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Review

Measurement of Vitamin D for Epidemiologic and Clinical Research: Shining Light on a Complex Decision

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Vitamin D is a fat-soluble vitamin that is synthesized in the skin with exposure to sunlight or is ingested from dietary supplements or food. There has been a dramatic increase in research on vitamin D, linking it with health outcomes as varied as reproductive function, infection, cardiovascular disease, and cancer. The study of vitamin D has generated much excitement, partly because there is an ideal intervention: Low levels may be common and can be remedied with widely available supplements. Determination of vitamin D status is complex and has advanced dramatically in the past 5 years. In this paper, we begin by describing important considerations for measurement of total 25-hydroxyvitamin D (25(OH)D), the biomarker traditionally assessed in epidemiologic studies. While 25(OH)D remains the most commonly measured biomarker, emerging evidence suggests that other related analytes may contribute to the characterization of an individual's vitamin D status (e.g., vitamin D-binding protein, bioavailable and free 25(OH)D, the C-3 epimer of 25(OH)D, 1,25-dihydroxyvitamin D, and 24,25-dihydroxyvitamin D). The measurement of these analytes is also complex, and there are important considerations for deciding whether their measurement is warranted in new research studies. Herein we discuss these issues and provide the reader with an up-to-date synthesis of research on vitamin D measurement options and considerations.

biomarkers; epimers; 25-hydroxyvitamin D; immunoassays; mass spectrometry; validity; vitamin D

Abbreviations: ARIC, Atherosclerosis Risk in Communities; ELISA, enzyme-linked immunosorbent assay; 3-epi 25(OH)D, 3-epi-25-hydroxyvitamin D; LC-MS/MS, liquid chromatography–tandem mass spectrometry; 25(OH)D, 25-hydroxyvitamin D; $25(OH)D_2$, 25-hydroxyvitamin D_2; $25(OH)D_3$, 25-hydroxyvitamin D_3; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; $24,25(OH)_2D$, 24,25-dihydroxyvitamin D; PTH, parathyroid hormone; VDBP, vitamin D-binding protein.

Vitamin D is a fat-soluble vitamin that is synthesized in the skin with exposure to sunlight. It can also be obtained from the diet, either from supplements or from food (such as dairy products) (Figure 1). The "active" form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), is the form that binds to the vitamin D receptor. However, the relatively short half-life of $1,25(OH)_2D$ (11–21 hours) (1) makes it a poor biomarker for assessing vitamin D status in epidemiologic studies. Instead, circulating total 25-hydroxyvitamin D (25(OH)D) concentration has traditionally been used to measure vitamin D status (2, 3).

Since 2000, there has been a dramatic increase in vitamin D research (Figure 2), linking it with health outcomes as varied as reproductive function, infection, cardiovascular disease, and cancer (4). The study of vitamin D has generated excitement, in part because there is an ideal intervention: Low levels may be common (5–9), they are amenable to correction through supplementation or sun exposure, and supplements are inexpensive and widely available. Moreover, the discovery that 25(OH)D is being converted to $1,25(OH)_2D$ within various tissues, including the brain, the uterus and placenta, and vascular smooth muscle cells (10–13), suggests that vitamin D is relevant to those tissues independently of the well-established calcium homeostasis pathway (5, 14).

In this paper, we describe the important considerations for measurement of total 25(OH)D, the most commonly assessed biomarker. Further, emerging evidence suggests that other vitamin D metabolites and related analytes may improve the characterization of an individual's vitamin D status, which is a composite of his/her ability to access and utilize vitamin D in physiological processes (15). The measurement of vitamin D-related analytes

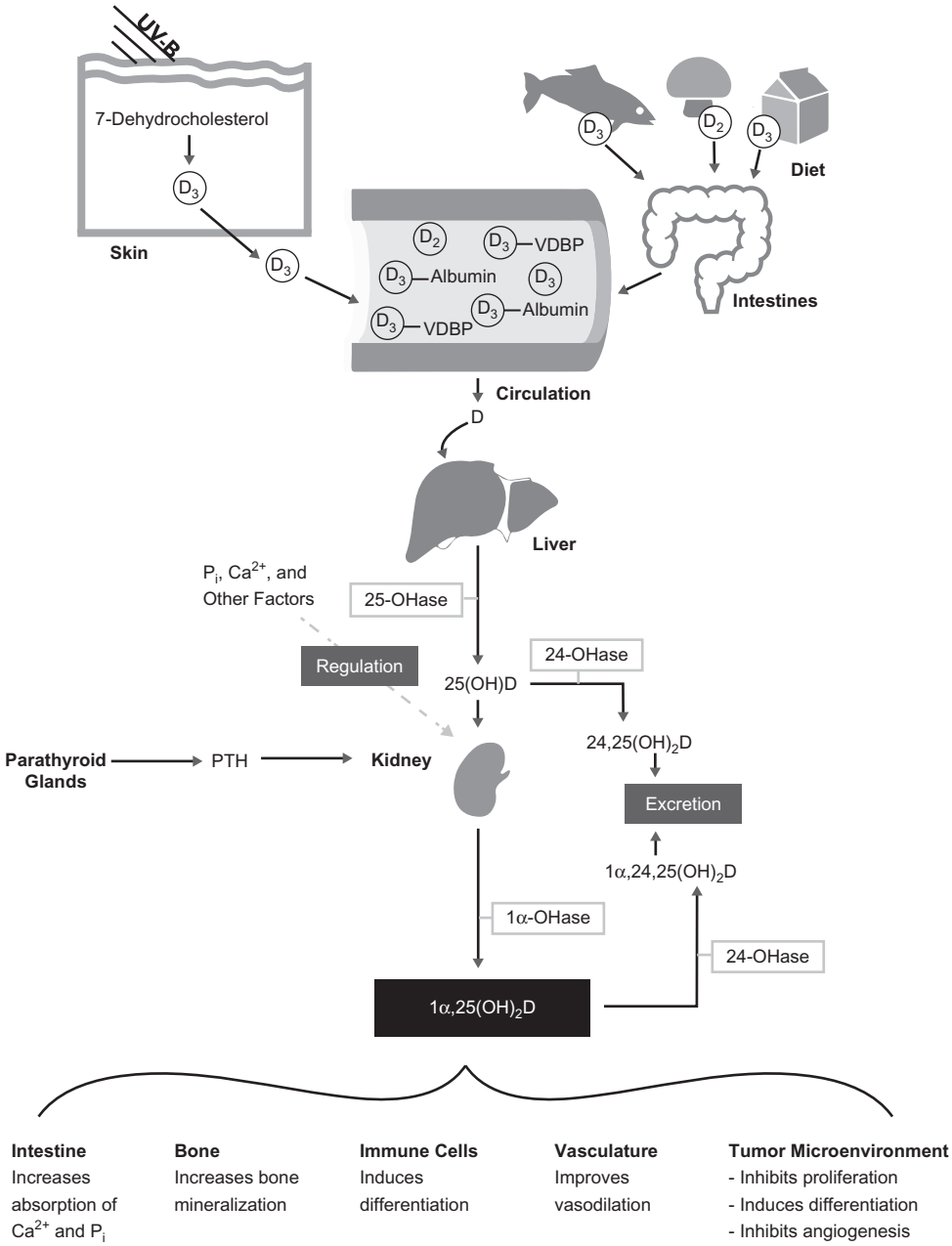

Figure 1. Vitamin D metabolism and functions. Vitamin D can be obtained through either sun exposure (top left) or diet (top right). When ultraviolet-B (UV-B) radiation strikes the skin, it stimulates the conversion of a cholesterol precursor into vitamin D_3 (D_3), which then enters the circulation. Vitamin D_3 can be absorbed from foods such as fatty fish and fortified milk products. Vitamin D_2 (D_2) is found in fungi, such as mushrooms. Vitamin D supplements can contain either vitamin D_2 or vitamin D_3. Most (85%–90%) of the vitamin D in blood is carried by vitamin D-binding protein (VDBP), although it can also bind to albumin (10%–15%) or be unbound or "free" (<1%). Both vitamin D_2 and vitamin D_3 follow the same metabolic pathway and are therefore represented with a "D" in the lower portion of the figure. Vitamin D is hydroxylated in the liver by the enzyme 25-hydroxyvitamin D 1-α-hydroxylase (25-OHase), to make 25-hydroxyvitamin D (25(OH)D), the metabolite most frequently used as a measure of vitamin D status. 25(OH)D can be further hydroxylated to 24,25-dihydroxyvitamin D ($24,25(OH)_2D$), which is then excreted, or to 1α,25-dihydroxyvitamin D ($1\alpha,25(OH)_2D$), which is also known as the active form of vitamin D; this form can bind the vitamin D receptor with the greatest affinity. Parathyroid hormone (PTH), calcium (Ca^{2+}), and phosphate (P_i) all act on the kidney to regulate calcium balance, partly through the conversion of 25(OH)D to its active form. The conversion of 25(OH)D to its active form can occur in a variety of tissues but is most well-known to occur in the kidney. The active form of vitamin D is responsible for several physiological changes across a variety of cell and tissue types. The effects of the active form are down-regulated by its conversion to 1α,24,25-dihydroxyvitamin D ($1\alpha,24,25(OH)_2D$), which is excreted from the body. (Adapted with permission from Macmillan Publishers Ltd.: *Nature Reviews Cancer* (119), copyright 2007).

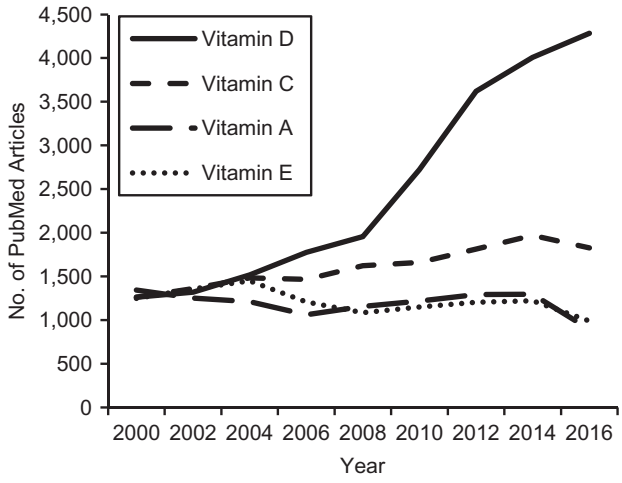

Figure 2. Numbers of publications related to vitamins A, C, D, and E in PubMed (National Library of Medicine, Bethesda, Maryland), 2000–2016.

is complex, and there are important issues to consider both for interpreting the published literature and for deciding whether their measurement is warranted in new research studies (Table 1). Herein we discuss these issues with the goal of providing the reader with an up-to-date synthesis of options and considerations for measuring vitamin D within the context of epidemiologic studies.

TOTAL 25(OH)D

Clinical recommendations for vitamin D sufficiency are based on total 25(OH)D (4). Similarly, most epidemiologic studies evaluating the association between vitamin D and health outcomes have focused on total 25(OH)D. Total 25(OH)D concentration is the sum of levels of the metabolites 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$). While these analytes are generally viewed as similar in terms of biological effect, they are derived from different sources, and there is some evidence suggesting that $25(OH)D_3$ has a longer half-life than $25(OH)D_2$ (15.1 days vs. 13.9 days) (16). In 2016, only 19% of the US population had detectable $25(OH)D_2$ levels (limit of detection, 2.05 nmol/L), with higher levels being seen among persons aged 60 years or more (17 nmol/L) in comparison with other age groups (<5 nmol/L) (17).

Total 25(OH)D can be measured in serum, plasma, whole blood, or blood spots. It has been shown to be extremely stable under a variety of laboratory preanalytical conditions (18–20) and with long-term storage (21–24). It can be measured using either liquid chromatography–tandem mass spectrometry (LC-MS/MS) or a ligand-binding assay (such as an immunoassay platform like a competitive enzyme-linked immunosorbent assay (ELISA) or a competitive chemiluminescent immunoassay, or competitive receptor-binding assays). In direct comparisons, immunoassay methods show bias (significant deviation from linearity) and increased variability relative to LC-MS/MS (25). In contrast to LC-MS/MS, immunoassays are not able to separately quantify the metabolites $25(OH)D_2$ and $25(OH)D_3$, and immunoassays are susceptible to cross-reactivity with 24,25-dihydroxyvitamin D ($24,25(OH)_2D$), a metabolite of 25(OH)D (26). LC-MS/MS is not without fault, however. For example, under typical chromatographic conditions, the epimeric form of $25(OH)D_3$ is not resolved from the native form (Figure 3). If a longer chromatographic separation or more selective column is not used, the epimer will be included in the measurement of total 25(OH)D concentration. (The relevance of the 25(OH)D epimer is discussed below.) Other shortcomings of LC-MS/MS, versus immunoassay, are that it requires more expensive equipment and expert staff, though reagent costs for LC-MS/MS are substantially lower.

Regardless of whether LC-MS/MS or a ligand-binding assay is used to measure 25(OH)D, it is vital to select a laboratory with appropriate quality controls in place. An indicator of outstanding laboratory performance is traceability to the National Institute of Standards and Technology (Gaithersburg, Maryland) (27), Ghent University (Ghent, Belgium) (28), and/or Centers for Disease Control and Prevention (Atlanta, Georgia) reference measurement procedures. Substantial variability and bias can exist in laboratory measurements of 25(OH)D (29). This led to the development of the Vitamin D Standardization Program, which was initiated by the National Institutes of Health's Office of Dietary Supplements. The Vitamin D Standardization Program aims to standardize vitamin D laboratory measurements such that results are accurate and comparable over time, location, and laboratory procedure (2, 3).

25(OH)D concentrations can be expressed in either ng/mL or nmol/L (1 ng/mL = 2.496 nmol/L). The Endocrine Society defines 25(OH)D sufficiency as 30–100 ng/mL, insufficiency as 21–29 ng/mL, and deficiency as <20 ng/mL (30). At the same time, the Institute of Medicine concluded that a 25(OH)D concentration of 16 ng/mL is adequate for bone health in 50% of the population, while 20 ng/mL is adequate for 97.5% of the population (4). Furthermore, as highlighted in a recent editorial (31), 97.5% of individuals require 25(OH)D concentrations *less* than 20 ng/mL. Although this is the intention of the Institute of Medicine report, in both research and clinical settings this cutpoint of 20 ng/mL is frequently misinterpreted as the minimum concentration for adequacy in any one individual, rather than the concentration at which 97.5% of the population has replete vitamin D stores (31). To additionally complicate the issue, questions have been raised about the appropriateness of these cutpoints for different racial/ethnic groups (9, 32–43).

An additional consideration for the measurement and interpretation of 25(OH)D concentrations is their inherent seasonality; typically a peak is observed in summer and a trough in winter, corresponding to usual variations in ultraviolet light exposure. For exposure-outcome relationships where the biological association is believed to be acute (e.g., 25(OH)D and sex hormone concentrations), the measured 25(OH)D concentration may be most appropriate. For exposure-outcome relationships where the influence of vitamin D on the outcome is believed to occur over a longer time frame (e.g., cancer and cardiovascular disease), estimating the annual average 25(OH)D concentration may be most appropriate (44). Several approaches have been used to account for seasonal variation in 25(OH)D concentrations. One approach is adjusting the exposure-outcome association for season or month of blood draw. Another approach is to

Table 1. Biomarkers of Vitamin D Metabolism and Status

Biomarker	Biospecimen	Measurement Options	Additional Considerations
Total 25(OH)D	Serum, plasma, whole blood, or blood spots	LC-MS/MS, immunoassay (such as an ELISA or chemiluminescent immunoassay)	Stable in specimens stored long-term Sum of the metabolites 25(OH)D_2 and 25(OH)D_3 Can be expressed in ng/mL or nmol/L (1 ng/mL = 2.496 nmol/L) In direct comparisons, immunoassay methods show bias and increased variability relative to LC-MS/MS Immunoassay shortcomings: variable reactivity toward 25(OH)D_2 and 25(OH)D_3 and unable to quantify each independently (however, the need for distinction is infrequent in epidemiologic investigations); susceptible to cross-reactivity with 24,25$(OH)_2$D; and susceptible to unidentified sample-specific matrix effects (interferences) LC-MS/MS shortcomings: unless using advanced platforms, the epimer will be measured as 25(OH)D; requires expensive equipment and expert staff
Free 25(OH)D	Serum, plasma	Immunoassay Calculated	May be most relevant in populations that show variation in VDBP concentrations (e.g., pregnant women, women using estrogens, or those with liver or kidney disease) Present at a very low concentration, making direct measurement challenging No gold standard measurement method An immunoassay exists to quantify free 25(OH)D, but it has not been rigorously validated Can be calculated using an equation which incorporates total 25(OH)D, VDBP, albumin, and (possibly) genotypic differences in VDBP; however, the validity of these equations has been questioned
Bioavailable 25(OH)D	Serum, plasma	Calculated	Sum of free and albumin-bound 25(OH)D Can be calculated using an equation which incorporates total 25(OH)D, VDBP, albumin, and (possibly) genotypic differences in VDBP; however, there is some discussion regarding the validity of these equations
Vitamin D-binding protein (VDBP)	Serum, plasma	Immunoassay LC-MS/MS	Monoclonal ELISA: The commercial assay that was most frequently used is no longer sold due to concerns about differential binding by genotype. Be skeptical of published literature using this assay. Polyclonal ELISAs: Are not biased by genotype. Several assays are now commercially available. It will be important to validate these against LC-MS/MS. LC-MS/MS: Unlikely to be biased by genotype. Has been rigorously validated; however, each laboratory must conduct its own validation processes. Presently being used by very few laboratories to measure VDBP.
3-epi-25(OH)D	Serum, plasma	LC-MS/MS	May be more relevant for research in infants and children; in adults, the absolute quantity of 3-epi-25(OH)D is small Under typical chromatographic conditions, the epimeric form of 25(OH)D_3 is not resolved from the native form Can be quantified via LS-MS/MS if a longer chromatographic separation or more selective column is used Cannot be detected with an immunoassay
1,25$(OH)_2$D	Serum, plasma	Immunoassay LC-MS/MS	Not an ideal epidemiologic biomarker given its short half-life, low concentration, and tight regulation by serum calcium, phosphate, and PTH Different concentrations by race/ethnicity Reduced concentration in kidney disease
24,25$(OH)_2D_3$	Serum, plasma	LC-MS/MS	Biomarker of 25(OH)D and 1,25$(OH)_2$D catabolism May serve as an indicator of tissue-level 1,25$(OH)_2$D activity Significantly reduced kidney disease

Abbreviations: ELISA, enzyme-linked immunosorbent assay; 3-epi-25(OH)D, 3-epi-25-hydroxyvitamin D; LC-MS/MS, liquid chromatography–tandem mass spectrometry; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; VDBP, vitamin D binding protein.

use the measured 25(OH)D concentrations to estimate the annual average 25(OH)D concentration using a cosinor model (44) or a residuals-based approach (45).

A final issue for 25(OH)D measurement is the suggestion that it should be accompanied by measurement of parathyroid hormone (PTH) level. However, the Institute of Medicine considers this approach controversial due to inconsistencies in the relationship between 25(OH)D and PTH, and because no clear threshold has been established for defining "sufficiency" using both 25(OH)D and PTH (4).

FREE AND BIOAVAILABLE 25(OH)D

It is possible that bioavailable or free 25(OH)D may better quantify vitamin D status than total 25(OH)D (15). For many

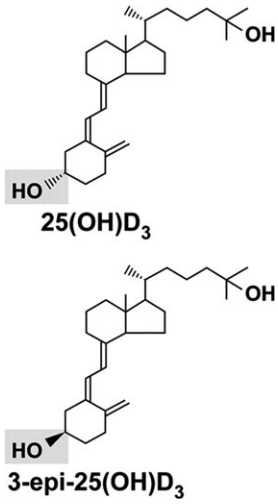

Figure 3. Chemical structure of 25-hydroxyvitamin D_3 ($25(OH)D_3$) and its epimeric form (3-epi-$25(OH)D_3$). (Reprinted from Lensmeyer et al. (96) by permission of The Endocrine Society).

hormones, such as testosterone, the free (unbound) hormone is considered the biologically relevant fraction (46, 47) because free hormones, particularly lipophilic steroid hormones, may passively diffuse across the cell membrane (48). However, for 25(OH)D the relevance of the free fraction is an open research question (49), the answer to which may depend on the outcome, or organ, or tissue of interest. Free 25(OH)D may be most relevant in populations that are expected to show variation in vitamin D-binding protein (VDBP) concentrations, such as pregnant women, women using estrogens, or persons with liver or kidney disease (50). On the other hand, when 25(OH)D is bound to VDBP, the entire complex can enter a cell by binding the transmembrane protein, megalin. Megalin is expressed in the kidney, but messenger RNA and/or protein expression has also been found in the parathyroid, placenta, epididymis, mammary epithelium, and thyroid (48). It is possible that extrarenal tissues that contain vitamin D receptors can also acquire 25(OH)D that is not bound to VDBP—in other words, free or bioavailable 25(OH)D (49, 51–53). In sum, it is possible that both free and bound 25(OH)D play a role in vitamin D signaling, depending on the organ system of interest.

Laboratory assays typically measure total 25(OH)D, which includes 25(OH)D that is bound to VDBP (which is the largest proportion—approximately 85%–90%), 25(OH)D that is bound to albumin (10%–15%), and 25(OH)D that is unbound (<1%) (54). Because 25(OH)D binds weakly to albumin ($K_a = 6 \times 10^5$ M^{-1} vs. $K_a = 7 \times 10^8$ M^{-1} for VDBP) (54, 55), it is thought that 25(OH)D dissociates from albumin during tissue perfusion (56). Thus, the albumin-bound and free fractions together are called "bioavailable" 25(OH)D.

Quantifying free 25(OH)D requires either an assay that targets the unbound 25(OH)D directly (54, 57, 58) or the measurement of 25(OH)D, VDBP, and albumin, which together provide the inputs necessary for calculating free and bioavailable 25(OH)D. Equations for the calculation of free and bioavailable 25(OH)D using VDBP and albumin have been modified from the equations used to calculate free testosterone (54, 59). There has been some discussion regarding 1) whether equations for testosterone are applicable to vitamin D (46) and 2) whether these equations should account for genotypic differences in VDBP (49).

To our knowledge, it is not possible to directly measure bioavailable 25(OH)D. Doing so would require a cell-based biological assay, which is technically challenging and hard to interpret. On the other hand, free 25(OH)D can be directly measured. In the past, this was challenging because free 25(OH)D is present at a very low concentration and the laboratory techniques were demanding and expensive (46). A recently developed immunoassay can directly measure free 25(OH)D (58); however, given its novelty, additional validation is needed.

To date, only a few publications have investigated the relationships between directly measured free 25(OH)D and calculated free 25(OH)D. In this literature (limited to studies that measured VDBP with either a polyclonal antibody or LC-MS/MS), the correlation between calculated free 25(OH)D and directly measured free 25(OH)D has been reported as strong ($r = 0.6$–0.8) (60–62), low ($r = 0.41$) (63), and nonsignificant (no point estimate reported) (64). While directly measured free 25(OH)D and calculated free 25(OH)D are correlated, the two methods do not consistently arrive at the same concentrations. In studies with both calculated and directly measured 25(OH)D, average calculated free 25(OH)D level has been higher (60–63), sometimes twice as high (60, 63), although Denburg et al. (65) reported that it was higher only among white participants and lower among black participants. In total, it is unclear whether calculated and directly measured free 25(OH)D are in fact measuring the same quantity. Further research is needed to explore the differences in calculated versus directly measured free 25(OH)D.

A logical question, given the importance of vitamin D for calcium balance, might be: Which correlates more strongly with markers of bone health—free 25(OH)D or total 25(OH)D? Some of the previous studies aimed at answering this question were flawed because their calculation of free 25(OH)D was based on a VDBP measure that was estimated with a monoclonal antibody (46), which has been shown to be biased (see further discussion below) (66). Thus, only more recent studies that incorporated a direct measurement of free 25(OH)D, or that quantified VDBP using either LC/MS-MS or a polyclonal immunoassay, can be leveraged to answer this question.

Of these studies, 6 (62, 63, 67–70) have reported correlations between PTH and both total 25(OH)D and free 25(OH)D (Table 2). Four of these (62, 67–69) reported correlations of similar magnitude between free 25(OH)D, total 25(OH)D, and PTH. Aloia et al. (63) reported that total 25(OH)D is more strongly correlated with PTH, and only among white women, not black women. The final study found a stronger correlation with free 25(OH)D than with total 25(OH)D (although the magnitude of the correlation between total 25(OH)D and PTH was similar to that seen in other studies) (70). In 1 additional study, Johnsen et al. (71) reported that among postmenopausal women, free, bioavailable, and total 25(OH)D were associated with PTH but only free and bioavailable 25(OH)D were correlated with bone mineral density. In a supplementation trial, change in intact PTH was associated with the change in directly measured free 25(OH)D but not total 25(OH)D

Table 2. Published Correlations Between Parathyroid Hormone and Either Free or Total 25-Hydroxyvitamin D[a]

First Author, Year (Reference No.)	Method of Free 25(OH)D Measurement	Correlation of PTH With Free 25(OH)D[b]		Correlation of PTH With Total 25(OH)D[b]		Which Is/Are More Strongly Correlated With PTH?
		Correlation Coefficient (*r*)	*P* Value	Correlation Coefficient (*r*)	*P* Value	
Aloia, 2015 (63)	Directly measured					Total 25(OH)D (free 25(OH)D is weakly correlated), and only among white women
White women		−0.16	>0.05	−0.33	<0.05	
Black women		0.01	>0.05	−0.002	>0.05	
Dastani, 2014 (68)	Calculated (54)	−0.26	$1.9e^{-33}$	−0.29	$1.3e^{-39}$	Both equally
Jemielita, 2016 (67)	Calculated (59)					Both, but total 25(OH)D is stronger in white women
White women		−0.12	0.14	−0.24	0.004	
Black women		−0.32	<0.0001	−0.30	0.0002	
Schwartz, 2014 (69)	Directly measured	−0.19	<0.02	−0.15	<0.05	Both
Schwartz, 2016 (70)	Directly measured	−0.28	0.02	−0.17	0.15	Free 25(OH)D (total 25(OH)D is weakly correlated)
Sollid, 2016 (62)	Directly measured	−0.17	<0.001	−0.21	<0.001	Both equally

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone.

[a] One additional study reported the associations between free, bioavailable, and total 25(OH)D with PTH as standardized β estimates, not correlations, and is reviewed in the text (71).

[b] Confidence intervals were not reported.

(72). Finally, a case-control study showed that osteoporotic men had lower free 25(OH)D but not lower total 25(OH)D, suggesting that free 25(OH)D may be a more useful measure of biological activity (73). Thus, based on the limited available data, there is no clear preference for free or total 25(OH)D when considering PTH or bone health. Further research is needed to explore the functional differences between free and total 25(OH)D and the racial/ethnic differences observed in these studies.

Similarly, the literature that compares free 25(OH)D and total 25(OH)D for other health endpoints is sparse. Only 1 study has examined free 25(OH)D in relation to preeclampsia, and those authors found no association (74). Free or bioavailable 25(OH)D may be more relevant for immune function. For example, one study has shown that dendritic cells can convert 25(OH)D to active vitamin D, but this process is hindered in the presence of VDBP (75). Further, monocytes cultured with 25(OH)D and increasing doses of VDBP showed lower production of cathelicidin, an antimicrobial protein (reviewed by Chun et al. (49)). Thus, for immune endpoints, free or bioavailable 25(OH)D may be the most relevant measure.

Calculated free and bioavailable 25(OH)D were also associated with development of end-stage renal disease in a nested case-control study of participants from the Atherosclerosis Risk in Communities (ARIC) cohort, but total 25(OH)D was unrelated (76). In a series of studies from a Finnish population (where the questionable monoclonal VDBP assay was used but the population was assumed to be mostly Caucasian), investigators have reported that the associations between 25(OH)D and several cancers (prostate cancer (77), renal cell carcinoma (78), colorectal cancer (79), and pancreatic cancer (80)) are modified by VDBP levels, suggesting that VDBP levels should be taken into account when examining associations between 25(OH)D and cancer. Finally, in a supplementation trial of vitamin D (in combination with statins; $n = 49$ men and women aged ≥60 years), at the end of the trial there was no correlation between total 25(OH)D and lipid levels; however, free 25(OH)D was inversely correlated with triglycerides, low-density lipoprotein cholesterol, and total cholesterol (81).

Interest in studying free and bioavailable 25(OH)D is recent, and studies examining the associations of these measures with health outcomes are rare. The literature that does exist is intriguing and warrants further investigation of free or bioavailable 25(OH)D, especially among persons with health conditions which may influence VDBP (i.e., pregnancy) and across populations that are diverse in terms of age and racial/ethnic background.

VITAMIN D-BINDING PROTEIN

Until recently, VDBP was measured relatively infrequently. However, as noted above, data on VDBP concentrations are needed to calculate levels of free and bioavailable 25(OH)D. The desire to measure VDBP has grown in parallel with interest in calculating bioavailable and free 25(OH)D concentrations.

VDBP assays have recently been the source of controversy. Within the past decade, numerous studies have used a monoclonal 2-site sandwich immunoassay in a 96-well ELISA format, which has been shown to be flawed. Two independent research groups have documented that the monoclonal ELISA binds differentially by VDBP genotype (50, 61, 66, 82); specifically, it improperly binds to the Gc1F/Gc1F haplotype of the gc-globulin (group-specific component) gene (*GC*) (i.e., rs7041 G and rs4588 A alleles), resulting in uniformly low values for

persons with this haplotype. Blacks are much more likely to have the Gc1F haplotype than are whites (e.g., in the community-based ARIC sample, it was present in 53.5% of blacks and 2.4% of whites (authors' unpublished data (P.L.L.))). When it is measured using an assay that is not biased by genotype, blacks and whites have similar concentrations of VDBP (50, 61, 63, 66, 82–84). To its credit, the manufacturer of the monoclonal ELISA (R&D Systems, Minneapolis, Minnesota) provided researchers (including two of us, P.L.L. and A.N.H.) with free assays for comparing the monoclonal ELISA with the gold-standard LC-MS/MS, and it has since removed the monoclonal ELISA from the market. However, in recent years the monoclonal ELISA was used regularly to measure VDBP, and numerous publications have reported findings based on this assay (either VDBP itself, or free and bioavailable 25(OH)D calculated using VDBP measured by this assay). This literature should be viewed with extreme skepticism. An open question is whether results from the monoclonal ELISA among populations where the Gc1F haplotype is infrequent can be considered valid.

Alternate ways to measure VDBP include polyclonal immunoassays and LC-MS/MS, both of which are less likely to be biased by genotype. The LC-MS/MS approach was developed recently, and it has been shown to possess all of the fundamental characteristics of a valid assay—namely precision, linearity, specificity, and stability (85). However, few laboratories are presently measuring VDBP by means of LC-MS/MS, and as noted above, LC-MS/MS requires expensive equipment and expert staff. Several polyclonal immunoassays for measuring VDBP have become commercially available. It will be important to rigorously validate these assays against the gold-standard LC-MS/MS.

3-EPI-25-HYDROXYVITAMIN D

All vitamin D metabolites can be epimerized (86). The C-3 epimer of vitamin D, 3-epi-25-hydroxyvitamin D (3-epi-25(OH)D or 3-epi), is formed when the hydroxyl group at position C-3 of the A-ring is converted from an α orientation to a β orientation (Figure 3) (86). When measured by mass spectrometry, because of their identical molar weight, the epimeric forms are included with the nonepimeric forms in the total 25(OH)D level, unless care is used to chromatographically separate them. When measured by immunoassay, the epimeric form of 25(OH)D is not included in the total 25(OH)D level, as the antibody does not recognize the epimeric form.

The biological significance of 3-epi-25(OH)D is unclear (17, 86, 87). The epimer is detectable across several diverse populations (17, 45, 87–101; also partially reviewed by Bailey et al. (86)), although levels of it appear to be higher in infants and children (17, 87, 88, 91–95, 99). 3-epi-25(OH)D is positively correlated with 25(OH)D (17, 45, 87, 92, 96–106). Most studies report correlations between 0.6 and 0.8 (17, 88, 92, 98–100, 102–106), but lower values (as low as 0.2 (87)) have also been reported, and Lutsey et al. (45) reported a slightly lower coefficient among blacks ($r = 0.36$) than among whites ($r = 0.54$). Study results are divided as to whether the increase of 3-epi with 25(OH)D is linear (87, 92, 98, 103) or whether the proportion of 3-epi increases with increasing 25(OH)D (96, 100, 104, 105). While levels of 3-epi and total 25(OH)D are correlated, the absolute quantity of 3-epi-25(OH)D in adults is small: a median concentration of 3.4 nmol/L in the National Health and Nutrition Examination Surveys (17). Similarly, in a recent review of 8 studies, Bailey et al. (86) reported a weighted mean 3-epi-25(OH)D concentration of 4.3 nmol/L for adults (1.7 ng/mL); however, for infants, the mean was 18.2 nmol/L (7.3 ng/mL), and the corresponding percentage of total 25(OH)D that was 3-epi was 21.

Although it is clear that 3-epi can be detected in a variety of populations, the source of the epimer is still unknown. For the most part, 3-epi-25(OH)D has not been found in vitamin D supplements (91, 105, 107) (Baily et al. (88) did find 3-epi-25(OH)D in a supplement). In randomized trials of vitamin D in pregnant women (88, 106) and preterm infants (107), levels of 3-epi increase with vitamin D treatment, which suggests endogenous formation. There is some evidence that vitamin D treatment increases levels of 3-epi-25(OH)D in lactating women and nonpregnant women (106), but the magnitude of the increase is small. Some have suggested that 3-epi-25(OH)D levels are higher in infants due to liver immaturity; however, this may not be the case. As Bailey et al. described, 1) in vitro studies have found that epimerization is tissue-specific, 2) epimers are absent in the adult liver disease population, and 3) cytochrome P-450 enzymes are not involved in epimerization (86). This was supported by a recent study that found, in a hypervitaminosis D population, that 3-epi-25(OH)D was not associated with liver function (105). The developmental advantages of increased 3-epi-25(OH)D in infants should be further explored (107).

The epimeric forms of vitamin D show reduced binding of the vitamin D receptor and VDBP compared with the nonepimeric forms; however, the epimeric forms are similar to the nonepimeric forms in suppressing PTH secretion (86). Given these inconsistencies, the decision to separate 3-epi-25(OH)D from its nonepimeric counterparts will depend on the biological pathways of interest. For example, for infants in whom bone growth may be of primary concern, it has been suggested that clinical decision-making should be based on a 25(OH)D measure that excludes 3-epi (86, 108, 109). Similarly, some authors suggest that the presence of 3-epi may conceal low 25(OH)D levels that may be relevant for clinical decisions or public health (92, 110), while other authors find very little difference in the classification of vitamin D deficiency when the epimer is included versus when it is excluded (45, 90, 111).

Few studies have examined the association of 3-epi-25(OH)D with health endpoints. In adults, there is some evidence that 3-epi-25(OH)D is inversely related to serum low-density lipoprotein cholesterol (while 25(OH)D is positively correlated) and positively associated with triglycerides (but negatively associated with 25(OH)D) (112). In a study of hypervitaminosis D patients, 3-epi was unrelated to C-reactive protein, calcium, liver and renal function, creatinine, and PTH (105). Finally, in preterm infants, 3-epi was correlated with gestational age at birth and negatively correlated with head circumference; this was true for both the absolute level of 3-epi and the level relative to total 25(OH)D (107). Additionally, infants who were receiving breast milk had a higher percentage of 3-epi-25(OH)D than infants who received formula exclusively (107).

The investigation of 3-epi-25(OH)D is in its infancy, and research is needed to clarify its importance. In future studies,

researchers should aim to determine the origin and function of 3-epi-25(OH)D. At this time, not all laboratories that measure 25(OH)D can separate 3-epi-25(OH)D, and researchers interested in the epimer should be sure to clarify this with their intended laboratory. Epidemiologic studies will be important for describing the health outcomes associated with 3-epi-25(OH)D and the populations that exhibit higher levels. Stored samples from randomized trials in disparate populations can be used to determine whether vitamin D supplementation increases levels of 3-epi-25(OH)D relative to its nonepimeric form and, if so, at what dose. The epimeric form of vitamin D metabolites may be more relevant for some organ systems and not others, but this remains to be seen. Finally, etiological studies of 3-epi-25(OH)D with endpoints that have been inconsistently associated with total 25(OH)D may help to clarify those inconsistencies.

BIOMARKERS OF THE FUTURE?—1,25(OH)$_2$D AND 24,25(OH)$_2$D$_3$

The analytes with the greatest potential to expand our understanding of the relevance of vitamin D for human health are total 25(OH)D, VDBP, 3-epi-25(OH)D, and bioavailable and free 25(OH)D (calculated or directly measured). However, other biomarkers, such as 1,25(OH)$_2$D, 24,25(OH)$_2$D$_3$, and cholecalciferol, are also of potential interest. Of course, biomarker discovery is actively under way as well.

Historically, 1,25(OH)$_2$D has rarely been measured for assessing vitamin D status in research, as it is tightly regulated by serum calcium, phosphate, and PTH, has a relatively short half-life, and has been difficult to measure accurately (113). However, it is now possible to measure 1,25(OH)$_2$D using a "gold-standard" mass spectrometry approach, thereby overcoming the analytical challenges of the past. Given its tight regulation and the fact that 1,25(OH)$_2$D can be synthesized locally without circulating throughout the body, the added benefit of measuring 1,25(OH)$_2$D in research settings remains uncertain.

24,25(OH)$_2$D$_3$ is the most abundant product of 25(OH)D$_3$ catabolism and may serve as an indicator of functional tissue-level 1,25(OH)$_2$D activity (36, 114–117). It has been more strongly correlated with PTH than 25(OH)D or 1,25(OH)$_2$D (115, 117). Additionally, the ratio of 24,25(OH)$_2$D$_3$ to 25(OH)D has been hypothesized to be a novel potential biomarker of tissue-level 1,25(OH)$_2$D$_3$ deficiency (116, 117). Genetic analyses have identified polymorphisms in the cytochrome P-450, family 24, subfamily A, member 1, gene (*CYP24A1*) as an important predictor of serum/plasma PTH concentrations in large populations (118).

In conclusion, vitamin D signaling is complex, may involve VDBP and/or multiple metabolites, and may vary by tissue or organ system. Investigation of the relevance of vitamin D for human health is evolving and advancing as researchers attempt to better characterize this complex pathway. While many studies of vitamin D exist, the literature is fraught with inaccurate measurements. Moreover, recent technological advances have led to the ability to measure other vitamin D metabolites and related compounds, but there are, as of yet, few studies of these novel biomarkers. Given these unresolved issues, further vitamin D research is critical.

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