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Variability in Free 25-OH Vitamin D Levels in Clinical Populations

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ABSTRACT

Our goal was to determine total and directly measured free 25-OH vitamin D serum levels in humans with a range of 25-OH vitamin D levels and clinical conditions associated with low and high vitamin D binding protein levels. Serum samples and clinical data were collected from 106 subjects: 62 without cirrhosis or pregnancy, 24 cirrhotic patients with albumin <2.9 g/dL, and 20 pregnant women. Total 25-OH D (LC/MS/MS) and "free" 25-OH D (immunoassay) were measured. Total 25-OH D was significantly lower in liver disease patients but free 25-OH D concentrations were significantly higher in this group ($p<.001$). Neither total nor free 25-OH D concentrations were significantly different in pregnant women vs. the comparator group. There were significant direct positive relationships between free 25-OH D and total 25-OH D concentrations for the entire dataset and for each group ($p<.0001$), however slopes of relationships differed in the cirrhotic group compared to pregnant women or the comparator group (free 25-OH D) $y = 2.522 + .289 * X$(total 25 OH-D), $r^2 = .507$, $p<.001$ cirrhotics; $y = 1.451 + .094 * X$; $r^2 = .772$, $p<.0001$ for pregnant women; and $y = 1.112 + .122 * X$; $r^2 = .715$, $p<.0001$ for the comparator group). Conclusions: directly measured free 25-OH D serum concentrations and relationships between total and free 25-OH D vary with clinical conditions, and may differ from those predicted by indirect estimation methods.

Key words: vitamin D, 25-OH vitamin D, free 25-OH vitamin D, cirrhosis , pregnancy

INTRODUCTION

There continues to be debate about assessment of vitamin D status in humans based on circulating vitamin D or metabolite concentrations. There is agreement, however, that adequate status should be defined by concentrations of serum 25-OH vitamin D (D) (1-3) as this metabolite reflects overall body storage of the immediate vitamin D precursor that is hydroxylated to active 1α,25-OH_2 vitamin D. Both circulating 1α, 25-OH_2 D and 25-OH D bind to albumin and D binding protein leaving only a small fraction unbound or "free". Receptor and drug theory posit that only the "free" compound is available to bind to receptor. Or in the case of 25-OH D, only free is available for conversion to active 1α, 25-OH_2 D and thus, may more closely reflect the biologic activity. The potential benefit of measuring "free or unbound" concentrations of D and its metabolites, especially in the presence of biologic conditions that alter levels of the carrier proteins, has been suggested. (4-7). Until recently, determination of serum free 25-OH D was an arduous undertaking involving some form of equilibrium dialysis or indirect estimation based on measurement of D binding protein, albumin, and 25-OH D (using D standards and assays that were variable) with equations derived from relatively small numbers of people (4) or modified from equations used for sex hormones (8) . An assay that directly measures serum free 25-OH D levels has been developed (Future Diagnostics B.V., Wijchen, The Netherlands). The purpose of this investigation was to determine total and directly measured free 25-OH D in humans with a range of 25-OH D levels and clinical conditions associated with a range of D binding proteins levels.

MATERIALS AND METHODS

Subjects. Stable subjects with liver disease and evidence of protein synthesis dysfunction defined as an albumin concentration of <2.9g/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 protocols approved by the University of California, San Francisco Committee on Human Research.

Laboratory Measurements. Total 25-OH D measurements were determined by CLIA certified liquid chromatography tandem mass spectrometry at Mayo Clinical Laboratories 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 D metabolites in human serum. The assay has ~10% CV at levels ≥10 ng/mL. Internal standard is NIST reference standard. Free 25-OH D concentrations were determined by immunoassay (Future Diagnostics B.V., Wijchen, The Netherlands, http://www.future-diagnostics.nl/) In this assay an anti-vitamin D antibody is coated on a microtiterplate. 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 non-occupied antibody binding sites in a second incubation. After washing 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.

Statistical Design and Data Analysis. Demographic and baseline characteristics of groups are presented as mean ± S. D. and compared using ANOVA. Relationships between total and free 25-OH D concentrations were tested by linear regression and between group differences by ANOVA.

RESULTS.

One hundred and six subjects participated. Characteristics by group (cirrhotic, pregnant, and comparator) and mean values for total and free 25-OH D are presented in Table 1. Cirrhotic patients had a mean Model for End-Stage Liver Disease MELD score of 16 ± 3 with Child Pugh score (9, 10) B in eight and C in twelve. Half of the pregnant women were in the second trimester of pregnancy and half were in the third. The comparator group had a mean Charlson co-morbidity score (11) of 3.7 ±3.4. In the comparator group, five women were taking oral contraceptives and two received post-menopausal hormone replacement; no other sex hormones were taken. Total 25-OH D was significantly lower in cirrhotic patients with protein synthesis dysfunction but free 25-OH D concentrations were significantly higher in this group. Neither total nor free 25-OH D concentrations were significantly different in pregnant women compared to the comparator group. There were

significant direct positive relationships between free 25-OH D and total 25-OH D concentrations for the entire dataset and for each group ($p<.001$), however the slopes of the relationships differed in the cirrhotic group compared to the pregnant women or the comparator group (free 25-OH D) $y = 2.522 + .289 * X$(total 25 OH-D), $r^2 = .507$, $p<.001$ cirrhotics; $y = 1.451 + .094 * X$; $r^2 = .772$, $p<.0001$ for pregnant women; and $y = 1.112 + .122 * X$; $r^2 = .715$, $p<.0001$ for the comparator group).

DISCUSSION

Variation in D binding protein levels and binding properties affect the free fraction of circulating 25-OH D. (4, 5, 12-14) Diseases such as cirrhosis that result in decreased protein synthetic capacity result in lower D binding protein concentrations and this would be anticipated to increase free 25-OH D concentrations. Conversely, the state of pregnancy, especially during the second and third trimesters when D binding protein concentrations increase, would be expected to result in decreased free 25-OH D concentrations. Indirect methods of calculating free D are highly dependent on D binding protein measurements and estimations will change in relation to changes in D binding protein. (4, 8,) As expected, we found patients with cirrhosis and low albumin concentrations had free 25-OH D levels that were higher for any given total 25-OH D level than in the pregnant women or comparator group. Surprisingly not only was the ratio of free to total 25-OH D higher but the actual measured level of free 25-OH D was higher in patients with cirrhosis. Likewise surprising was that women in their second and third trimester of pregnancy did not have lower free total 25-OH vitamin D concentrations than the comparator group despite predicted increased DBP levels. However, these results are consistent with the observation made by Bikle et al. (15) that the affinity of DBP for the vitamin D metabolites appears to be decreased during pregnancy perhaps to compensate for the increased DBP concentrations without decreasing the free metabolite levels.

CONCLUSIONS. Directly measured circulating free 25-OH vitamin D concentrations are a better means of assessing vitamin D sufficiency than total 25-OH D measurements in clinical conditions in which DBP and albumin levels are altered. Moreover, estimations of percent free 25-OH D levels

based on DBP and albumin levels that assume a fixed affinity constant for the binding of 25 OH D to these proteins will be misleading in conditions in which this assumption is invalid.

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Table 1. Results by Group

	Cirrhotics	Pregnant	Comparison Group
Number (n)	24	20	62
Women / Men (n)	15/9	20/0	31/31
Age (y)	57.2 (8)*	30.7 (7)	54.5 (17)
Race (Cauc/AfrAmer/Asian/Other)	16/2/2/4	15/4/1/0	42/14/5/1
Body Mass Index (M^2)	32.1 (7.4)	31 (5.2)	29.2 (7)
Albumin (g/dL) #	2.4 (0.4)	3.6 (0.3)	n.a.
Calcium (mg/dL) #	8.4 (0.5)	9.1 (0.6)	9.4 (0.4)
Total 25-OH D (ng/mL)#	14 (7.3)**	26.7 (10)	21.7 (12.7)
Free 25-OH D (pg/mL)#	6.6 (3)**	4 (1.1)	3.8 (1.9)^
Per Cent Free	.054 (.023)**	0.016 (.004)	.018 (.007)^

Data are mean (S.D.) ** p<.001 for group differences for liver disease vs. other groups. ^ ns for differences between pregnant women and women in comparator group.# for conversion to S.I. units: albumin- multiple by 10 for g/L; calcium multiply by 0.25 for mmol/L; for 25-OH D multiply by 2.496 (for nmol/L or pmol/L)

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