A Comparison of Measured and Calculated Free 25(OH) Vitamin D Levels in Clinical Populations

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Objective: Our goal was to compare direct quantitation of circulating free 25-hydroxyvitamin D (25(OH)D)levels to calculated free 25(OH)D levels and their relationships to intact PTH (iPTH), a biomarker of 25(OH)D effect, in humans with a range of clinical conditions.

Patients and Methods: Serum samples and clinical data were collected from 155 people: 111 without cirrhosis or pregnancy (comparison group), 24 cirrhotic patients with albumin <2.9 g/dL, and 20 pregnant women (second and third trimester). Total 25(OH)D (LC/MS/MS), free 25(OH)D (immunoassay), vitamin D binding protein (DBP) (immunoassay), albumin, and iPTH (immunoassay) were measured.

Results: Total 25(OH)D, DBP, and albumin were lowest in patients with cirrhosis, but measured free 25(OH)D was highest in this group ($P < .001$). DBP was highest in pregnant women ($P < .001$), but measured free 25(OH)D did not differ from the comparison group. Calculated free 25(OH)D was positively correlated with measured free 25(OH)D ($P < .0001$) but explained only 13% of the variability with calculated values higher than measured. African Americans had lower DBP than other ethnic populations within all clinical groups ($P < .03$), and differences between measured and calculated free 25(OH)D were greatest in African Americans ($P < .001$). Measured free 25(OH)D was correlated with total 25(OH)D ($P < .0001$; $r^2 = 0.51$), but calculated free 25(OH)D was not. Similarly, both measured free 25(OH)D ($P < .02$) and total 25(OH)D ($P < .05$) were correlated with iPTH, but calculated free 25(OH)D was not.

Conclusions: Calculated free 25(OH)D levels varied considerably from direct measurements of free 25(OH)D with discrepancies greatest in the data for African Americans. Differences in DBP binding affinity likely contributed to estimation errors between the races. Directly measured free 25(OH)D concentrations were related to iPTH, but calculated estimates were not. Current algorithms to calculate free 25(OH)D may not be accurate. Further evaluation of directly measured free 25(OH)D levels to determine its role in research and clinical management of patients is needed. (***J Clin Endocrinol Metab*** **99: 1631–1637, 2014)**

Vitamin D plays a role in the regulation of hundreds of genes involved in bone and mineral metabolism, the renin-angiotensin-aldosterone system, the immunologic system, the cardiovascular system, muscle metabolism and strength, cellular proliferation and differentiation, and survival of cells in disorders such as cancer (1–3). Recognition of the important role of vitamin D in health and disease has created the incentive to optimize vitamin D status in people. Adequate status is currently defined by total concentrations of serum 25-hydroxyvitamin vitamin

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Abbreviations: CV, coefficient of variation; CYP, cytochrome P450; DBP, vitamin D binding protein; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH_2)D, 1α,25-dihydroxyvitamin D; Gc, group-specific component; iPTH, intact PTH.

D (25(OH)D) (4–6) because this metabolite is hydroxylated to biologically active 1α,25-dihydroxyvitamin D ($1,25(OH_2)D$). Only small fractions of circulating $1,25(OH)_2D$ and 25(OH)D are in the unbound, or free, state. Nonetheless, the free-hormone hypothesis attributes biologic activity of hormones to the unbound or free fractions rather than protein bound concentrations in the circulation.

The potential benefit of measuring free or unbound concentrations of 25(OH)D and its metabolites has been suggested and is being evaluated (7–10). Until recently, determinations of serum free 25(OH)D involved laborious forms of equilibrium dialysis or indirect estimation based on measurement of vitamin D binding protein (DBP), albumin, and 25(OH)D (using 25(OH)D standards and assays that were variable) with equations derived from relatively small numbers of people (7) or modified from equations used for sex hormones (11). An assay that directly measures serum free 25(OH)D levels has been developed (Future Diagnostics BV). Our primary goal was to compare directly measured circulating free 25(OH)D concentrations with calculated free 25(OH)D levels in humans with and without conditions associated with alterations in albumin and DBPs. A secondary goal was to compare relationships between directly measured and calculated free 25(OH)D concentrations with a biomarker of vitamin D effect.

Subjects and Methods

Subjects

Stable subjects with cirrhosis and evidence of protein synthetic dysfunction defined as an albumin concentration <2.9 g/dL, women in their second and third trimester of pregnancy, and medically stable community-dwelling adults without evidence of liver disease or pregnancy provided informed consent and venous blood samples as part of research protocols approved by the University of California, San Francisco, Committee on Human Research.

Laboratory measurements

Total 25(OH)D measurements were determined by Clinical Laboratory Improvement Amendments-certified liquid chromatography tandem mass spectrometry at Mayo Clinical Laboratories, Rochester, Minnesota, with participation in National Institutes of Health Office of Dietary Supplements-funded National Institute of Standards and Technology (NIST) quality assurance program for analysis of 25(OH)D metabolites in human serum. The assay has ~10% coefficient of variation (CV) at levels ≥10 ng/mL. Internal standard is the NIST reference standard.

Albumin was measured at Heartland Assays, Inc by Bromcresol green (BCG) dye-binding procedure (albumin reagent set from Pointe Scientific, Inc).

Vitamin D binding protein was measured using the Quantikine Human Vitamin D Binding Protein Immunoassay kit (Catalog number DDBP0, R&D Systems, Inc) at Heartland Assays, Inc.

Free 25(OH)D levels

Calculated free 25(OH)D was determined using the method reported by Bikle et al (7) and by modification of the Vermuelen method for free testosterone estimation (11). The equations and affinity constants used in these calculations are provided in the Supplemental Appendix (published on The Endocrine Society's Journals Online website at http://jcem.endojournals.org). Direct measurement of free 25(OH)D concentrations was made by immunoassay (Future Diagnostics BV; http://www.future-diagnostics.nl/) In this assay, an anti-vitamin D antibody is coated on a microtiter plate. Serum samples and calibrators are pipetted into the wells of the microtiter plate. Free 25(OH)D is captured by the antibody during a first incubation. After washing, a biotin-labeled 25(OH)D analog is allowed to react with the unoccupied antibody binding sites in a second incubation. After a second washing step and incubation with a streptavidin-peroxidase conjugate, bound enzyme is quantitated using a colorimetric reaction. Intensity of the signal is inversely proportional to the level of free 25(OH)D in the sample. The assay was calibrated against a symmetric dialysis method. The calibrator range was 0.0 to 35.0 pg/mL. The limit of blank and limit of detection of the assay were determined according to the Clinical Laboratories and Standards Institute (CLSI) guideline EP17-A (12). The limit of blank from 60 replicates was 0.7 pg/mL. The limit of detection was determined from the pooled SD from 12 measurements of 5 low samples. The limit of detection was 1.9 pg/mL. Imprecision was determined on 3 samples during 20 consecutive days with 2 runs per day according to the CLSI EP05-A2 guidelines (13). At 23.6 pg/mL, the CV was 3% between runs and 1.1% between days with total imprecision CV of 5.6%; at 13.2 pg/mL, the between-run CV was 4.3% and between-day CV was 1% with a total imprecision CV of 6.9%. At 5.02 pg/mL, the between-run CV was 6.2% and between-day CV was 4.5% with a total imprecision CV of 15.7%. The antibody in this assay detects $25(OH)D_2$ at a level that is 60% that of $25(OH)D_3$.

Intact PTH (iPTH) was measured at San Francisco General Hospital Clinical Laboratories using the Siemens ADVIA Centaur assay, a 2-site sandwich immunoassay using direct chemiluminometric technology.

Statistical design and data analysis

Demographic and clinical characteristics and assay results of groups are presented as mean ± SD and compared using ANOVA. Linear regression was used to test for relationships between directly measured and calculated free 25(OH)D concentrations, between free 25(OH)D and total 25(OH)D, and between free 25(OH)D measurements and iPTH. Data were transformed before analysis if nonnormally distributed. Post hoc tests of fold change differences using different equilibrium association constants (ka) for DBP were made by unpaired *t* test, and $25(OH)D_2$ presence and dose effects on errors in fold change were tested by χ^2.

Results

Participants

A total of 151 subjects participated. Demographic characteristics by group (cirrhotic, pregnant, and compar-

ator group) and mean values for estimated glomerular filtration rate, albumin, calcium, DBP, 25(OH)D measurements, and iPTH are presented in Table 1. Patients with cirrhosis had a mean Model for End-Stage Liver Disease (MELD) score of 16 ± 3 with Child Pugh score B in one-third and C in two-thirds (14, 15). Half of the pregnant women were in the second trimester of pregnancy, and half were in the third. The comparison group of 107 included 28 healthy normal subjects under the age of 50 years and 79 medically stable adults. Of the 79 medically stable adults, 58 did not have diabetes, heart failure, liver disease, renal disease, or pregnancy; 56 had hypertension and 15 had diabetes. No sex hormones were taken by any of the women or men.

Laboratory measures

Total 25(OH)D, DBP, and albumin were lowest in patients with cirrhosis, but measured free 25(OH)D was highest in this group ($P < .001$, Table 1 and Figure 1). DBP was highest in pregnant women ($P < .001$), resulting in the lowest calculated free 25(OH)D, but measured free 25(OH)D did not differ from the entire comparison group (Table 1) or from nonpregnant, noncirrhotic women not taking estrogen or progesterone (4 ± 1.1 vs 3.5 ± 2.0 pg/mL, respectively). Total 25(OH)D levels also did not differ between the pregnant and comparison group.

Race significantly affected DBP levels ($P < .03$), with African Americans having the lowest levels (152 ± 107 μg/mL), followed by Asians (166 ± 83 μg/mL), and with Caucasians having the highest (301 ± 210 μg/mL). Excluding data from the cirrhotic and pregnancy groups, DBP remained highest in Caucasians and lowest in African Americans (259 ± 91 compared with 121 ± 71 μg/mL, $P < .01$) Despite DBP differences, directly measured free 25(OH)D was not affected by race when all subjects were considered ($P = .15$) or after exclusion of data from the cirrhotic subjects or pregnant women (3.6 ± 1.8 vs 3.5 ± 1.3 pg/mL in Caucasians vs African Americans, respectively, $P = .7$)

Calculated and measured free 25(OH)D

Calculations using a method described by Bikle et al (7) or one modified from the Vermuelen method (11) produced almost identical estimates ($y = -0.13 + 1.031x$; r^2

Table 1. Study Participant Characteristics[a]

	Comparison Group	Liver Disease	Pregnant Women	Normal Values
n	107	24	20	
Age, y	58 ± 16[a]	57.1 ± 7.9	30.7 ± 6.9[c]	
Gender (men/women), n	63/44	15/9	0/20[c]	
Race (African American, Asian, Caucasian, other), n	31/13/61/2	2/2/16/4	4/1/15/0	
Weight, kg	84.6 ± 20.9	93.2 ± 17.9	81.1 ± 20.9	
Height, cm	169 ± 9.1	173.4 ± 10.7	158.7 ± 6.7	
BMI, kg/m^2	29.6 ± 7.1	31.0 ± 5.2	32.1 ± 7.4	<25
Estimated glomerular filtration rate, mL/min/1.73 m^2[b]	82 ± 25	69.9 ± 29.3	81.6 ± 25.6	>60
Albumin, g/dL	4.2 ± 0.4	2.6 ± 0.5[c]	3.3 ± 0.3	3.6–5.1
Calcium, mg/dL	9.5 ± 0.4	8.4 ± 0.5[c]	9.1 ± 0.6	8.6–10.4
Calcium (corrected for albumin), g/dL	9.3 ± 0.4	9.5 ± 0.5	9.7 ± 0.5	8.6–10.4
DBP, μg/mL	218 ± 57	112.2 ± 64.0[c]	460.3 ± 229.5[c]	300–600
Total 25(OH)D, ng/mL	26.2 ± 11.4	14.0 ± 7.3[c]	26.7 ± 10.0	25–80[d]
Directly measured free 25(OH)D, pg/mL	4.5 ± 1.6	6.3 ± 3.2[c]	4.0 ± 1.1	
Calculated free 25(OH)D, pg/mL[e]	7.6 ± 4.2	9.6 ± 4.9	5.7 ± 3.7	
Calculated free 25(OH)D, pg/mL[f]	7.7 ± 4.3	9.8 ± 5.1	5.8 ± 3.8	
iPTH, pg/mL	75.7 ± 39.2	51.1 ± 63.4	21.8 ± 18.0[c]	14–72

Abbreviation: BMI, body mass index.

[a] Unless indicated otherwise, data are mean ± SD.

[b] by MDRD formula.

[c] Statistically significant group differences ($P < .001$, ANOVA for continuous variables or χ^2 for sex distribution).

[d] For Mayo Clinical Laboratories assay.

[e] Ref. 7.

[f] Modified from Ref. 11.

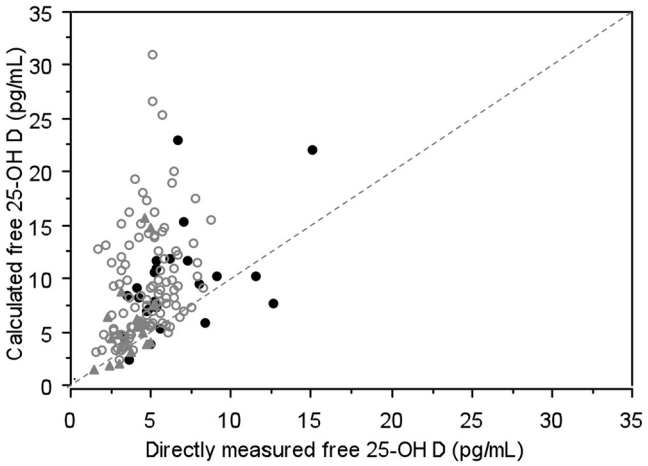

Figure 1. Directly measured free 25(OH)D concentrations are plotted on the x-axis, and calculated free 25(OH)D estimates (based on albumin, DBP levels, and published affinity constants) are plotted on the y-axis. Triangles represent data from pregnant women, closed circles represent data from liver failure patients, and open circles represent data from the comparison group. The dotted line represents the line of identity for 1:1 correlation. Although the measures were significantly related ($P < .0001$, $r^2 = 0.13$), calculated free 25(OH)D concentrations were higher than directly measured free 25(OH)D concentrations.

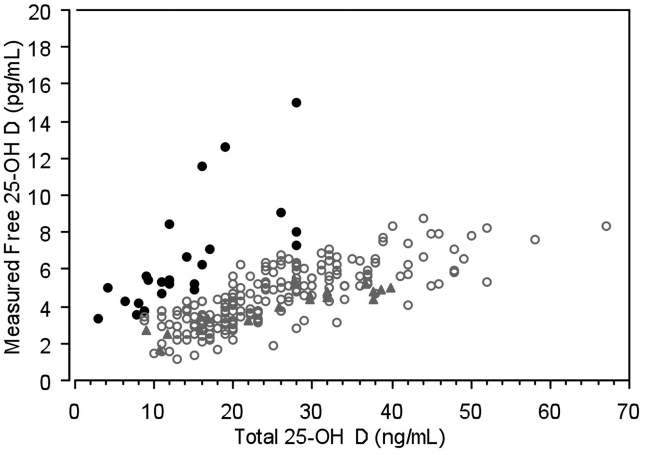

Figure 2. Total 25(OH)D concentrations are plotted on the x-axis, and directly measured free 25(OH)D levels are plotted on the y-axis. Triangles represent data from pregnant women, closed circles represent data from liver failure patients, and open circles represent data from the comparison group. Directly measured free concentrations were related to total 25(OH)D concentrations ($P < .0001$), but the relationship varied slightly for each clinical group with free 25(OH)D concentrations highest in liver failure patients despite lower total 25(OH)D concentrations. Relationships between total 25(OH)D and pregnant women did not differ from the comparator group.

= 1.0; $P < .0001$ for the relationship between estimates using the 2 methods) and results (see Table 1). Therefore, further results will be presented for the simpler Bikle method (7). Figure 1 presents data comparing directly measured free 25(OH)D with calculated free 25(OH)D for individuals and by clinical grouping. Calculations overestimated free 25(OH)D levels compared with directly measured free 25(OH)D with larger fold differences in non-Caucasians compared with Caucasians (African Americans, 2.9 ± 1.9-fold more than Asians, 2.1 ± 1.0 more than others,1.5 ± 0.8, Caucasians, and 1.5 ± 0.6-fold greater than Caucasians; $P < .001$). Differences between estimates were also greater in the comparison group than in the cirrhotic patients or pregnant women (2.1 ± 1.3 in the comparison group vs 1.6 ± 0.7 in cirrhotics and 1.4 ± 0.8 for pregnant women, $P < .05$).

Relationships with 25(OH)D

Figure 2 presents individual and group data for free 25(OH)D and total 25(OH)D. There were significant direct positive relationships between measured free 25(OH)D and total 25(OH)D concentrations for the entire dataset and for each group ($P < .0001$). The relationship, however, was weaker in the cirrhotic subjects compared with the other groups (cirrhotic subjects: free 25(OH)D, y = 2.522 + 0.289x, where x = total 25(OH)D, $r^2 = 0.507$, $P < .001$; pregnant women: y = 1.451 + 0.094x; $r^2 = 0.772$, $P < .0001$; and for the comparator group, y = 1.349 + 0.124x; $r^2 = 0.722$, $P < .0001$).

Relationships with DBP

Directly measured free 25(OH)D was not correlated with DBP for the entire sample or within subgroups (P = .35–.76). Calculated free 25(OH)D was highly inversely correlated with DBP ($P < .0001$, $r^2 = 0.36$) as expected because the calculations include DBP.

Relationships with iPTH

Significant inverse relationships were detected between iPTH and measured free 25(OH)D (iPTH = 83.4–16.7 × ln free 25(OH)D; $r^2 = 0.036$, $P < .02$) but not between iPTH and calculated free 25(OH)D ($P = .46$, r^2=0.006). Similarly, measured free 25(OH)D but not calculated free 25(OH)D was correlated with calcium concentrations ($r^2 = 0.035$, $P < .004$; and $r^2 = 0.004$, $P = .47$, respectively). As expected, total 25(OH)D concentrations were also inversely correlated with iPTH (iPTH = 100.4–12.74 × ln total 25(OH)D; $r^2 = 0.023$, $P < .05$) and positively related to calcium concentrations ($r^2 = 0.021$, $P < .08$).

Discussion

The free hormone hypothesis postulates that protein-bound ligands cannot freely cross the cell membrane to interact with cytoplasmic or nuclear binding proteins, whereas unbound free small lipophilic ligands can cross cell membranes and access cytoplasmic or nuclear bound proteins to exert effects. A large amount of biochemical, cellular, and physiologic data strongly support the free

hormone hypothesis and in vivo activity of sex and thyroid hormones is routinely evaluated in relation to free hormone concentrations. Vitamin D is increasingly recognized as a prohormone, with its active metabolite, $1,25(OH)_2D$, the hormone. It would be expected that free vitamin D metabolites would be more closely related to vitamin D effects than total vitamin D metabolite concentrations as has been reported (9, 10). The major circulating metabolite of vitamin D is 25(OH)D, the substrate for the enzyme cytochrome P450 (CYP) 27B1 that converts 25(OH)D to $1,25(OH)_2D$. Circulating concentrations of $1,25(OH)_2D$ are orders of magnitude lower than circulating concentrations of 25(OH)D, and a number of tissues express CYP27B1 and so are able to convert 25(OH)D to the pharmacologically active $1,25(OH)_2D$. Therefore, it follows that circulating levels of free 25(OH)D represent the driving free hormone of the vitamin D system, especially for many of the nonclassical, intracrine actions of vitamin D.

The major binding and transport protein for vitamin D and its metabolites is DBP, which binds 85% of 25(OH)D. Albumin and lipoproteins account for another 15% due to a much lower affinity despite their much higher serum concentrations. Variation in DBP levels and binding properties have been reported in humans (7, 8, 16–19). Diseases such as cirrhosis result in decreased protein synthetic capacity and lower DBP concentrations that increase the percent free 25(OH)D concentrations resulting in free concentrations that are similar to those seen in normal subjects despite lower total 25(OH)D concentrations (7). DBP has also been reported to be lower in African Americans compared with Caucasians, and this could result in normal free 25(OH)D levels despite lower total 25(OH)D concentrations (20). Conversely, circulating DBP is increased during pregnancy, especially during the second and third trimesters, and would be expected to result in decreased percentages of free 25(OH)D concentrations.

Phenotypic variations in DBP based on isoelectric focusing migration have been recognized with more recent descriptions of polymorphisms in the DBP gene that alter the binding affinity for vitamin D ligands (21–23). Three alleles, group-specific component (Gc) 1F, 1S, and 2 have been observed in all human groups studied. However, the prevalence of alleles show distinct racial distribution patterns with African American and Asian populations more likely to carry the higher-affinity (Gc1F; CAT haplotype) form of DBP, whereas Caucasians more frequently have the lower-affinity DBP genotypes (Gc1S and Gc2) (24, 25). The relative difference of affinity constants of Gc1F and Gc1s in humans is about 2-fold (22). Unfortunately, current equations for calculation of free 25(OH)D assume 1 DBP binding affinity constant ($7 \times 10^8 M^{-1}$).

Directly measured free 25(OH)D was highest in patients with cirrhosis when compared with either pregnant women or the comparator group despite lower total 25(OH)D. In contrast to expectations, free 25(OH)D concentrations did not differ in pregnant women vs the comparator group, even when sex-matched and with exclusion of women on sex hormones in the comparator group. These results are consistent with the observation made by Bikle et al (26) that affinity of DBP for the vitamin D metabolites appears to be decreased during pregnancy, perhaps compensating for increased DBP concentrations to maintain the free metabolite levels. Whether this reflects the influence of changes in the hormonal milieu during pregnancy on DBP affinity is not known. The other unexpected finding was that free 25(OH)D levels were not affected by race, despite lower DBP levels in African Americans. The lower DBP levels may explain the lower total 25(OH)D levels generally found in African Americans and contribute to a higher free concentration than would be expected even with the greater prevalence of high-affinity DBP forms found in African Americans.

Our primary goal was to compare directly measured circulating free 25(OH)D concentrations with calculated free 25(OH)D levels. A direct positive statistically significant correlation was found that accounted for only 13% of the variation. In general, the calculations overestimated the directly measured free 25(OH)D concentration. The differences were most pronounced with mean 3-fold differences in African Americans compared with 1.5-fold differences in Caucasians. The equations to estimate free 25(OH)D rely heavily on DBP concentrations with a single assumed affinity constant. As a post hoc analysis, we recalculated free 25(OH)D levels assuming a 1.9-fold higher DBP affinity constant for 25(OH)D in African Americans compared with Caucasians based on relative Gc frequencies and equilibrium association constant (ka) differences (24, 25, 27). This reduced the mean overestimation in African Americans to 1.6 ± 1.2-fold, which did not differ from that in Caucasians (1.5 ± 0.6-fold), supporting the premise that differences in DBP binding affinity may have been responsible for the overestimation differences between the races. Nonetheless, there remained a considerable 1.5- to 1.6-fold overestimation of calculated compared with directly measured free 25(OH)D levels. The other variables in the equations for calculating free 25(OH)D are albumin concentration, albumin binding affinity for 25(OH)D, DBP, and total 25(OH)D concentration. Albumin has a much lower affinity constant for 25(OH)D metabolites and is thought to bind only 15% of circulating 25(OH)D and contributes far less to the calculations, suggesting albumin-related factors are not a likely source of 50% differences. Inaccuracies in measure-

ment of DBP could result in inaccurate calculated free 25(OH)D estimates. Concentrations of DBP were within the ranges reported in humans with a variety of clinical conditions (28–31); however, they were somewhat lower than reported in normal subjects (26).

Additional sources of estimation error could have resulted from use of a single DBP affinity constant because affinity constants for DBP differ for $25(OH)D_3$ and $25(OH)D_2$, or from errors in measurement of total 25(OH)D. A highly accurate tandem mass spectrometry assay with NIST 25(OH)D standards was used, making issues with 25(OH)D measurement unlikely and providing measurements of $25(OH)D_2$ as well as $25(OH)D_3$. $25(OH)D_2$ was detected in 6% of the samples. In post hoc analyses, neither the presence nor magnitude of $25(OH)D_2$ concentrations were related to the magnitude of overestimation. Calculation of free 25(OH)D did not account for potential 25(OH)D binding to chylomicrons because this is thought to be only a very small fraction, chiefly during the postprandial state, and all our samples were fasting samples.

A secondary goal was to compare relationships between directly measured and calculated free 25(OH)D concentrations with a biomarker of 25(OH)D effect. A significant inverse relationship between directly measured free 25(OH)D and iPTH was observed, whereas no relationship could be detected between calculated 25(OH)D levels and iPTH. Similarly, relationships between calcium and directly measured free 25(OH)D were observed, but none were found for calculated free 25(OH)D. These findings favor the use of the direct measurement of free 25(OH)D over calculated estimates of free 25(OH)D with the assays and equations used. They also call into question results on relationships between biomarkers of vitamin D activity and calculated free 25(OH)D reported using the same methodologies. Relationships detected for measured free 25(OH)D and iPTH and calcium were at least as strong or stronger than relationships detected for total 25(OH)D and these biomarkers of 25(OH)D effects.

Our study had some limitations. We did not directly measure albumin or DBP binding affinities for 25(OH)D, nor did we assess 25(OH)D metabolites other than 25(OH)D that compete for binding to DBP. However, except in the case of vitamin D toxicity, these other metabolites contribute little to the binding of 25(OH)D to DBP (32). The comparator group included patients with chronic stable disease and elderly patients with conditions such as inflammation with higher circulating actin levels that could have altered DBP binding. In addition, any administered medications could have competed for binding with albumin. However, these conditions would not have resulted in the lower directly measured value compared with calculated free 25(OH)D differences. Moreover, this group provides a relevant clinical population in which vitamin D measurements are frequently made.

In summary, directly measured free 25(OH)D concentrations were not accurately predicted using current algorithms or based on clinical conditions known to alter DBP concentrations. Differences in DBP binding affinity likely contributed to estimation errors between the races. Directly measured free 25(OH)D concentrations were related to iPTH and calcium, but calculated estimates were not. Correlations between directly measured 25(OH)D and total 25(OH)D were weakest in patients with cirrhosis. These findings suggest that current algorithms to calculate free 25(OH)D may not be accurate, and direct measurement of free 25(OH)D concentrations warrants further evaluation in the clinical setting.

Acknowledgments

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