emergence of reference LC–MS/MS methods, the availability of international standards, and standardization programs such as the Vitamin D Standardization Program (VDSP) (4).

[a]Department of Clinical Chemistry, University of Liège, CIRM, CHU de Liège, Liège, Belgium; [b]Clinical Biochemistry Department, KAT General Hospital, Athens, Greece; [c]Department of Clinical Chemistry, Endocrine Laboratory, Amsterdam Gastroenterology & Metabolism, Vrije Universiteit Amsterdam and University of Amsterdam, Amsterdam UMC, Amsterdam, the Netherlands; [d]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria; [e]Laboratoire d'Exploration Fonctionnelles, Hôpital Necker-Enfants Malades, Paris, France.
*Address correspondence to this author at: Department of Clinical Chemistry, Tour 6, 2ème étage, CHU de Liège, Domaine du Sart-Tilman, 4000 Liège, Belgium. E-mail Etienne.cavalier@chuliege.be.
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The concurrence of less-than-anticipated toxicity, a high prevalence of deficiency in the general population, and the potential pleiotropic effects coming to light shifted vitamin D from a prescription-only medication, in many countries, to a common consumer product, available over the counter or in fortified foods. Vitamin D became a fashionable topic, and more and more experts began recommending supplementation with increasingly high doses without necessarily measuring the consequences of these actions.

However, the medical community awaited the results of large double-blind randomized controlled studies (RCT) using sometimes quite high doses of vitamin D supplements before making a final judgment on the value of systematic supplementation. The results of these studies, although subject to nuanced interpretation, were generally negative or inconclusive, curbing the enthusiasm for vitamin D and even giving rise to "vitamin D bashing," possibly negating significant benefits of vitamin D supplementation.

In this review, after a short reminder of vitamin D metabolism, we will discuss the analytical methods and their limitations, update on emerging vitamin D metabolites [potentially more relevant than measuring 25(OH)D alone], and then assess the latest controversies and the practical clinical relevance of vitamin D.

Overview of Vitamin D Metabolism

The term vitamin D includes lipophilic steroid compounds with similar structure and the same biological effects (5). The 2 primary compounds, vitamin D3 or cholecalciferol, synthesized in the skin on exposure to ultraviolet-B rays, and vitamin D2 or ergocalciferol, obtained through plant irradiation, share similarities except for their side chain structures.

Vitamin D and its metabolites demonstrate a strong binding affinity to plasma vitamin D binding protein (VDBP). The half-life of plasma VDBP is approximately 2.5–3.0 days (6). Specific pathological conditions, such as critical illness and inflammatory states, are linked to reduced plasma levels of VDBP. Conversely, certain physiological conditions, such as pregnancy, may lead to an elevation in VDBP levels (1).

Vitamin D2 and D3 are pro-hormones requiring a series of enzymatic steps for their conversion into biologically active forms (7, 8). The first one is the conversion of vitamin D to its 25-hydroxylated form, 25(OH)D, in the liver. The principal vitamin D-25-hydroxylase is the microsomal cytochrome P4502R1 (*CYP2R1*). The half-life of 25(OH)D is about 10–40 days for D_3 and slightly lower for D_2.

The second hydroxylation step involves the activation of 25(OH)D to its biologically active form, occurring in the proximal tubule of the kidney as well as in numerous extrarenal tissues. While most tissues can only absorb the free form of 25(OH)D, the renal proximal tubular cells can uptake the bound forms. This uptake is facilitated by the megalin–cubulin complex, an active receptor-based transport mechanism that reabsorbs VDBP or the VDBP-25(OH)D complex, preventing urinary loss of VDBP and 25(OH)D (9).

Several tissues express megalin–cubulin, but it remains uncertain whether they significantly contribute to a greater internalization of 25(OH)D than would be expected for free 25(OH)D. In the kidney, 25(OH)D undergoes conversion to $1,25(OH)_2D$, also known as calcitriol, catalyzed by the enzyme *CYP27B1* (or 1α-hydroxylase). The expression of *CYP27B1* is tightly regulated, stimulated by parathyroid hormone (PTH), a low calcium diet, a low phosphate diet, and insulin-like growth factor-1 (IGF-1), and inhibited by fibroblast growth factor 23 (FGF23). Furthermore, $1,25(OH)_2D$ downregulates its own synthesis.

Calcitriol, produced by the kidney, enters the bloodstream, binds to VDBP with a half-life of approximately 4–6 hours, and is delivered to target tissues such as bone, intestine, parathyroid glands, and kidney, where it exerts genomic effects. Notably, calcitriol also exerts autocrine activities as it can be synthesized in various tissues expressing *CYP27B1*, contributing to the extra-skeletal effects of vitamin D. However, there is no evidence suggesting that this locally synthesized calcitriol significantly contributes to plasma levels.

In the metabolism of vitamin D, another crucial step is catabolism. The levels of both calcidiol and calcitriol are tightly regulated by the mitochondrial enzyme 24-hydroxylase (*CYP24A1*). This enzyme catalyzes the hydroxylation at positions C23 and C24 of both calcidiol and calcitriol. Expressed in most cells, *CYP24A1* is induced by elevated $1,25(OH)_2D$ concentrations, serving as a negative feedback mechanism to prevent hypercalcemia. *CYP24A1* initiates a 5-step inactivation pathway, leading to the production of calcitroic acid, an inactive product ultimately excreted through the bile. It is suggested that $1,24,25(OH)_3D$ may also be produced through the activation of $24,25(OH)_2D$ by *CYP27B1* (10). Notably, *CYP24A1* also catalyzes an alternative pathway, known as the 23-hydroxylase pathway, leading to the formation of the end product $1,25\text{-}(OH)_2D\text{-}26,23$ lactone. Figure 1 provides a concise summary of vitamin D metabolism.

Measurement Methods for Vitamin D in the Clinical Laboratory Context

Most clinical laboratories assess 25(OH)D levels, while only specialized laboratories undertake the

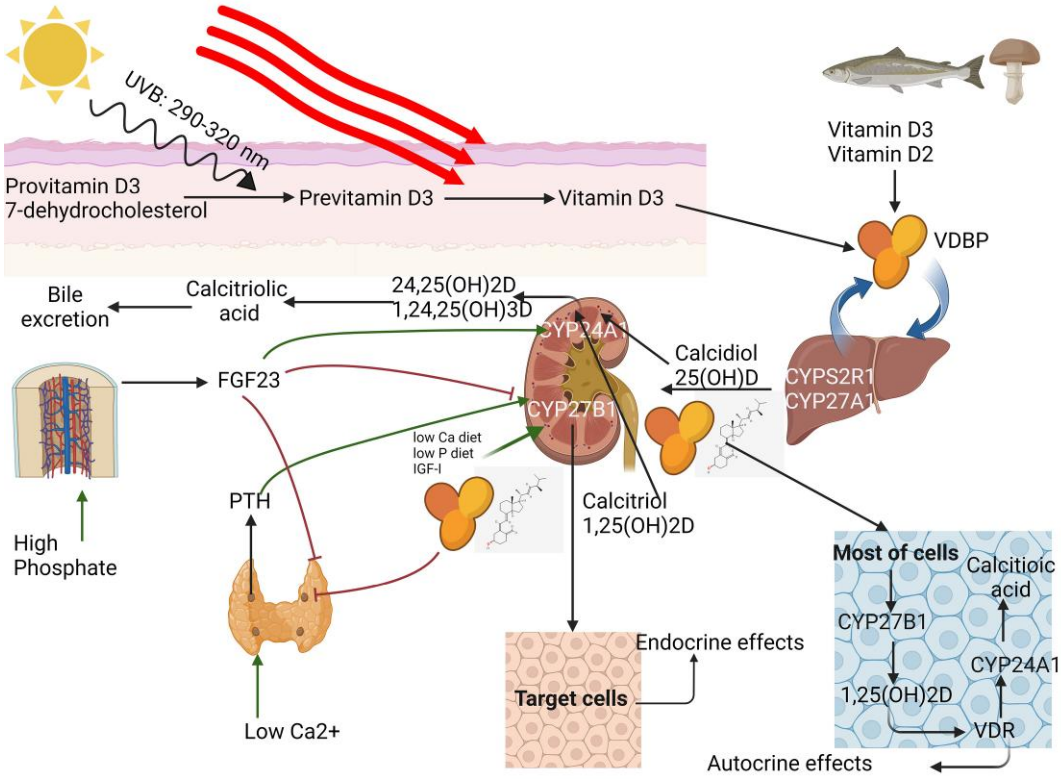

Fig. 1. Summary of the metabolism of vitamin D. Vitamin D2 and vitamin D3 are transported in the blood by the VDBP and hydroxylated in the liver to form 25(OH)D, the concentration of which represents an individual's vitamin D status. Regulation of this hepatic hydroxylation is weak, and, the more vitamin D is ingested (D2 or D3) or synthesized in the skin (D3 only), the more 25(OH)D is produced. 25(OH)D is hydroxylated again to produce $1,25(OH)_2D$ (also called calcitriol), the active vitamin D metabolite. This second hydroxylation can take place either in the proximal renal tubule, or in many other tissues. Renal hydroxylation, which is tightly regulated by calciotropic hormones, PTH and FGF23, allows production of the $1,25(OH)_2D$ "hormone" (i.e., which enters the blood and acts on distant target tissues binding to its receptor, the VDR). Peripheral hydroxylation seems independent of the calciotropic hormones but is dependent on the amount of circulating 25(OH)D. It forms $1,25(OH)_2D$ that binds the VDR in the local tissue (where this $1,25(OH)_2D$ has been formed), acts locally (in an intracrine manner), and does not participate to the calcium/phosphorus/bone metabolism. It is now demonstrated that in some tissues, circulating $1,25(OH)_2D$ can bind membrane proteins (probably variants of the VDR) with subsequent activation of different intracellular enzymes and/or modification of the intracellular calcium flux. Of note, an inactivating pathway through a 24-hydroxylase exists both in the kidney and peripheral tissues.

measurement of metabolites such as $1,25(OH)_2D$ and $24,25(OH)_2D$. Measurement of vitamin D metabolites is typically conducted using either automated ligand binding assays or liquid chromatography, frequently coupled with tandem mass spectrometry (LC–MS/MS).

25(OH)D MEASUREMENTS

Significant variations in quality exist among 25(OH)D assays, primarily stemming from technical disparities. Chromatography-based methods (HPLC and LC–MS/MS) exhibit higher specificity, capable of distinguishing between 25(OH)D3 and 25(OH)D2. In contrast, ligand binding assays (immunoassays or protein binding assays) are less specific. These assays often report an ambiguous sum of 25(OH)D3 and 25(OH)D2, as the antibodies employed typically vary in their affinity for both metabolites (11). This leads to unwanted results in the diagnostic and research setting, especially in countries where ergocalciferol is prescribed. Also, the ligand binding assays may suffer from cross-reactivity with other vitamin D metabolites such as $24,25(OH)_2D$ (12).

LC–MS/MS methods demonstrate high specificity, distinguishing between 25(OH)D3, 25(OH)D2, and other metabolites. Notably, all vitamin D metabolites can undergo epimerization at position C3 through the action of the enzyme 3-epimerase, resulting in the conversion of the hydroxyl group at position C3 of the A ring from alpha to beta orientation. While the separation of the 25(OH)D epimer can be challenging, it holds significance, especially in newborns in whom its concentrations can be notably high and diminish with age. Therefore, it is crucial to ascertain whether the chosen method is capable of effectively separating the epimer or not (13). In general, immunoassays do not cross-react with the epimer.

In addition to the challenge of cross-reactivity, certain ligand binding assays appear to encounter accuracy issues when handling samples from specific patient groups. Given that the measurement of total 25(OH)D is essential, the complete release of all 25(OH)D from the VDBP must occur before the measurement process. Complications may arise when VDBP levels deviate from the normal range, potentially resulting in erroneously low or high 25(OH)D results (14). This is specifically problematic in patients with significantly elevated or decreased VDBP concentrations such as in pregnant women, patients with liver failure, or at the intensive care unit. Also, in several other patient groups with normal VDBP concentrations such as hemodialysis (15) and osteoporosis patients (16), immunoassays encounter accuracy problems that are probably related to alterations of the sample matrix.

Finally, it is crucial to address the issue of standardization in 25(OH)D assays. Recognizing this concern, the VDSP was established in 2010, complemented by the initiation of the International Vitamin D Standardization and Certification Program (VDSCP) led by the Centers for Disease Control (CDC) (17). The CDC VDSCP began providing information on individual sample pass rates, indicating the proportion of the 40 samples meeting the defined bias criterion (<5%). By June 2020, 34 methods, including those from in vitro diagnostics manufacturers and in-house methods from medical laboratories, were certified and standardized against the Reference Method Procedure for the year 2019. However, the "individual sample pass rate" varied significantly among methods in 2019, ranging from 45% to 88% for LC–MS/MS methods (with a mean pass rate of 63%) and from 8% to 68% for ligand biding assays (with a mean pass rate of 30%) (18). Nevertheless, the VDSP efforts led to an improvement in the standardization of 25(OH)D measurements in reference samples. In certain patient samples, however, standardization remains an unresolved issue for immunoassays (14).

As mentioned earlier, LC–MS/MS methods are preferred for the measurement of 25(OH)D, though it is important to note variations in the quality of these methods as well. As many 25(OH)D LC–MS/MS methods are laboratory-developed tests, the quality is contingent on the proficiency of the laboratory. Ongoing initiatives involving fully automated LC–MS/MS approaches are anticipated to broaden the range of available methods in the market. However, it is noteworthy that the Cascadion SM Clinical Analyzer (Thermo Fisher Scientific), although briefly available on the market, did not demonstrate superiority over existing ligand binding assays (19).

VITAMIN D METABOLITE MEASUREMENTS

The measurement of serum $1,25(OH)_2D$ concentrations, given their very low levels, has historically posed challenges. Traditional methods such as manual competitive protein binding assays and radioimmunoassays encountered specificity issues, particularly with cross-reactivity. While automated immunoassays offer improved performance in this regard, they cannot distinguish between $1,25(OH)_2D2$ and $1,25(OH)_2D3$, presenting challenges in regions with widespread D2 supplementation. In recent years, LC–MS/MS methods have been developed by various laboratories to measure $1,25(OH)_2D$, addressing sensitivity concerns through 2D chromatography, derivatization, and immunopurification. The last not only enhances sensitivity but also improves specificity, crucial for avoiding isobaric interferences. However, method discrepancies exist among LC–MS/MS studies due to challenges in separating 1β-25-dihydroxyvitamin D3 from its epimer. Unfortunately, there are no reference methods for measuring $1,25(OH)_2D$, hindering the evaluation of existing methods' quality (1, 20). A reference method that allows standardization is only available for $24,25(OH)_2D$ (21). Among the other metabolites, measuring $1,24,25(OH)_3D$ seems of interest for a more precise evaluation of the vitamin D metabolome (10). Multiplexing the metabolites reflecting the activation and degradation pathways of vitamin D, alongside with 25(OH)D, will help to improve understanding of the complexity of this pivotal pathway.

FREE OR BIOAVAILABLE 25(OH)D MEASUREMENT

Free and bioavailable 25(OH)D have emerged as alternative biomarkers for assessing vitamin D metabolism. In typical conditions, <0.1% of circulating 25(OH)D is free and capable of passive diffusion into target cells. Certain researchers posit that this free fraction may predominantly account for most of vitamin D's effects (22). Notably, the absence of VDBP does not necessarily induce functional vitamin D deficiency (VDDef), even in the presence of significantly low concentrations of 25(OH)D and $1,25(OH)_2D$. This observation lends support to the free hormone hypothesis (23). Furthermore, the median concentration of free 25(OH)D in patients with liver cirrhosis is approximately 2-fold higher than

that of unaffected controls, despite exhibiting lower total 25(OH)D concentrations (24). In individuals without health complications, approximately 85% of circulating vitamin D metabolites exhibit a high-affinity binding to VDBP, while the remaining 15% bind to albumin or lipoproteins with a comparatively lower affinity, facilitating easier dissociation of vitamin D metabolites from these carriers. Consequently, vitamin D molecules bound to albumin and lipoproteins may play a role in intracellular availability of vitamin D. Building on this understanding, the free hormone hypothesis has been broadened to include the bioavailable hormone fraction, encompassing the sum of both free and albumin-bound hormone. Nevertheless, ongoing debates persist regarding the true availability of this fraction for metabolism (25). Assessing free and bioavailable 25(OH)D is a complex process involving the measurement of 25(OH)D, VDBP, and albumin. The obtained results are then input into formulas to compute the values for free and bioavailable 25(OH)D (26). The quantification of bioavailable 25(OH)D faces significant uncertainty owing to the inherent analytical variability of all 3 compounds involved. Notably, VDBP is a highly polymorphic protein, and its genotype can introduce considerable bias, especially in the context of immunoassays (27). The absence of reference intervals for free and bioavailable 25(OH)D poses an additional hurdle in the clinical application of these biomarkers. Alongside technical limitations, there is insufficient clinical evidence to robustly support a broader use of free and bioavailable vitamin D measurements (28). In several investigations exploring associations between these markers and diverse medical conditions, no added value of free and bioavailable 25(OH)D was identified when compared to total 25(OH)D (29–34). This observation may not be unexpected when considering that 25(OH)D is not the active compound itself but rather a prohormone. Consequently, free 25(OH)D is not subject to regulation by feedback loops. In summary, both free and bioavailable 25(OH)D do not appear to offer significant potential for enhancing the assessment of vitamin D status and the diagnosis of vitamin D deficiency.

Association of Vitamin D with Bone Health and Mineral Metabolism

ROLE OF VITAMIN D IN THE REGULATION OF CALCIUM AND PHOSPHATE HOMEOSTASIS

Calcitriol exerts direct effects on the 4 organs crucial for calcium/phosphate metabolism. In the intestine, it stimulates active calcium absorption through its influence on Transient Receptor Potential cation channel subfamily V member 6, a calcium channel facilitating calcium entry into the enterocyte; Calbindin-D9K, responsible for transporting calcium from the apical to the basolateral membrane of the enterocyte; and plasma membrane Ca^{2+}-ATPase isoform 2c, a calcium-ATPase enabling calcium exit from the enterocyte to the plasma. Additionally, calcitriol upregulates the expression of sodium-dependent phosphate transport protein 2B, a sodium phosphate cotransporter encoded by the SLC34A2 gene, located at the apical membrane of the enterocyte, enhancing the intestinal absorption of phosphate (35).

In the parathyroid glands, calcitriol inhibits the expression of the PTH gene and exerts antiproliferative effects on parathyroid cells, limiting parathyroid hyperplasia, notably in cases of persistent secondary hyperparathyroidism observed in conditions such as chronic kidney disease (CKD). Its impact on bone is multifaceted. The vitamin D receptor (VDR) is present in osteoblasts and osteocytes. Calcitriol stimulates the synthesis of anabolic bone proteins such as osteocalcin, osteopontin, and Low-density lipoprotein Receptor-related Protein 5, but also triggers the secretion of Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL), a potent resorptive agent, while inhibiting osteoprotegerin, the decoy receptor of RANKL, and Runt-related Transcription factor 2, a bone anabolic protein. Consequently, calcitriol may exert both anabolic and catabolic effects on bone. Contrary to expectations, mice with a selective deletion of the VDR in the intestine, while maintaining a functional VDR in other tissues, did not exhibit hypocalcemia and rickets under conditions of very low calcium intake. Instead, these mice remained normocalcemic but developed osteoporosis with fractures and hyperosteoidosis. Additionally, there is a notable increase in calcitriolemia, stimulating bone resorption and inhibiting bone mineralization (36). Therefore, in situations characterized by deficient intestinal calcium absorption, the conventional role of calcitriol in promoting bone mineralization through the stimulation of calcium absorption and optimization of the calcium/phosphate product may undergo a shift. Its primary emphasis may transition to maintaining normal calcemia, potentially at the expense of the skeleton. Ultimately, calcitriol stimulates osteocytes to release FGF23, a potent hypophosphatemic hormone that diminishes phosphate reabsorption in the proximal tubule and inhibits the renal synthesis of calcitriol. The significance of vitamin D in calcium/phosphate metabolism is underscored by the hypocalcemic and hypophosphatemic state associated with a deficiency in vitamin D (37), or vitamin D action (38), and by the hypercalcemic state associated with vitamin D or calcitriol excess, or defect in its catabolism (Table 1).

EFFECTS OF VITAMIN D SUPPLEMENTATION ON PHOSPHOCALCIC METABOLISM AND PARATHYROID FUNCTION

Excessive intake of vitamin D can lead to hypercalcemia, hypercalciuria, and decreased PTH levels, posing renal

Table 1. Causes, effects on calcium/phosphate metabolism, and clinical features of the main pathologies due to an anomaly of vitamin D metabolism.

Pathology	Cause(s)	Consequences
Vitamin D-deficient rickets/ osteomalacia	Severe vitamin D deficiency, often associated with low calcium intake. May be due to the combination of very low dietary intake and lack of sunlight exposure. May also be due to intestinal malabsorption.	The presence of markedly low serum levels of 25(OH)D and calcitriol is indicative, accompanied by hypocalcemia, hypophosphatemia, hypocalciuria, and elevated or very high PTH levels, along with increased alkaline phosphatase. The clinical presentation of rickets typically involves fractures, diffuse musculoskeletal pain, and bone deformation. This clinical scenario is reversible through the correction of vitamin D deficiency and the adoption of suitable calcium intake. However, achieving reversal may pose increased challenges in severe cases of malabsorption.
VDDR, type 1A	Inactivating mutation of the *CYP27B1* gene that encodes 1-alpha hydroxylase. Inability to produce calcitriol.	Following vitamin D supplementation, achieving normal 25(OH)D levels coincides with undetectable or very low calcitriol. The associated biochemical profile includes hypocalcemia, hypophosphatemia, hypocalciuria, and elevated or very high PTH levels, along with increased alkaline phosphatase. Clinically, this manifests as the typical presentation of rickets, featuring fractures, diffuse musculoskeletal pain, and bone deformation. Importantly, this clinical picture is effectively reversible with the administration of an appropriate 1-hydroxylated vitamin D compound.
VDDR, type 1B	Inactivating mutation of the *CYP2R1* gene that codes for the main 25-hydroxylase. Inability to produce 25(OH)D. The rarest of the genetic VDDR.	The biochemical and clinical presentation closely resembles that of vitamin D deficiency rickets. However, in this case, it exhibits either unresponsiveness (homozygous) or poor/moderate responsiveness to vitamin D supplementation. A positive outcome is observed with treatment involving calcidiol (25(OH)D).
VDDR, type 2	Inactivating mutation of the VDR1 gene which codes for the VDR. This induces a resistance to all effects of calcitriol.	Despite normal 25(OH)D levels (post-supplementation) and serum calcitriol, there is still an observed hypocalcemia, hypophosphatemia, hypocalciuria, elevated PTH, and increased alkaline phosphatase. Alopecia is a common manifestation in more than two-thirds of patients. The clinical presentation aligns with severe rickets, featuring fractures, diffuse musculoskeletal pain, and bone deformation. Notably, this form of rickets remains unresponsive to treatment with any 1-hydroxylated vitamin D compound. However, variable improvement may be seen with high calcium intake.

Continued

Table 1. (continued)

Pathology	Cause(s)	Consequences
Vitamin D intoxication	Excessive intake of vitamin D or calcidiol (25(OH)D). No vitamin D intoxication by excess sunlight exposure.	Very high 25(OH)D levels accompanied by low or low-normal calcitriol with hypercalcemia, high or high-normal phosphatemia, hypercalciuria, and reduced serum PTH. Clinical manifestations include signs of hypercalcemia, with a notable risk of urolithiasis and nephrocalcinosis if left unaddressed. BMD tends to decrease initially but improves with the correction of the underlying disorder.
Hypercalcemia of granulomatosis especially sarcoidosis	Granuloma can synthesize and secrete calcitriol independently of the usual regulator (PTH). Hypercalcemia is thus due to excessive calcitriol, which stimulates bone resorption and intestinal absorption of calcium.	Serum 25(OH)D levels are typically within "normal" range (though not usually high), accompanied by elevated serum calcitriol. This scenario is associated with hypercalcemia, high or high-normal phosphatemia, hypercalciuria, and low serum PTH, often with an elevation in angiotensin-converting enzyme. Clinical manifestations include signs of hypercalcemia, with a notable risk of urolithiasis and nephrocalcinosis if left unaddressed. BMD may decrease initially but tends to improve with the correction of the underlying disease.
Idiopathic infantile hypercalcemia	Inactivating mutation of the *CYP24A1* gene that codes for the 24-hydroxylase. Inability to inactivate calcitriol. Hypercalcemia is due to excessive effect of calcitriol. Can be considered as a hypersensitivity to vitamin D.	Serum 25(OH)D levels are typically within a "normal" range, occasionally reaching high-normal levels, coupled with elevated calcitriol (sometimes high). A low VMR, calculated as $24,25(OH)_2D/25(OH)D \times 100$, is indicative. This presentation is associated with hypercalcemia, high or high-normal phosphatemia, hypercalciuria, and low serum PTH, with observable clinical signs of hypercalcemia. Notably, there is a heightened risk of urolithiasis and nephrocalcinosis with a rapid onset. Initially identified in newborns, this condition is now more frequently diagnosed in older children and even adults.

VDDR, vitamin D-dependent rickets.

and cardiovascular risks. Nevertheless, it is generally deemed safe for adults to consume vitamin D doses up to 4000 IU per day (2, 3). Whereas a previous report suggested that vitamin D supplementation increases the risk of hypercalcemia (39), it is worth noting that, more recently, excess hypercalcemia was not reported in the vitamin D groups of mega-trials performed mostly in vitamin D-sufficient patients who received vitamin D supplementation up to 4000 IU/day (see Table 2). Furthermore, in individuals with moderate VDDef, vitamin D supplementation typically does not alter serum calcium levels but often results in a decrease in serum PTH. Consequently, we advocate for the establishment of PTH reference values in populations of vitamin D-replete subjects—a crucial consideration in clinical chemistry that markedly reduces the upper limit of the PTH reference range (40). A parallel recommendation was proposed for serum calcium reference values by Roizen et al. (41). They demonstrated in a large population of children that the lower limit, although not the upper limit, of the 95% interval of serum calcium values was higher in vitamin D-replete subjects compared to unselected subjects. Additionally, routine practice in CKD patients involves the use of native vitamin D or calcifediol

supplementation to mitigate secondary hyperparathyroidism: a well-recognized risk factor for adverse outcomes, particularly in those undergoing chronic dialysis (42).

IMPACT OF VDDEF, AND VITAMIN D SUPPLEMENTATION ON BONE MINERAL DENSITY, OSTEOPOROSIS, FALLS, AND FRACTURES

Very severe VDDef is responsible of bone mineralization defects and may cause rickets and osteomalacia (37). On the other hand, the debate continues regarding whether mild VDDef, without evident mineralization defects, may contribute to bone fragility and worsen osteoporosis. Numerous studies examining the association between VDDef and bone mineral density (BMD) loss or the risk of fractures have indicated a significant connection. However, more recent studies have instead reported a U-shaped relationship, suggesting an increased risk for both low and high 25(OH)D levels (50). A decreased risk of fracture was not found in recent mega-trials of vitamin D supplementation in apparently healthy, mostly vitamin D sufficient, subjects (see Table 2). However, supplementation with a combination of moderate doses of cholecalciferol (800–1000 IU/day) and calcium slightly, but significantly, decreased the risk of nonvertebral fracture, especially hip fracture in frail persons at risk of osteoporosis (51). As falls are a main cause of peripheral fractures in the elderly, several studies have tested the effect of vitamin D supplementation on the risk of falls. While vitamin D deficiency is associated with an increased risk of falls, interventional studies produced conflicting results. In brief [reviewed in (50)], supplementation with moderate vitamin D doses (800–1000 IU/day) decrease the risk of falls in vitamin D-deficient elderly patients but not in vitamin D-sufficient people. Doses <800 IU/day do not seem to reduce falls while large bolus doses increase falls. Finally, it seems that optimal vitamin D status improves the efficacy of antiresorptive drugs such as bisphosphonates (52). For these reasons, experts in musculoskeletal health still strongly recommend supplementation for persons at risk of VDDef.

Extra-musculoskeletal Effects of Vitamin D: Are They Real?

Studies on the extra-skeletal effects of vitamin D can be categorized into 3 main groups: experimental studies, observational studies, and randomized controlled trials (RCTs). Experimental studies involve the administration of vitamin D or its metabolites to animals or cultured cells, offering insights into potential mechanisms of action on specific pathologies, although findings may not be directly applicable to humans. Observational studies assess the association between serum 25(OH)D concentration or vitamin D intake and the incidence, severity, or progression of a particular disease. While many of these studies have reported a significant association between VDDef or low vitamin D intake and increased risks for various pathologies, it is crucial to recognize that an association does not imply causality, which requires validation through RCTs.

A multitude of RCTs have been conducted, and their results, incorporated into meta-analyses, present a somewhat inconclusive picture. While some RCTs demonstrated the beneficial effects of vitamin D, most yielded "neutral" outcomes, indicating no significant difference between vitamin D and a placebo. In rare instances, vitamin D was even found to be less favorable than a placebo. Mega-trials and meta-analyses, often employing an intent-to-treat (ITT) analysis, commonly conclude that vitamin D lacks significant effects. However, it is important to acknowledge that the ITT analysis, typically the gold standard for evaluating a "classic" drug, may have limitations when assessing the effects of vitamin D:

1. In an RCT evaluating the effects of a traditional drug, the baseline blood concentration of the drug is zero by definition. Conversely, in an RCT assessing the effects of vitamin D in unselected subjects or patients, it is expected that many participants may not be vitamin D deficient, as evident in several recent mega-trials (see Table 2) (43–48).
2. In a meta-analysis assessing the outcomes of multiple RCTs involving a specific "classic" drug, the dosage and timing of administration remain consistent across all the tested RCTs. However, this is not the scenario with vitamin D, where the dosage can vary by a factor of 20 or more, and the mode of administration may occur daily, weekly, monthly, quarterly, or even annually. This diversity in dosing regimens and administration schedules introduces additional complexities when interpreting the collective findings of vitamin D RCTs in a meta-analysis.
3. When evaluating the impact of a "classic" drug on a specific disease, the study typically enrolls only patients afflicted by that particular condition (e.g., a candidate antihypertensive drug is tested in hypertensive patients). However, in certain meta-analyses assessing the effects of vitamin D supplementation on specific diseases, some RCTs conducted in ostensibly healthy groups were also incorporated (see the example of hypertension in Table 3).

The factors mentioned previously, including the widely variable duration of the RCTs and the observation that, even when administered to vitamin D-deficient patients, supplementation did not correct VDDef in many cases, should encourage a cautious interpretation of meta-analytical results. While the ITT analysis remains the gold standard for definitive conclusions, it is worthwhile to consider available prespecified secondary or post hoc

Table 2. Brief description of recent mega-trials (>2000 participants). Overall, the results show that, compared to a placebo, vitamin D supplementation with relatively high doses does not prevent hard-disease endpoints, such as cardiovascular disease, cancer, fractures, or falls, in subjects unselected according to their vitamin D status, most of whom are vitamin D sufficient. 1 μg of vitamin D is equal to 40 IU.

Study	Population: Number of subjects (mean age at enrollment)	Intervention	Mean basal 25(OH) D in nmol/L (SD)	Summary of results (ITT analysis) of primary and prespecified secondary objectives
VITAL (43)	25 871 subjects (67 years)	2000 IU/d D3 for a median of 5.3 years	77 (25)	Vitamin D did not significantly reduce the primary endpoints of total invasive cancer incidence and of major CVD events. Vitamin D did not reduce the secondary endpoints: other cardiovascular endpoints, all-cause mortality, falls, fractures.
Do-Health (44)	2157 subjects (75 years)	2000 IU/d D3 for a median of 3 years	56 (21)	No significant benefit of vitamin D for the 6 primary outcomes: change in systolic and diastolic BP, Short Physical Performance Battery, Montreal Cognitive Assessment, nonvertebral fractures and infections.
D2D (45)	2423 patients with prediabetes (60 years)	4000 IU/d D3 for 2 years	70 (25.5)	Vitamin D3 supplementation did not result in a significantly lower risk of diabetes, the primary objective, although a trend was observed.
D-Health (46)	21 315 persons (60–84 years)	60 000 IU/month D3 for a median of 5 years	76% had a predicted 25(OH)D ≥50 nmol/L	The rate of major cardiovascular events, the primary objective, was lower in the vitamin D group (HR: 0.91) although the *P* value was not significant. Myocardial infarction but not stroke was significantly reduced. As a secondary objective, vitamin D did not reduce all-cause mortality, and exploratory analyses excluding the early follow-up period were consistent with an increased risk of death from cancer in the vitamin D group.

Continued

Table 2. (continued)

Study	Population: Number of subjects (mean age at enrollment)	Intervention	Mean basal 25(OH) D in nmol/L (SD)	Summary of results (ITT analysis) of primary and prespecified secondary objectives
VIDA (47)	5018 subjects (66 years)	200 000 IU D3 start followed by 100 000 IU/months for a median of 3.6 years	63 (24)	Vitamin D did not reduce the main endpoints of incident CVD, acute respiratory infections, nonvertebral fractures, falls, and all cancer.
TIPS-3 (48)	5713 subjects (63.9 years)	60 000 IU/months D3 for a median of 4.6 years		Vitamin D did not reduce fracture, the primary outcome, and the composite of CV death, myocardial infarction stroke, cancer, fracture, or fall, the secondary outcome. Higher mortality, a prespecified outcome was observed in the vitamin D group ($P = 0.03$).
Finnish Vitamin D trial (49)	2495 persons (43% women) age 68.2 years in mean	Placebo, 1600 IU/day, or 3200 IU/d for a mean follow-up of 4.3 years	74.8 (18.2) nmol/L in a subcohort of 600 participants	In the ITT analysis, vitamin D did not significantly reduce the primary endpoints of total invasive cancer incidence and of major CVD events. Vitamin D did not reduce the secondary endpoints of all-cause mortality.

CVD, cardiovascular disease; BP, blood pressure.

analyses, particularly when derived from "Individual Patient Data" (IPD) meta-analysis. However, it is crucial to recognize that these analyses can only be regarded as "hypothesis-generating" and should be confirmed by future RCTs that ought to employ more stringent inclusion criteria, potentially limiting participants to those who are deficient or severely deficient.

It is worth noting that conducting a RCT that directly compares the effects of vitamin D to a placebo in vitamin D-deficient patients is currently considered ethically challenging by some institutional review boards who could recommend supplementing the control group (which thus ceases to be a "placebo" group) with a small vitamin D dose. This approach may significantly limit the ability to confirm or refute the aforementioned results. Notably, beneficial effects of vitamin D have frequently been reported in subgroups of vitamin D-deficient subjects who received daily vitamin D supplementation (Table 3) (53, 55–59). In summary, there is evidence suggesting that vitamin D may contribute to the reduction of respiratory infections and cancer mortality (though not incidence). Additionally, with a lower level of supporting evidence, it may play a role in mitigating the progression from a prediabetic state to type 2 diabetes, as well as influencing pregnancy pathologies like preeclampsia or gestational diabetes, hypertension, and autoimmune diseases.

Definition of Vitamin D Status and Consequences for Supplementation

Current guidelines unanimously recommend measuring 25(OH)D for the assessment of patients' vitamin D status. Results are usually interpreted using universal cutoffs that are primarily based on bone health. However, recommended cutoffs vary not only between countries and regions, but also among experts within the same area (28), Nonetheless, the most commonly suggested 25(OH)D cutoff to define vitamin D sufficiency, particularly in the general population, is set at 50 nmol/L (20 ng/mL), while a value below 30 nmol/L (12 ng/mL) is considered indicative of severe deficiency (60).

Table 3. Some examples of recent RCTs or meta-analyses of RCTs of vitamin D supplementation where positive results, mainly (but not only) issued from secondary or subgroup analyses.

Outcome	Publication reference	Brief description of the study	Main results, including both ITT results and secondary or subgroups analyses
Respiratory infections	Martineau et al. (53)	IPD meta-analysis of 25 RCTs (10 933 subjects analyzed).	Vitamin D reduced the risk of ARI among all participants (adj OR 0.88, 95% CI: 0.81–0.96). In subgroup analysis, protective effects were seen in those receiving daily or weekly vitamin D (adj OR: 0.81, 0.72–0.91) but not in those receiving bolus doses (adj OR 0.97, 0.86–1.10). Among those receiving daily or weekly vitamin D, protective effects were stronger in those with baseline 25-hydroxyvitamin D levels <25 nmol/L (adj OR 0.30, 0.17 to 0.53) than in those with baseline 25-hydroxyvitamin D levels ≥25 nmol/L (adj OR 0.75, 0.60–0.95). In an updated meta-analysis of more studies (43 RCTs, 48 488 subjects for the primary outcome) by the same group (54), a slight but significant reduction in the risk of ARI was confirmed (OR: 0.92; 0.86–0.99). However, no significant beneficial effect was observed in vitamin D-deficient participants. Beneficial effect was observed in subgroup of patients who received daily vitamin D dosage (OR: 0.78; 0.65–0.94). Although impressively larger than their previous meta-analysis, the authors acknowledged as a limitation that their updated meta-analysis was mostly a trial-level, and not an IPD meta-analysis.
Cancer mortality	Kuznia et al. (55)	Meta-analysis of vitamin D supplementation in 14 RCTs (104 727 patients; 2015 cancer deaths) and IPD meta-analysis of 7 RCTs (94.068 patients).	Vitamin D supplementation with daily dosing (10 trials), but not infrequent large bolus (4 trials), reduced total cancer mortality (OR: 0.88; 0.78–0.98 for daily dosing vs OR: 1.07; 0.91–1.24 for intermittent dosing). Subgroup analyses suggested that patients aged 70 years or more or those in whom initiation of vitamin D supplementation was initiated before cancer diagnosis benefited more of vitamin D supplementation.

Continued

Table 3. (continued)

Outcome	Publication reference	Brief description of the study	Main results, including both ITT results and secondary or subgroups analyses
Pregnancy pathologies	Palaccios et al. (56)	Meta-analysis of supplementation with vitamin D vs placebo/no intervention (22 trials involving 3725 pregnant women).	Supplementation with vitamin D during pregnancy reduces the risk of pre-eclampsia (RR 0.48, 95% CI 0.30–0.7; 4 trials, 499 women) and gestational diabetes (RR 0.51, 95% CI 0.27–0.97; 4 trials, 446 women); and probably reduces the risk of having a baby with low birthweight (less than 2500 g) (RR 0.55, 95%CI 0.35–0.87; 5 trials, 697 women).
Type 2 diabetes (T2DM)	Zhang et al. (57) Dawson-Hughes et al. (58)	Meta-analysis of 8 trials with a total of 4896 subjects with prediabetes. Intratrial vitamin D exposure was calculated. HR for diabetes among participants who had intratrial 25(OH)D levels of <50, 75–99, 100–124, and ‡125 nmol/L were compared with those with levels of 50–74 nmol/L.	Vitamin D significantly reduced the risk of T2DM (RR] 0.89 [95% CI 0.80–0.99]). Benefit was found in nonobese subjects but not in obese subjects. The HR for diabetes among participants treated with vitamin D who maintained intratrial 25(OH)D levels of 100–124 and ≥125 nmol/L were 0.48 (0.29–0.80) and 0.29 (0.17–0.50), respectively, compared with those who maintained a level of 50–74 nmol/L. Daily vitamin D supplementation to maintain a serum 25(OH)D level ≥100 nmol/L is a promising approach to reducing the risk of diabetes in adults with prediabetes.
Autoimmune diseases	Hahn et al. (59)	A secondary analysis of the VITAL study (see Table A). Endpoint was all incident autoimmune diseases confirmed by medical record review: rheumatoid arthritis, polymyalgia rheumatica, autoimmune thyroid disease, psoriasis, and all others.	Vitamin D reduced autoimmune diseases by 22%. 123 participants in the Vitamin D group and 155 in the placebo group had a confirmed autoimmune disease (HR 0.78, 95% CI 0.610.9–9, $P = 0.05$).

BP, blood pressure; ITT, intent-to-treat; IPD, individual patient data; OR, odds ratio; RR, risk ratio; CI, confidence interval.

Many experts, as well as international guidelines (2, 50, 60), recommend that a 25(OH)D of 125–150 nmol/L (50–60 ng/mL) should not be exceeded.

However, the concept of universal cutoffs may be flawed as a result of several factors. First, the same 25(OH)D concentration can have differential effects in specific patient groups. For example, despite 40% lower 25(OH)D concentrations, Black individuals have comparable or even higher BMD and lower fracture risk than White individuals (61). Even if numerous factors other than vitamin D are determinants of bone health, this may suggest that comparable 25(OH)D concentrations are associated with variable metabolic activity. Second, the *Gc* gene, responsible for encoding VDBP, exhibits significant polymorphism. VDBP alleles have been observed to vary in their affinity for

Table 4. Clinical use of vitamin D, adapted from various position papers (28, 42, 50, 62, 63, 69–73).

Vitamin D supplementation	
Should we screen the population to detect vitamin D deficiency?	The answer is clearly no. 25(OH)D determination should only be used to monitor patients in whom a medical treatment by vitamin D has been envisaged by the clinician, to be sure that it reaches a defined target that can vary according to the clinical condition (69).
Which frequency of supplementation should be preferred?	Daily doses are preferred over large spaced doses since they are less likely to activate the *CYP24A1* pathway and FGF23 secretion, and are more physiological. Large spaced doses have been shown also to increase the risk of falls and other unexpected manifestations. If the patient prefers avoiding daily intakes, weekly dosages can be envisaged and monthly ones can also be accepted, but they should not be the rule (70).
Should everybody get a supplementation?	There is no evidence that a systematic supplementation of the whole population could contribute to a general healthcare improvement. Nevertheless, a systematic supplementation, especially in winter months, can be envisaged in some particular populations, such as black-skinned individuals living in northern latitudes, nursing home residents, or patients suffering from bone and muscle diseases (42).
What should be the daily considered supplementation dose?	A daily dose of 800–1000 IU is considered as safe and will increase 25(OH)D concentrations >50 nmol/L in most of the individuals. However, some individuals such as those having a bypass or suffering from malabsorption might benefit from higher dosages (42).
Should we use vitamin D3 or vitamin D2 for supplementation?	Both forms will increase the 25(OH)D concentrations in the same manner. However, several studies have shown that serum level of 25(OH)D will increase more effectively with vitamin D_3 than with D_2. Vitamin D_2 has shorter plasma half-life and a lower affinity for the VDBP, the hepatic vitamin D hydroxylase and the VDR (71).
Should we use cholecalciferol or calcifediol?	In some patients such as those with VDDR type 1b, liver failure, and those with all kinds of malabsorption, calcifediol may be an alternative to cholecalciferol for the treatment/prevention of VDDef (73)
Vitamin D metabolites determination	
Which method(s) should be used for 25(OH)D determination?	Thanks to the standardization efforts of the VDSP, most of the assays for 25(OH)D are providing generally consistent results, at least from a clinical point of view. However, in some patients, such as those undergoing hemodialysis or pregnant women, the serum matrix differs in its composition, which leads to so-called "matrix effects." In such patients, the results of most immunoassays are less accurate and can lead to significant divergences compared to techniques that bypass the matrix thanks to a physico-chemical treatment of the sample, such as LC–MS/MS (or even former radioimmunoassays). In such patients, these techniques may be preferred, even if such methods are not available everywhere, and they require highly trained skilled personnel (62).

Continued

Table 4. (continued)

Vitamin D metabolites determination	
When should 1,25(OH)$_2$D be measured?	1,25(OH)$_2$D circulates in the picomolar range, has a very short half-life and is tightly regulated. As such, it should never be used to evaluate vitamin D status of the patients and be requested for this indication, even in CKD or hemodialyzed patients. Its determination can be very useful is some rare clinical cases such as in the differential diagnosis of rickets, in the exploration of sarcoidosis (or, more largely, of any granulomatosis) or in the exploration of unexplained hypercalcemia (72).
When should 24,25(OH)2D be measured?	Besides the rare but dramatic idiopathic infantile hypercalcemia, 24,25(OH)2D should be measured in patients who present with hypercalcemia (and the secondary diseases associated with this condition) with a decreased PTH. Such patients also often present unexplained elevated 25(OH)D (and 1,25(OH)$_2$D) concentrations, even without taking any supplementation or sunbathing. If the 24,25(OH)$_2$D is very low (or undetectable) in association with an elevated 25(OH)D, a genetic test for *CYP24A1* mutation should be considered (28).
Should the VMR be measured instead of 25(OH)D?	Recent literature has shown that the VMR could be a better indicator than 25(OH)D alone of functional vitamin D deficiency. Nevertheless, the cost of measuring both metabolites compared to the cost of supplementation and the limited availability of methods able to reliably measure 24,25(OH)$_2$D prevents the wide use of the VMR in clinical practice (63).

25(OH)D. Consequently, both VDBP concentration and genotype play a role in influencing 25(OH)D levels, without necessarily impacting bioavailable vitamin D (6). Third, despite certification of many immunoassays by the VDSCP, the variable analytical performance of widely used immunoassays with bias of up to ±20% is another argument against the use of fixed cutoffs (62). Therefore, the choice of the method has a great impact on the classification of patients, especially when serum 25(OH)D ranges between 40–60 nmol/L.

The simultaneous measurement of 25(OH)D, 24,25(OH)$_2$D, and potentially other metabolites, allows a dynamic assessment of vitamin D metabolism that may overcome some of the limitations associated with the use of fixed 25(OH)D cutoffs for VDDef (63). Specifically, in the presence of critically low vitamin D, 25(OH)D catabolism is assumed to be down-regulated through a reduction of 24-hydroxylase activity, inducing low circulating 24,25(OH)$_2$D concentration and a low 24,25(OH)$_2$D/25(OH)D ratio (vitamin D metabolite ratio, VMR) (63, 64). Recent evidence suggests that the combined use of these markers can identify functionally relevant VDDef with accelerated bone metabolism and increased all-cause mortality (63). While such a dynamic assessment of vitamin D metabolism suggests superior diagnostic specificity for bone metabolism and all-cause mortality, data for other entities that have been linked to vitamin D deficiency, such as malignancies, infectious disease, autoimmune disease, hypertension, or cardiovascular disease are still missing. One limitation of the VMR is related to the current limit of quantification of the 24,25(OH)$_2$D measurement, which means that calculation of the VMR is impossible in individuals with undetectable 24,25(OH)$_2$D, a frequent condition in those with low 25(OH)D. This problem should be resolved in the near future with improvement of the methods of measurement of 24,25(OH)$_2$D.

A serum 25(OH)D concentration below 50 nmol/L should not be considered as a "magic" value suitable for everyone in the general population to define VDDef, as illustrated by the various published 25(OH)D thresholds associated with control of PTH concentration (65). It allows, however, on the one hand a pragmatic approach to the evaluation of the frequency of insufficient vitamin D stores in various areas or groups of patients, and, on the other, determination of the amount of vitamin D intake that enables vitamin D sufficiency in a maximum of individuals. Concerning the first point, a pooled analysis of 14 population studies from different European countries showed that

13.0%, and 40.4% of 55 844 individuals had average serum 25(OH)D concentrations <30 and <50 nmol/L over the course of the year, respectively (66). Interestingly, the 25(OH)D values of the 14 studies were obtained by using the same VDSCP certified LC–MS/MS method, meaning that the data were comparable across the various countries involved. There was considerable variation in prevalence of VDDef among countries (66), with a lower prevalence in the more northern latitude countries likely attributable to higher rates of vitamin D supplement and/or food fortification use. This may be extended to North American countries as evidenced by the relatively high mean 25(OH)D observed in the participants of recent mega-trials such as the VITAL (43) or D2D studies (45). Population studies allow identification of risk factors for VDDef, the most important being overweight/obesity, dark-skin, old age, lack of sunlight exposure, or chronic diseases. It is worth noting that season is important, especially at latitudes above 40° (66). For example, 25(OH)D < 30 nmol/L was reported in 17.7% and 8.3% of those sampled during the October–March and April–November periods, respectively.

Apart from rare exceptions, which need to be confirmed (58, 59), supplementation of vitamin D-sufficient patients and/or use of intermittent large bolus doses failed to show any benefit, while benefits where only observed in vitamin D-deficient subjects/patients who were given daily vitamin D (Table 3). This indicates that while data may not endorse vitamin D supplementation in individuals already vitamin D sufficient, hypovitaminosis D itself emerges as a standalone risk factor for various diseases, necessitating correction. This significance lies in the fact that, although modest, this risk factor is easily modifiable through simple, economical, and safe supplementation: unlike other common risk factors such as obesity, aging, tobacco/alcohol abuse, or chronic diseases, which are challenging or impossible to correct. If the aforementioned results, predominantly derived from secondary/post hoc analyses, are validated by forthcoming trials, it would underscore the importance of a population-wide approach to prevent VDDef in those susceptible to hypovitaminosis D. In such a scenario, a recent IPD meta-analysis should be taken into consideration. This meta-analysis, conducted on studies assessing the increase in serum 25(OH)D using certified LC–MS/MS methods with various daily vitamin D doses, yielded conclusions on the necessary daily vitamin D intake. The findings indicated that to sustain winter 25(OH)D levels above 50 nmol/L in 97.5% of apparently healthy White individuals (67) and dark-skinned individuals (68) living above a northern latitude of 40°, approximate daily vitamin D doses of 30 μg (1200 IU) and 67 μg (2680 IU) were required, respectively.

In summary, Table 4 addresses critical considerations related to vitamin D supplementation and the determination of vitamin D metabolites, offering concise insights that aim to inform and streamline decision-making processes in clinical practice and research.

Conclusion

Vitamin D is a subject that has sparked controversy like few other scientific topics. Its metabolism, which may seem simple at first glance, is actually very complex, and the new methods for measuring vitamin D metabolites using LC–MS/MS are gradually shedding light on a particularly well-regulated dynamic process. Despite more than a decade of effort in standardizing the measurement methods for 25(OH)D, which have yielded significant results, there is still some inaccuracy in measurements by ligand binding assays, especially in specific populations such as dialysis patients or pregnant women. However, not all methods using LC–MS/MS are considered "reference methods," and when used by laboratories with little experience in such complex methods, they may provide results that are just as inaccurate, or even worse, than immunoassays. Finally, recent mega-trials have significantly tempered the enthusiasm for vitamin D. Nevertheless, these studies are not beyond criticism, and beneficial effects of vitamin D demonstrated in secondary analyses restricted to vitamin D-deficient patients deserve further trials. The goal is not to switch from one extreme position to another but to keep a scientific and critical approach to this "old" molecule that, while definitely not a panacea, still deserves an appropriate place in nutrition and therapeutic management.

Nonstandard Abbreviations: 25(OH)D, 25-hydroxyvitamin D; VDSP, Vitamin D Standardization Program; VDSCP, International Vitamin D Standardization and Certification Program; CKD, chronic kidney disease; VDR, vitamin D receptor; RANKL, Receptor Activator of Nuclear factor Kappa-B Ligand; BMD, bone mineral density.

Human Genes: *CYP27B1*, cytochrome P450, family 27, subfamily B, polypeptide 1; *CYP24A1*, cytochrome P450, family 24, subfamily A, member 1; *CYP2R1,* cytochrome P450, family 2, subfamily R, polypeptide 1.

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