Editor's note: With this issue, we initiate a symposium on the epidemiology of vitamin D. Our Guest Editor, Dr. Cedric Garland, has overseen the project and will provide an overview and commentary at its completion. The current contribution deal with measurement and clinical interpretation of Vitamin D, and with some aspects of the relationship of Vitamin D and cancer. Subsequent issues will continue the discussion of cancer, with attention to the risks of vitamin D and perspectives on its use for cancer prevention.

Vitamin D Status: Measurement, Interpretation, and Clinical Application

MICHAEL F. HOLICK, PhD, MD

Vitamin D, the sunshine vitamin, is now recognized not only for its importance in promoting bone health in children and adults but also for other health benefits, including reducing the risk of chronic diseases such as autoimmune diseases, common cancer, and cardiovascular disease. Vitamin D made in the skin or ingested in the diet is biologically inert and requires 2 successive hydroxylations first in the liver on carbon 25 to form 25-hydroxyvitamin D [25(OH)D], and then in the kidney for a hydroxylation on carbon 1 to form the biologically active form of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)$_2$D]. With the identification of 25(OH)D and 1,25(OH)$_2$D, methods were developed to measure these metabolites in the circulation. Serum 25(OH)D is the barometer for vitamin D status. Serum 1,25(OH)$_2$D provides no information about vitamin D status and is often normal or even increased as the result of secondary hyperparathyroidism associated with vitamin D deficiency. Most experts agree that 25(OH)D of <20 ng/mL is considered to be vitamin D deficiency, whereas a 25(OH)D of 21-29 ng/mL is considered to be insufficient. The goal should be to maintain both children and adults at a level >30 ng/mL to take full advantage of all the health benefits that vitamin D provides.
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KEY WORDS: Vitamin D, 25-Hydroxyvitamin D, Sunlight, Parathyroid Hormone, Cancer.

INTRODUCTION

The association of sunlight and vitamin D for the promotion of bone health began with the industrialization of northern Europe. The lack of adequate sun exposure resulted in an epidemic of children with severe growth retardation and bony deformities that was commonly known as rickets (1). In 1919, Huldschinsky et al. (2) reported that exposure to ultraviolet radiation cured rickets. This was followed by Hess and Unger in 1921 (3), who observed that exposure to sunlight cured rickets.

In the 1930s, it was appreciated that ultraviolet irradiation of yeast extract was effective in producing an antirachitic substance known as vitamin D. This vitamin D was structurally identified and called vitamin D$_2$. Vitamin D$_3$ was identified by the irradiation of 7-dehydocholesterol. Because vitamin D$_2$ was inexpensive to produce, vitamin D$_2$ was used widely for the fortification of foods, including milk and bread in the United States and Europe. When 7-dehydrocholesterol was easily extracted from lanolin from sheep's wool, vitamin D$_3$ was inexpensively made and was used in food fortification and for supplements.

In the early 1950s, there was an outbreak of hypercalcemia, caused by what was thought to be the overfortification of milk with vitamin D and, as a result, most European countries forbid the fortification of milk and other dairy products with vitamin D. In the United States, milk and orange juice are fortified with vitamin D$_3$, whereas a majority of multivitamin supplements and pharmaceutical preparations contain vitamin D$_2$ (1, 4).

The appreciation that vitamin D (D represents either D$_2$ or D$_3$) required a hepatic hydroxylation on carbon 25 to produce 25-hydroxyvitamin D [25(OH)D] (Fig. 1) led to the development of a binding protein assay using the vitamin D binding protein (DBP) to measure circulating levels of 25(OH)D in the circulation (5–7). The identification of 1,25-hydroxyvitamin D as being the biologically active form

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Selected Abbreviations and Acronyms

25(OH)D = 25-hydroxyvitamin D
1,25(OH)$_2$D = 1,25 dihydroxyvitamin D
PTH = parathyroid hormone

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of vitamin D led to the development of a binding protein assay using the vitamin D receptor as the binder to measure circulating levels of 1,25(OH)$_2$D (8–10).

25(OH)D ASSAYS

The first assays for 25(OH)D used the competitive protein binding format with the vitamin D binding protein (DBP)

as the binder. The advantage of this assay was that DBP recognized 25(OH)D$_2$ equally as well as 25(OH)D$_3$. The major limitation of this assay was that the assay measured 25(OH)D in a serum sample that contained other vitamin D metabolites, including 24,25-dihydroxyvitamin D [24,25(OH)$_2$D], 25,26-dihydroxyvitamin D, and the 25,26-dihydroxyvitamin D-26, 23-lactone (11). However, typically these more polar metabolites of 25(OH)D circulate at less than 10–15% of the total concentration of 25(OH)D and were usually insignificant.

In 1985, a radioimmunoassay (RIA) was developed for 25(OH)D (11, 12). This assay (Diasorin@) recognized 25(OH)D$_2$ equally as well as 25(OH)D$_3$ (11, 12). However, like the DBP competitive protein binding assay, the RIA for
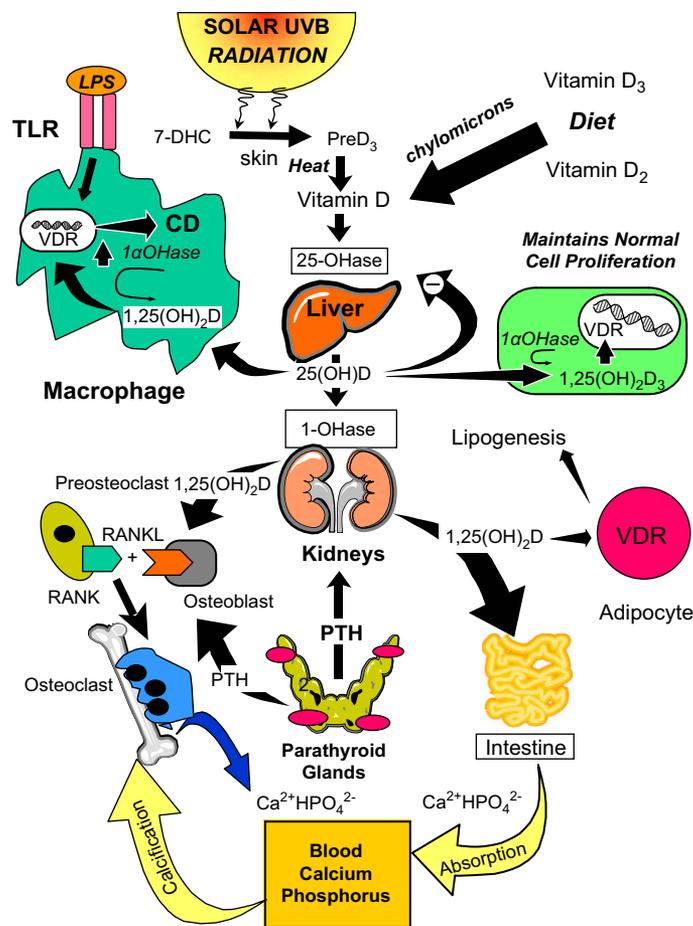
FIGURE 1. The metabolism and biologic function of vitamin D. During exposure to sunlight, 7-dihydrocholesterol (7-DHC) is photolyzed to previtamin D$_3$ (preD$_3$). Body heat converts preD$_3$ to vitamin D$_3$. Vitamin D$_2$ and vitamin D$_3$ in the diet and vitamin D made in the skin enters the circulation and either is stored in the body's fat adipocytes or enters the liver and is converted to 25-hydroxyvitamin D [25(OH)D]. For regulation of calcium metabolism, 25(OH)D is converted in the kidneys to 1,25-dihydroxyvitamin D [1,25(OH)$_2$D]. 1,25(OH)$_2$D interacts with its vitamin D receptor (VDR) in the small intestine and on osteoblasts to regulate calcium and phosphorus metabolism. 25(OH)D is metabolized in various tissues and cells for regulating cellular proliferation and differentiation as well as inducing cathelicidin D(CD) in macrophages. The induction of 1,25(OH)$_2$D in the macrophage is controlled by the 2/1 toll-like receptors (TLR) and its interaction with lipopolysaccharide (LPS). In addition, circulating concentrations of 1,25(OH)$_2$D may help increase insulin production and decrease renin production and alter adipocyte lipogenesis. Used with permission; copyright Michael F. Holick.

25(OH)D also recognized 24,25(OH)$_2$D and other polar metabolites to the same extent. Thus, both the DBP and the RIA assays typically overestimated 25(OH)D levels by approximately 10–20%. However, because the coefficient of variations (CV) for the intra and interassay variations were 8–15%, this was within the CV for the assay. More recently IDS developed an RIA which has a 100% specificity for 25(OH)D$_3$ and only 75% specificity for 25(OH)D$_2$.

To remove interfering vitamin D metabolites, simple preparative chromatography was developed to separate 25(OH)D from more polar metabolites that interfered with the assay. In the mid-1970s, high-performance liquid chromatography (HPLC) was applied to the 25(OH)D assay (11, 13). This assay included a lipid extraction of the serum followed by preparative chromatography and the 25(OH)D fraction was applied to HPLC and the UV absorption of 25(OH)D was used to measure its concentration. HPLC was considered to be the gold standard but was a very cumbersome assay, and, thus, was not routinely used by reference laboratories for clinical samples.

The advances in liquid chromatography tandem mass spectroscopy (LC-MS) were applied for the direct measurement of 25(OH)D in human serum. This assay quantitatively measured both 25(OH)D$_2$ and 25(OH)D$_3$ (14, 15).

1,25-DIHYDROXYVITAMIN D ASSAYS

Once 1,25(OH)$_2$D was identified, an assay using the chicken intestinal vitamin D receptor was developed as a competitive protein binding assay to measure circulating levels of 1, 25(OH)D (8). It was observed that the bovine thymus was an excellent source for the VDR and competitive binding protein assay for 1, 25(OH)$_2$D was developed using bovine VDR as the binding protein (9, 11). Radioimmunoassays were later developed to measure 1,25(OH)D. The Diasorin assay was reported to measure 1,25(OH)D$_3$ equally as well as 1,25(OH)D$_2$. However, the IDS assay recognized 1,25(OH)$_2$D$_3$ more effectively than 1,25(OH)$_2$D$_2$.

DETERMINATION OF VITAMIN D STATUS

25(OH)D is the only vitamin D metabolite that is used to determine whether a patient is vitamin D deficient, sufficient or intoxicated (1, 4, 16, 17). 25(OH)D is the major circulating form of vitamin D that has a half life of approximately 2–3 weeks. 25(OH)D is a summation of both vitamin D intake and vitamin D that is produced from sun exposure (1, 4).

Although 1,25(OH)D$_3$ is the biologically active form of vitamin D and, thus, would be thought to be the ideal measure for vitamin D status, it is not. There are several reasons for thi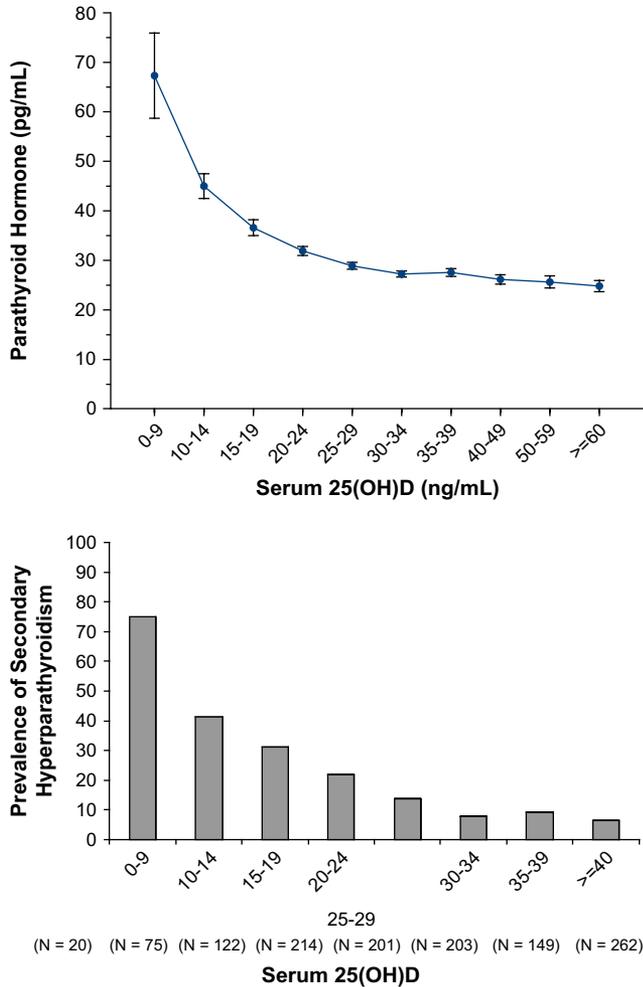s. The circulating half life of circulating 1,25(OH)D is only 4–6 hours. Circulating levels of 1,25(OH)D are a thousand fold less than 25(OH)D. As a patient becomes vitamin D deficient, there is a decrease in intestinal calcium absorption which lowers ionized calcium transiently. This signal is recognized by the calcium sensor in the parathyroid glands to increase the production and secretion of parathyroid hormone (PTH) (18). PTH regulates calcium metabolism by increasing tubular reabsorption of calcium in the kidney, increasing mobilization of calcium from the skeleton, and by increasing the renal production of 1,25(OH)D (1, 4, 16). Thus, as a patient becomes vitamin D insufficient and deficient, the increase in PTH levels result in normal or elevated levels of 1,25(OH)D. This makes the 1,25(OH)$_2$D assay useless as a measure of vitamin D status. The 1,25(OH)D assay, however, has been effectively used to help in the diagnosis of several inherited and acquired disorders in calcium metabolism as they relate to alteration in the renal or extra renal production of 1,25(OH)D (4, 16, 19).

DEFINITION OF VITAMIN D INSUFFICIENCY AND DEFICIENCY

There is no absolute consensus as to what a normal range for 25(OH)D should be. Part of the difficulty is how a normal range is determined, i.e., typically it is done by obtaining blood from several hundred volunteers and deeming them to be normal and to perform the measurement of the analyte and do a distribution with a mean $\pm$ 2SD as the normal range. However, since it is now recognized that 30–50% of both the European and US population are vitamin D insufficient or deficient, the previously reported normal ranges of 10-55 ng/ml are totally inadequate (1, 4, 16, 20–23). Chapuy et al. (24) reported that a dot plot of serum 25(OH)D levels as a function of PTH levels provided an insight as to what the serum 25(OH)D levels should be to be considered sufficient. They observed that the PTH levels began to plateau at their nadir when 25(OH)D levels were between 30 and 40 ng/mL. A similar observation was made by Thomas et al. (25) and Holick (14) (Fig. 2).
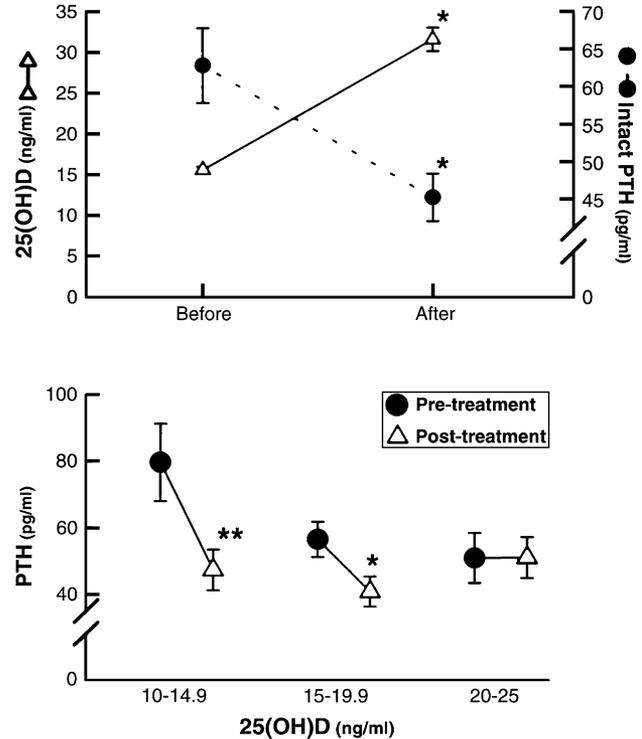
Malabanan et al. (26) did provocative testing by giving healthy adults who had a 25(OH)D of between 11 and 25 ng/mL, 50,000 IU of vitamin D once a week for 8 weeks. At the end of 8 weeks, it was observed that 25(OH)D levels increased on average by more than 100%. An analysis of the change in PTH levels for each of the subjects revealed that on average, the mean decrease on PTH levels declined by 55% in subjects who had 25(OH)D between 11 and 15 ng/mL and decreased by 35% for those with 25(OH)D levels of between 16 and 19 ng/mL. Those subjects who had 25(OH)D > 20 ng/mL had no significant change in their PTH level (Fig. 3). Thus, based on the provocative testing, it was suggested that vitamin D deficiency should be defined

FIGURE 3. A, Serum levels of 25(OH)D (-⊗-) and PTH (-o-) before and after therapy with 50,000 IU of vitamin D_2 and calcium supplementation once a week for 8 weeks. B, Serum levels of PTH levels in patients who had serum 25(OH)D levels of between 10 and 25 ng/mL and who were stratified in increments of 5 ng/mL before and after receiving 50,000 IU of vitamin D_2 and calcium supplementation for 8 weeks. Reproduced with permission (26).

FIGURE 2. A, Mean (±SE) serum PTH (picograms per milliliter) by serum 25(OH)D subgroups. Subject PTH concentrations (picograms per milliliter) relative to serum 25(OH)D concentrations sorted by subgroups delineated by predefined cutoffs for analyses of 25(OH)D inadequacy. Serum PTH values began to increase with 25(OH)D concentrations less than 29.8 ng/ml. B, Percent of subjects with secondary hyperparathyroidism by 25(OH)D level. The percent of subjects with secondary hyperparathyroidism (PTH > 40 pg/mL) sorted by subgroups with serum 25(OH)D concentrations delineated by predefined cutoffs for analyses of 25(OH)D inadequacy. Reproduced with permission (14).

as 25(OH)D >20 ng/mL. Heaney et al. (27) measured the efficiency of intestinal calcium absorption in women who had on average a 25(OH)D of 20 ng/mL and then in the same women who received 25(OH)D₃ to increase their blood level on average to 32 ng/mL. They reported a 45–65% increase in the efficiency of intestinal calcium transport when women were able to achieve a 25(OH)D of >32 ng/mL.

With all of this information collectively, most experts now agree that vitamin D deficiency should be defined as a 25(OH)D of <20 ng/mL. Vitamin D insufficiency is now

recognized as a 25(OH)D of 21–29 ng/ml. The preferred level for 25(OH)D is now recommended by many experts to be >30 ng/mL (1, 4, 28).

The upper limit of normal has also been questioned (4, 29, 30). An upper limit of 55 ng/mL seemed to be inadequate, especially since lifeguards who are exposed to a lot of sunlight typically have reported levels of 100–125 ng/mL (4, 16, 29). There has never been a reported case of vitamin D intoxication from sun exposure, and lifeguards have not been reported to be vitamin D intoxicated. On the basis of the literature, it appears that vitamin D intoxication does not occur until blood levels are >150–200 ng/mL (31, 32). Vitamin D intoxication is defined as a 25(OH)D >150 ng/m– that is associated with hypercalcemia, hypercalciuria and, often, hyperphosphatemia.

On the basis of all of this information, many of the reference laboratories are now using a normative range for 25(OH)D to be 20–100 ng/mL. However, several reference laboratories are also recognizing the recommendation by some experts that a preferred level of >30 ng/mL is most desirable.
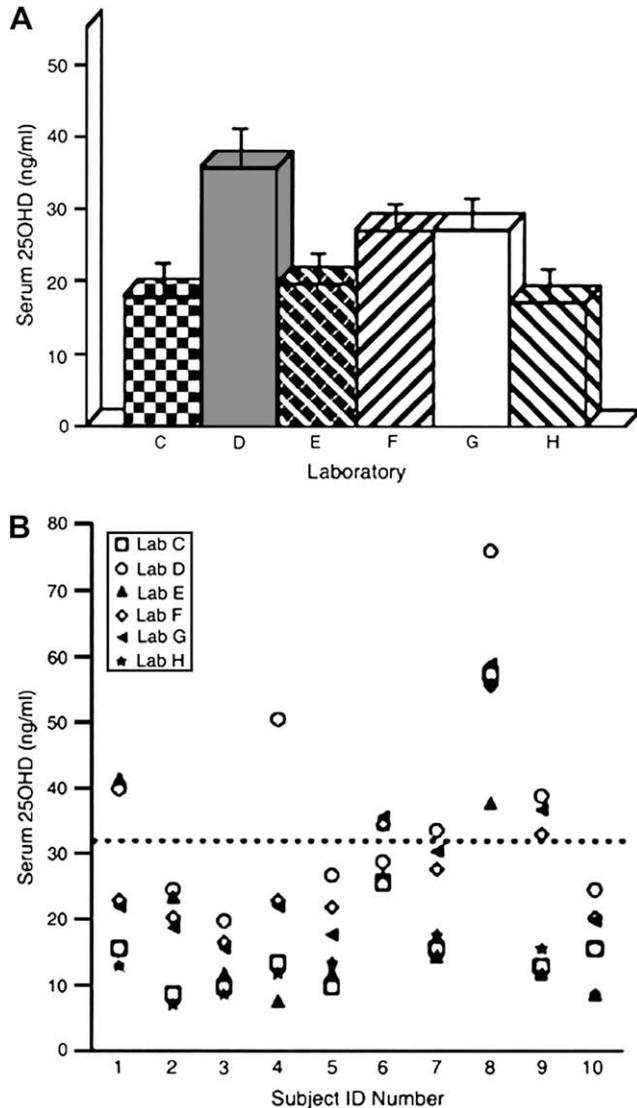
FIGURE 4. Laboratory personnel basal serum 25(OH)D concentration. A, In these 10 individuals, the mean serum 25(OH)D varied widely [from 17.1 (4.6) to 35.6 (5.2) ng/mL; $P < 0.005$] between laboratories (*error bars* represent SEM). B, Similarly, marked within-individual variation was observed in 25(OH)D measurement in different laboratories. Whether an individual has hypovitaminosis D (arbitrary threshold, 32 ng/mL shown as *dashed line*) depends on which laboratory was used. Note that laboratory H used HPLC and was considered to be the "gold standard" against which others were compared. Reproduced with permission (33).

At least two of the reference laboratories are now using LC-MS routinely to measure 25(OH)D. Because they are able to quantitatively measure $25(OH)D_2$ and $25(OH)D_3$, they report them out as individual levels. They also report the total 25(OH)D which is summation of $25(OH)D_2$ and $25(OH)D_3$. Physicians only need to be aware of the total 25(OH)D level.

CONCLUSION

The only way to determine whether a person is vitamin D deficient or sufficient is to measure their circulating level of 25(OH)D. There are a variety of assays used to measure 25(OH)D. The radioimmunoassays and competitive protein binding assays for 25(OH)D are useful in detecting vitamin D deficiency and sufficiency. However, these assays are fraught with technical difficulties, especially if they are not run routinely (Fig. 4) (33). Several reference laboratories have now switched to LC-MS ,which measures both $25(OH)D_2$ and $25(OH)D_3$ quantitatively. The total 25(OH)D, i.e., $25(OH)D_2$ plus $25(OH)D_3$, is what physicians need to be aware of for their patients. A level >30 ng/mL is now considered to be the preferred healthful level that all children and adults should maintain throughout the year.

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