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Methods for determining vitamin D and its metabolites. Threshold value of bone manifestations

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Summary

The vitamin D endocrine system (VDES), through the mediation of calcitriol, regulates more than 3% of all the genes of the organism, with multiple effects both at the bone and extra-osseous level. The total concentration of circulating 25OHD, (expression of the sum of the concentrations of 25OHD3 and 25OHD2), constitutes a robust and reliable biomarker of the nutritional status of the VDES, used by health authorities and Scientific Societies in America and Europe. The current methods to measure VDES metabolites are classified into two types: physical detection methods, which include high pressure liquid chromatography (HPLC) and tandem mass spectrometry liquid chromatography (LC-MS/MS) and immunoassay methods. Even today, there is no uniform international consensus that defines vitamin D deficiency and sufficiency for bone health. People at risk of 25OHD deficiency should always be tested for deficiency or insufficiency and intensity. However, there is no reported benefit of general population screening in healthy people.

Endocrine system metabolism of vitamin D

Since the time of its discovery a century ago, there have been advances into what was erroneously called "vitamin" D. It is now acknowledged that it is not a vitamin, though we continue to use that term out of custom and tacit consensus. In fact, it is an endocrine system, the vitamin D endocrine system (VDES), similar to that of other steroid hormones. Cholecalciferol or "vitamin" D_3, is the threshold (physiological) nutrient of the system, synthesized from 7-dehydrocholesterol, which is produced, and found, from single-celled organisms to the skin of higher animals, including human. This route represents around 90% of the physiological contribution to the body, the rest is obtained through diet. There is another isoform, of nutritional or pharmacological contribution, ergocalciferol, "vitamin" D_2 or produced by ultraviolet irradiation of ergosterol contained in fungi, yeasts, etc...[1].

To be hormonally active, "vitamin" D_3 requires sequential metabolic activations through the action of the enzyme 25 hydroxylase (*CYP2R1* and others) mainly in the liver, not hormonally regulated, but subject to various influences, it becomes calcifediol (or 25OHD3) which has a long half-life of two to three weeks. 25OHD3 is a substrate for, through the action of the enzyme 1 alpha hydroxylase (*CYP27B1*), synthesizing 1,25 dihydroxyvitamin D_3 (calcitriol; 1,25(OH)2D3), a system hormone, in the kidney for its systemic endocrine action and in multiple cells and tissues of the organism for its local auto/paracrine action. 1,25(OH)2D3 has a short half-life and is hormonally regulated to maintain a constant concentration within a narrow range. 1,25(OH)2D stimulates 24 hydroxylase (*CYP24A1*) to form 24.25 hydroxyvitamin D_3 or 1.24.25 trihydroxyvitamin[1].

Calcitriol or 1,25(OH)2D3 binds with high affinity to its receptor (VDR), whereas 25OHD3, 24.25 hydroxyvitamin D_3 or 1.24.25 trihydroxyvitamin D and other metabolites have a much lower affinity. VDR belongs to the superfamily of steroid nuclear receptors that use the same heterodimeric partner (RXR) and co-activators or repressors, and bind to similar hexanucleotide sequences in DNA (elements that respond to repeated direct hormone) separated by three or four nucleotides, respectively[1]. The metabolites of VDES, which are poorly soluble in water, need to bind for their transport to their transporter protein "vitamin D-binding protein" (or DBP), with different degrees of affinity, higher for calcitriol and lower for calcifediol, 24.25 hydroxyvitamin D_3 or cholecalciferol or albumin[1].

The VDES, through the mediation of calcitriol, regulates more than 3% of all the genes of the organism, with multiple effects, interacting not only on bone health, and phospho-calcium homeostasis, but on multiple physiological processes in muscle, innate immune system and adaptive, cardiovascular system; controlling cell growth and differentiation, hormonal secretion, xenobiotic metabolism and numerous biological processes throughout the organism[1] (Figure 1).

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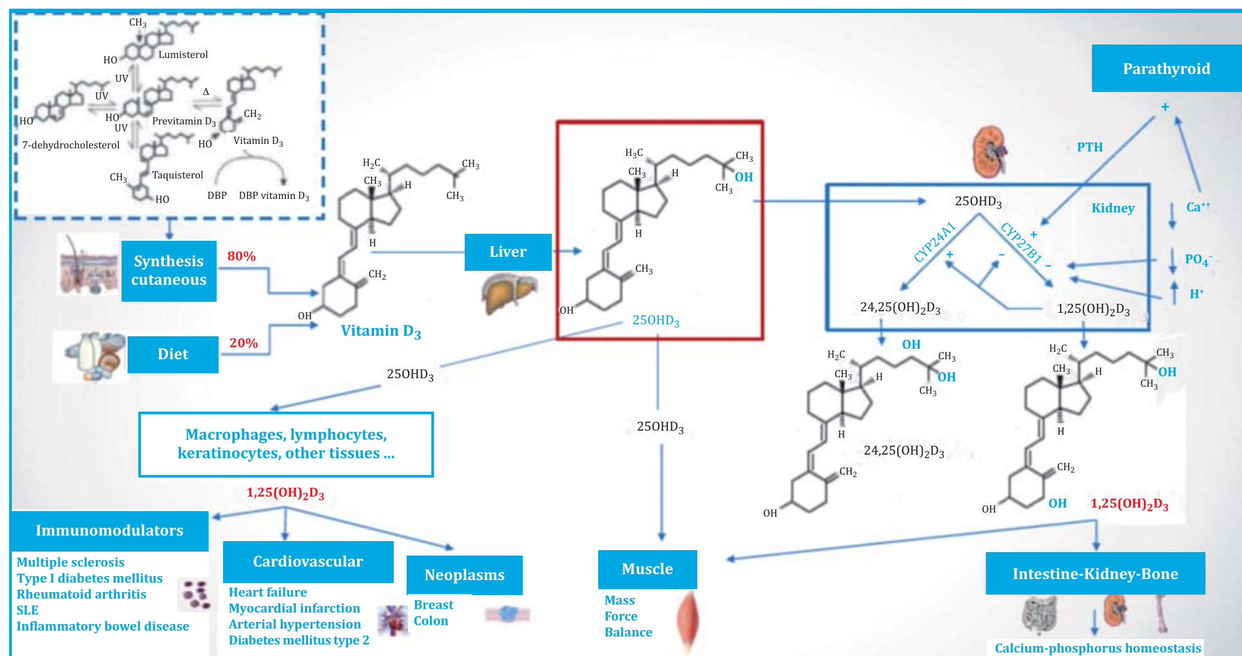
Rev Osteoporos Metab Miner. 2021;13(Supl 2):S4-10

Figure 1. Endocrine system of vitamin D



Therefore, nowadays, the functional deficiency of the system should be related not only to rickets or osteomalacia and osteoporosis, but also to a greater potential risk of suffering cardiovascular, autoimmune, diabetes, oncological, and infectious diseases, among others[1,2]. Currently, we know that "vitamin D" deficiency is very prevalent, even in developed countries or with great potential for acquiring it, due to sun exposure, or due to the ease of accessing supplementation, as is the case in Spain[3].

Therefore, the demand for measurement of vitamin D metabolites used for clinical diagnosis and research of the role of VDES in human health has increased significantly in the past twenty years[4].

Measurement of endocrine metabolites of vitamin D

Serum is the usual matrix used for the measurement of the metabolites of VDES. It has the advantage of not being contaminated with anticoagulants used to obtain plasma, such as heparin, EDTA, or citrate. The assays of the metabolites of the system are very sensitive to the interferences generated by these substances and an appropriate validation must be carried out when considering using plasma for the determinations[4].

Although vitamin D_3 is the threshold nutrient of the VDES, the direct measurement of circulating vitamin D_3 (and/or D_2), strictly speaking, does not constitute a good marker of its nutritional status. Immediately after its cutaneous synthesis or intestinal absorption, it rapidly disappears from the circulation. From then on, it reappears as 25OHD, intensely linked to DBP, which has a long half-life and a higher concentration and is also the essential substrate for the synthesis of 1,25(OH)2D3, the hormone of the system[1,4].

Therefore, the measurement of the total concentration of circulating 25OHD, (expression of the sum of the concentrations of 25OHD3 and 25OHD2), constitutes a robust and reliable biomarker of the nutritional status of the VDES, used by health authorities and scientific societies in America and Europe to establish the status of normality, the definition of deficiency of "vitamin" D and the degrees of insufficiency of the same, on which to establish values of dietary reference intake for "vitamin" D, as well as the control in the population of the deficiency, insufficiency or excess of "vitamin"[5-7]. It should be noted that, in Spain, where, with very specific exceptions, vitamin D_2 is not taken, when 25OHD results are given in practice, 25OHD3 levels are being indicated.

Sometimes in routine clinical practice the quantification of serum levels of 1,25(OH)2D3 is requested to evaluate the nutritional status of the VDES. This constitutes an erroneous and inappropriate practice. The 1,25(OH)2D measurement is not a reliable marker for that goal. By regulating its circulating levels, strictly, in an endocrine way, the organism tends to maintain its values within a very narrow range of normality (20-50 pg/mL, more than a thousand times lower than the serum concentration of 25OHD), even in situations of intense substrate deficiency (25OHD) essential for its synthesis. Therefore, it should never be used to assess the nutritional status of the VDES. The determination of 25OHD is the marker of the nutritional status of the system, or what we colloquially call vitamin D.

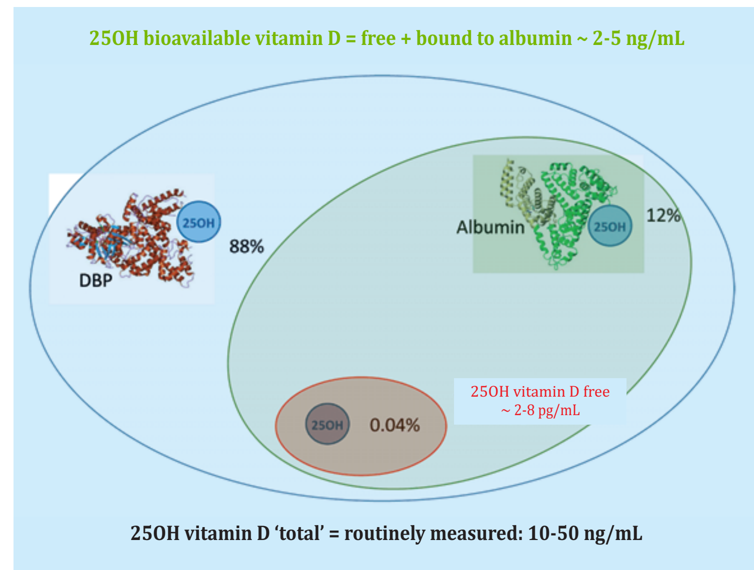
However, 1,25(OH)2D quantification may be useful as a second-level test in the evaluation of VDES, especially in patients with severe kidney disease[8], and allows us to identify a series of conditions, including 1α-hydroxylase deficiency or vitamin D-dependent rickets type 1, due to 1α-hydroxylase enzyme defect, vitamin D-dependent rickets type 2, or VDR defect, and in a series of granulomatous or lymphoproliferative diseases accompanied by hypercalcemia. Also in the diagnosis of hypo and pseudohypoparathyroidism. Measurement of 1,25(OH)2D also helps to distinguish between hypophosphatemic syndromes mediated and non-mediated by FGF23[9].

Quantification of the free 25OHD3 fraction, which represents about 0.04% of the total 25OHD concentration, is not routine clinical practice. The free and albumin-

bound fraction is called 25OHD bioavailable[10]. The set of three fractions is called total 25OHD, although the term "total" is often referred to in the literature as the sum of the 25OHD2 and 25OHD3 forms. Directly measured free 25OHD concentrations generally range from 1.2 to 7.9 pg/mL and are strongly correlated with total 25OHD concentrations and have been reported to account for 0.02% to 0.09% of total concentrations of 25OHD[10] (Figure 2).

In the so-called free hormone hypothesis, it is posited that only the free hormone crosses the cell membrane. To the extent that this is valid, it raises the question whether it is the free concentration that should be measured rather than the total 25OHD, especially in circumstances where the levels and/or affinities of the binding proteins are physiologically altered (eg, pregnancy), or pathophysiologically (liver disease, nephrotic syndrome, acute disease), or by genetic mutations of DBP[10,11].

Figure 2. Bioavailability of vitamin D



Pregnancy or taking contraceptives leads to an increase in DBP levels by approximately 50%, while, for example, liver failure and chronic kidney disease lead to a decrease in DBP concentration, also around 50%. In the case of a high concentration of binding proteins, the free 25OHD fraction is lower, and vice versa in the case of a low concentration of binding proteins. Under these conditions, the measurement of free 25OHD could be a better marker of vitamin D nutritional status than the classic measurement of total 25OHD[10,11]. Direct measurement of free 25OHD has been available since 2013 using a super sensitive ELISA[12]. The method has a detection limit of <3 pg/mL and a measurement range that covers 0.2-35 pg/mL. Repeatability and reproducibility are representative of ELISA technology.

The determination of 24.25(OH)2D has aroused little clinical interest and not much in research areas. This metabolite is formed by hydroxylation of 25OHD by the CYP24A1 enzyme of the cytochrome P450 family, and has long been considered a pure catabolite of the VDES catabolic pathway (Figure 1).

The determination of 24.25(OH)2D is useful in the diagnosis of idiopathic infantile hypercalcemia where it is very high (80-100 ng/mL) and has the potential utility in the identification of other diseases, alone or as part of a ratio of 24.25(OH)2D/25OHD. This proportion is less than 0.09 in patients with vitamin D insufficiency and/or deficiency (serum levels of 25OHD <20 ng/mL)[13]. Historically, the ratio of PTH to 25OHD has been used to estimate the nutritional adequacy of VDES, recently it has been proposed that the molar ratio of 25OHD/24.25(OH)2D has greater potential. The decrease in 25OHD catabolism can also be measured by a lower concentration of 24.25(OH)2D and is associated with an increased risk of secondary hyperparathyroidism and possibly death[14].

C3-epi-25OHvitamin D, also called the C3 epimer, is a stereoisomer that is differentiated by a single chiral center. The hydroxyl function at position 3 of the molecule is reversed while the other chiral centers remain unchanged. C3-epi-25OHD is formed through an epimerization pathway, parallel to the conventional metabolic pathway[15]. C3-epi-25OHD is more abundant in infants under one year of age and is less extensive in adolescents and adults. For many years, it was questioned whether C3-epi-25OHD was as important as its analog 25OHD in the biological activity of vitamin D in the body. However, several groups have reported varying concentrations and prevalences, hindering an assessment of their true importance[16,17].

Techniques for determining the metabolites of VDES

The first measurements of 25OHD date back to the early 1970s, using competitive protein binding assays.

Current methods for measuring the metabolites of VDES are basically classified into two types:

1) Physical detection methods, including high pressure liquid chromatography (HPLC) and tandem mass spectrometry liquid chromatography (LC-MS/MS);

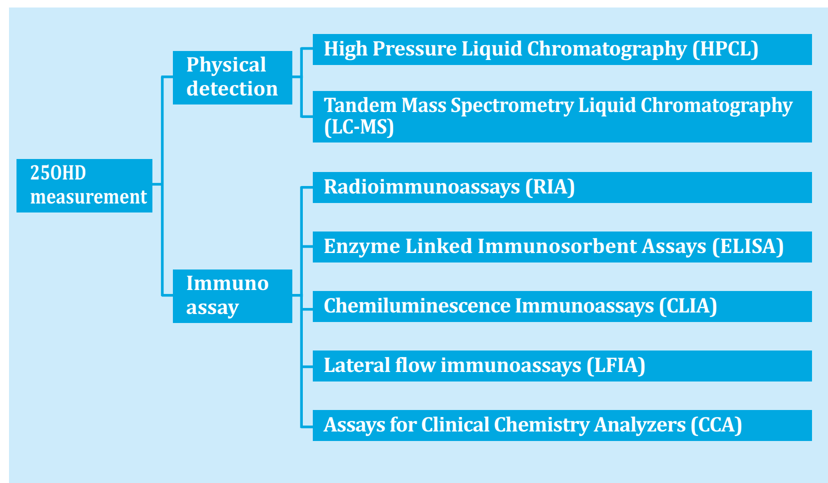
2) Immunoassay methods, encompassing radioimmunoassays (RIA), now obsolete, and enzyme-linked immunosorbent assays (ELISA), chemiluminescence immunoassays (CLIA), lateral flow immunoassays, and clinical chemistry analyzer assays (CCA).) (Figure 3).

Physical detection methods

High-pressure or high-performance liquid chromatography (HPLC) and LC-MS/MS are the physical detection methods used, each with their strengths and weaknesses[4]. In the former, ultraviolet (UV) detection thanks to its strong absorption at 264 nm is a powerful detection method for metabolites of VDES, but various metabolites of VDES exhibit similar UV patterns and need to be completely separated by the LC step to achieve success. be detected, be adequately quantified. Tandem mass spectrometry (LC-MS/MS) fragments molecules that have the same mass and similar affinities that make their chromatographic separation difficult, producing different fragmentation patterns for each individual compound that allow detection and quantification of metabolites separately. This is the case, among others, of 1,25(OH)2D and 24,25(OH)2D.

The benefits of using these measurement techniques of 25OHD as gold standard for determining the nutritional status of VDES are their high sensitivity (<1 ng/mL

Figure 3. Methods for measuring 25OH vitamin D



for LC-MS), high precision and excellent reproducibility profile (the coefficients of variation range from 2% to 7-8%). Thanks to its excellent results, the LC-MS is now recognized as the benchmark for 25OHD measurement.

HPLC and LC-MS also have drawbacks, mainly of a technical nature. Instrument cost and maintenance are also often an obstacle. Both require access to high-quality water, solvents, and chemicals, and serum or plasma needs to be cleaned before being tested[4].

The presence in serum of C3-epi-25OH remains a problem even for many HPLC and LC-MS methods[4,9]. Due to its similar UV pattern and identical monoisotopic mass and fragmentation patterns it cannot be adequately separated from 25OHD by UV or MS detection techniques. Therefore, the development of an LC protocol is required that completely separates the C3-epi metabolite from the desired 25OH vitamin D. Although technical solutions exist and are used by various laboratories, vitamin D C3-epi-25OH still interferes in many HPLC and LC-MS4 methods.

Competitive immunoassays for the measurement of 25OH vitamin D and other metabolites of the system

In competitive immunoassays, each metabolite, for example the 25OHD present in the sample, competes with a labeled 25OHD with a limited number of binding sites on an antibody. They differ in competitive 25OHD labeling and detection method. The RIA was based on radioactive iodine labeling 25OHD and used gamma-ray counters for detection. In the ELISA, the labeling is carried out with an enzyme, the detection is based on a colorimetric reaction and is quantified by measuring the absorbance in an ELISA reader. CLIA methods are also based on enzyme-labeled 25OHD, but detection is based on the emission of light by a specific substrate and is quantified using a photometer.

Technically, immunoassays require the release of 25OHD from its transporter proteins DBP and albumin, followed immediately by the binding of 25OHD by the antibody and its competition with the labeled 25OHD, being able to use polyclonal, monoclonal antibodies in immunoassays, even using different types of DBP.

After 25OH, vitamin D has been released from its binding proteins, different biological molecules can participate in immunoassays, including polyclonal antibodies, monoclonal antibodies and VDBP.

The specificity of the antibodies, that is, the cross-reactivity against the various metabolites of the VDES is one of the key aspects for the quantification of 25OHD and other metabolites in immunoassays because vitamin D3 coexists in serum, exceptionally in Spain 25(OH)D2; 24.25(OH)2D3/; 25.26(OH)2D and C3-epi-25OHD. Polyclonal antibodies generally lack the necessary specificity, limiting the quality of determinations.

For any type of method to be used in the assessment of vitamin D status, it should be an objective of state agencies, scientific societies and laboratories involved to participate in efficient standardization processes, such as DEQAS (Vitamin D External Quality Assessment Scheme), to obtain results with precision and accuracy, fundamental in research and assistance, that allow us to adequately define deficient and insufficient levels of vitamin D, in all age ranges, sex and for any health goal.

Currently the Vitamin D Standardization Program (VDSP) recognizes three Reference Measurement Procedures (RMP) (Ghent, NIST and CDC), which are all LC-MS methods[4].

Definitions of vitamin D deficiency and sufficiency for bone health

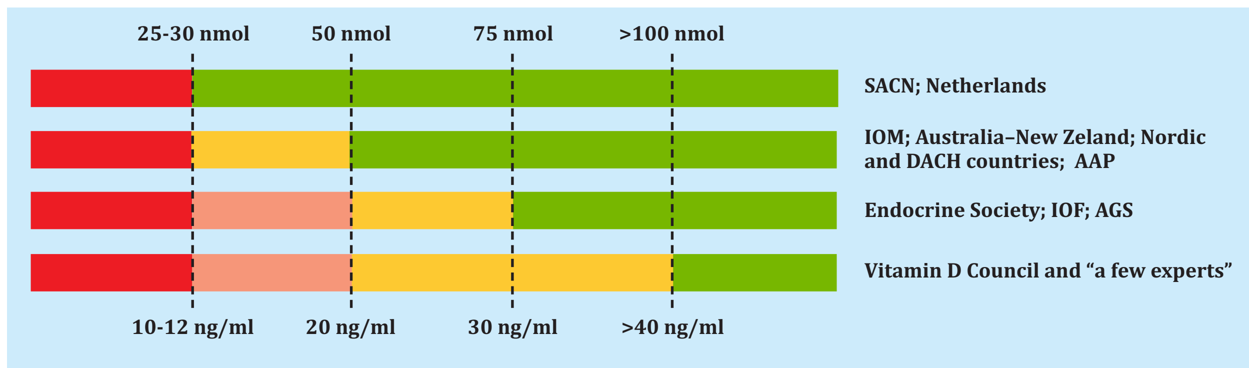
Even today, we do not have a uniform international consensus that defines vitamin D deficiency and sufficiency for bone health[18]. A serious problem in obtaining these definitions is that they depend to a great extent on the precision of the quantification of blood levels of 25OHD and the discussions on this aspect continue, without constructively advancing[19].

In 1998, the United Kingdom was the first country to adopt a serum cut-off point of 25OHD to define deficient status, Committee on Medical Aspects of Food and Nutrition Policy (COMA). More recently, the UK Scientific Advisory Committee on Nutrition (SACN), assessing musculoskeletal health goals (rickets, osteomalacia, falls, muscle strength and function), proposed that the risk of poor musculoskeletal health was higher with serum concentrations of 25OHD less than ~10-12 ng/ml.

On this basis, the SACN defined in 2016 that serum concentrations of 25OHD below 12 ng/ml were deficient for all age groups, and concluded that this threshold is the only one that has been shown to be beneficial for any related health outcomes. with 25OHD levels and that there was insufficient evidence to define that higher 25OHD levels were optimal for bone or even overall body health[20]. Although the Netherlands adopted these recommendations, this is not the opinion of the majority of societies or experts[18].

The US Institute of Medicine (IOM) (later renamed the National Academy of Medicine), selected in 2011 calcium absorption, bone mineral density (BMD) and rickets in children or osteomalacia in adults, to establish levels serum levels of 25OHD and facilitate the development of its recommendations for vitamin D intake, the so-called Dietary Reference Intakes (DRI)21. They defined a serum 25OHD concentration of 12 ng/ml (30 nmol/L) as the

Figure 4. Definitions of vitamin D deficiency and sufficiency for bone health



threshold below which a clinical vitamin D deficiency (severe deficiency) can occur, and established 25OHD levels of 12-20 ng/ml (30-50 nmol/l), to define an inadequate status, which represents an uncertain range that may or may not be sufficient for a given individual. It proposes a 25OHD concentration of 20 ng/mL (50 nmol/L) as the sufficiency threshold, in terms of bone health, for 97.5% of the population. It defines sufficiency for 25OHD levels between 20 and 30 ng/ml (50-75 nmol/l), and indicates that serum levels greater than 20 ng/ml of 25OHD would satisfy the physiological needs in vitamin D, without there being any added benefit. above 30 ng/ml (75 nmol/l), but warns that there could be potential harm with levels above 50 ng/ml (>125 nmol/l)[22]. Subsequently, the Nordic countries, the Swiss Federal Nutrition Commission and the European Food Safety Authority (EFSA) adopted the IOM 2011 guidelines for interpreting 25OHD concentrations[20]. Although it is important to note that the 20 ng/ml (50 nmol/L) suggested by the US National Academy of Medicine, were not proposed for the diagnosis of vitamin D deficiency, but were indicated to support the relationship between intake of vitamin D and the status of 25OHD, on which the dietary recommendations are established[21,22].

The US Endocrine Society (ES)[7] published another set of guidelines in 2011 which have become the focus of debate and controversy with the UK and IOM guidelines. The ES established through its Working Group on vitamin D in the United States a concentration of 25OHD to define deficiency as serum levels of 25OHD <20 ng/ml (50 nmol/l), insufficiency 21-29 ng/ml (52.5-72.5), sufficiency 30-100 ng/ml (75-250 nmol/l)) and possible damage> 100 ng/ml (> 250 nmol/l)), respectively. In summary, the ES has defined serum 25OHD levels of 20 ng/mL (50 nmol/L) as the deficiency threshold and 75 nmol/L (30 ng/mL) as the sufficiency threshold, for 97.5% of the population[7]. Several medical societies and non-governmental organizations have adopted the ES guidelines (Figure 4)[20].

The ES guidelines[7] were quite different from those proposed by the UK guidelines in 199120 or IOM in 2011[21,22] and thus sparked an intense debate, which has continued ever since. The Endocrine Society stated that its guidelines were designed for clinical practice and are directed primarily at patients with a wide variety of diseases and generally at increased risk of 25OHD deficiency, rather than the healthy population (the main group target of most government organizations). However, they do not offer consistent arguments to justify why the optimal vitamin D status in patients would be different from that of the healthy population[7]. To add to the confusion, in 2016, the SACN objected by stating that its guidelines were not for use in clinical practice, but rather public health guidelines for the healthy general population, not sick[20].

Both guidelines agree that the recommendations will require reconsideration in the future as additional standardization data for quantification of 25OHD levels and ongoing randomized trials become available. A minority of experts and grassroots organizations recommend even higher 25OHD levels (above 40 ng/ml (100 nmol/l)), based on the concept that "optimal vitamin D" status is best defined using the presumed Vitamin D status of early Homo sapiens living in equatorial Africa. These proposed target levels also imply that more than 90% of the current human population would be "vitamin D deficient or insufficient" and would require high-dose oral vitamin D supplementation. However, we must consider that it is very possible that the elevated serum concentrations of 25OHD found in primitive African tribes do not represent optimal serum concentrations, but rather the maximum tolerated in evolution to avoid chronic vitamin D toxicity[23].

In addition to the recommendations cited for the general population and with different conditions[24], the main randomized clinical trials of osteoactive anti-osteoporosis drugs used vitamin D and calcium supplements in both arms of these studies, indicating that vitamin supplements should be administered D/calcium to all patients receiving bisphosphonates or denosumab[25,26].

Quantification of 25OHD levels

People at risk of 25OHD deficiency should always be tested for deficiency, or insufficiency and intensity of these (Table 1). As in these patients, it is expected that the vitamin D replacement treatment will produce a rapid favorable health effect. From a public health perspective, determining 25OHD levels is absolutely cost-effective.

Although decreased serum levels of 25OHD in healthy people are frequently described worldwide, there is no evidence of a benefit in general population screening in healthy people for 25OHD deficiency, therefore screening studies for 25OHD are recommended. vitamin D deficiency in patients belonging to risk populations[7].

Therefore, measuring 25OHD is also recommended in osteoporotic patients with or without a history of non-traumatic fractures (particularly before starting treatment with osteoactive, anticatabolic or anabolic agents[25,26]), in the processes listed in table 1[27]. Patients with the clinical

Table 1. Cases that must always be analyzed to detect 25OHD deficiency

• Rickets-osteomalacia	• Drugs:
• Osteoporosis	- Anticonvulsants
• Chronic kidney disease	- Glucocorticoids
• Liver failure	- Anti-HIV
• Hyperparathyroidism	- Antifungals, eg. Ketoconazole
• Malabsorption syndromes:	- Cholestyramine
- Cystic fibrosis	• Dark skin color
- Inflammatory bowel disease	• Pregnant and lactating women
- Crohn's disease	• Elderly with a history of falls
- Bariatric surgery	• Elderly with a history of fractures non-traumatic
- Radiation enteritis	• Obese (children and adults BMI: >30 kg/m^2)
	• Granulomas
	• Sarcoidosis
	• Tuberculosis
	• Histoplasmosis
	• Beriliosis
	• Lymphomas

diagnosis of rickets or osteomalacia; elderly with a history of falls; pregnant and lactating women, obese (children and adults); people with insufficient sun exposure; patients with malabsorption syndromes (congenital or acquired); maldigestion and undergoing bariatric surgery; chronic kidney disease, liver failure, cystic fibrosis; primary and or secondary hyperparathyroidism. It is also worthwhile to evaluate 25OHD levels in patients undergoing treatments that interfere with the metabolism of the vitamin D endocrine system (anticonvulsant drugs, glucocorticoids, AIDS drugs, antifungals and cholestyramine, among others) and in granulomas and some lymphomas (in these cases, it is also advisable to evaluate serum levels of 1,25(OH)2D).

Conflict of interest: The author declares that he has no conflicts of interest.

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