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Context: The optimal measure of vitamin D(D) status is unknown.
Objective: Directly measure circulating free 25(OH)D concentrations and relationships to total 25(OH)D in a clinically diverse sample of humans.
Design: Cross-sectional analysis
Setting: Seven academic sites
Patients: 1661 adults: (healthy(n=211), pre-diabetic(n=479), outpatients(n=783), cirrhotic(n=90), pregnant(n=20), nursing home(n=79))
Interventions: Merge research data on circulating free 25(OH)D (directly measured immunoassay), total 25(OH)D (LC/MS/MS), D binding protein (DBP by radial (polyclonal) immunodiffusion assay)), albumin, creatinine, iPTH and DBP haplotype
Main outcome measures: Distribution of free 25(OH)D (ANOVA with Bonferroni correction for post hoc comparisons) and relationships between free and total 25(OH)D (mixed effects modeling incorporating clinical condition, DBP haplotype with sex, race, eGFR, BMI and other covariates).
Results: Free 25(OH)D was 4.7±1.8 pg/mL (mean ±SD) in healthy and 4.3 ±1.9 pg/mL in outpatients with 0.5-8.1 pg/mL and 0.9-8.1 pg/mL encompassing 95% of healthy and outpatients, respectively. Free 25(OH)D was higher in cirrhotics (7.1 ±3.0 pg/mL, p<.0033) and nursing home residents (7.9± 2.1pg/mL, p<.0033) compared to other groups and differed between whites and blacks (p<.0033) and between DBP haplotypes (p<.0001). Mixed effects modeling of relationships between free and total 25(OH)D identified clinical conditions (cirrhotic>nursing home>prediabetic > outpatient > pregnant), and BMI (lesser effect) as covariates affecting relationships but not eGFR, sex, race or DBP haplotype.
Conclusions: Total 25(OH)D, health condition, race and DBP haplotype affected free 25(OH)D, but only health conditions and BMI affected relationships between total and free 25(OH) D. Clinical importance of free 25(OH)D needs to be established in studies assessing outcomes.

Free 25(OH) D levels were affected by clinical conditions as well as race, BMI, or DBP haplotype. Relationships between free and total 25(OH)D were only affected by clinical conditions and BMI. .

1. Introduction

The adequacy of vitamin D status is usually assessed by measurement of total circulating 25(OH) vitamin D (25(OH)D) levels. Total circulating 25(OH)D includes 25(OH)D bound to vitamin D binding protein (DBP) estimated to be about 85% of total with about 10-15% bound to albumin and a very small fraction as free or unbound 25(OH)D. As DBP is the main carrier for 25OHD and other vitamin D metabolites, its concentration and affinity are the main drivers of the free concentration of 25(OH)D and other D metabolites. If the free hormone hypothesis applies to vitamin D biology, only free 25(OH)D is available for conversion to active $1\alpha,25(OH)_2$ D that interacts with the vitamin D receptor regulating hundreds of genes in most cells. It has been shown that health conditions such as cirrhosis that is associated with protein synthetic dysfunction resulting in decreased DBP as well as albumin and pregnancy that is associated with increased protein synthesis and DBP in the second and third trimesters alter levels of free 25(OH)D inversely to the changes in DBP. (1-3). There is uncertainty regarding DBP genetic variant effects on free 25(OH)D levels but *in vitro* DBP affinity constants for 25(OH)D that differ between DBP haplotypes would predict altered 25(OH)D binding and differing free 25(OH)D levels. (4-7) Altered albumin concentrations such as the lower levels reported in the frail elderly or nursing home residents (8) could also alter free 25(OH)D concentrations, albeit to a smaller extent than changes in DBP. Thus, total 25(OH)D may not accurately reflect levels available for cellular uptake with the exception of cells in the kidney or parathyroid capable of megalin/cubilin-mediated internalization of DBP-bound 25(OH)D. (9)

Primary goals of this work were to combine data from human investigations involving direct measurement of free 25(OH)D to a) describe the distribution of circulating free 25(OH)D concentrations in adult humans with and without various conditions or disease states known to alter DBP, with differing DBP haplotypes, with a wide range of body weights as higher BMI such as seen in those with obesity, metabolic syndrome or prediabetes has been shown to alter total 25(OH)D and calculated free 25(OH)D,(10) in the very elderly such as nursing home patients or women with osteoporosis likely to receive D supplementation or receive exogenous female sex hormones in whom free 25(OH)D data are not available; and, b) to determine relationships between free and total 25(OH)D in these clinical conditions and disease states, and different DBP haplotypes. Our findings provide a measure of the normal range of free 25(OH)D concentrations as well as new observations on factors that do and do not alter relationships between free and total 25(OH)D in clinical populations.

2. Subjects and Methods

A. Subjects.

Investigators who directly measured free 25(OH)D in clinical investigations contributed de-identified data. Adult groups sampled included: healthy subjects, medically stable community-dwelling outpatients enrolled in longitudinal or D dosing studies, pre-diabetics, medically stable nursing home residents >65 years of age, stable subjects with cirrhosis, and pregnant women (second or third trimester).(2, 11-26) Subjects provided informed consent for research approved by the Institutional Review Board of the respective organizations. For investigators, sites, and subject description see Appendix.

B. Laboratory Measurements

1. Free 25(OH)D Levels**.** Direct measurement of free 25(OH)D concentrations was by immunoassay (Future Diagnostics B.V., Wijchen, The Netherlands, http://www.future-diagnostics.nl/) as described. (23) In brief, an antibody to 25(OH)D is pre-coated onto a microtiterplate and serum samples and calibrators added. Free 25(OH)D is captured during this first incubation step, and after washing, a second incubation with biotin-labeled 25(OH)D analog reacts with non-occupied antibody binding sites (competitive immunoassay). Finally, after washing and incubating with a streptavidin- peroxidase conjugate, absorbance [A450nm] is measured using a plate spectrophotometer, where concentration of free 25(OH)D in the sample is inversely proportional to absorbance in each sample well. Assay calibration was against a symmetric dialysis method. (see http://www.future-diagnostics.nl/) Limit of detection (LOD) for blank serum is 0.7 pg/mL; at 5.02 pg/mL, between-run coefficient of variation (CV) was 6.2% and between-day CV was 4.5% with a total imprecision CV of 15.7%. Biotin at 4mg/dL was tested for assay interference and mean % interference was 1% at 6.5 pg/mL, 4% at 10.6 pg/mL and 1% at 15.7 pg/mL: free 25(OH)D. Assays were performed at Future Diagnostics B.V. except for measurements in pre-diabetics performed in Tromso using the Future Diagnostics B.V. kit with the same technique calibrated over the range of 0.1-35 pg/mL with LOD of 2.8 pg/mL, with inter- and intra-assay CVs <10%. (25)
2. Total 25(OH)D was determined by liquid chromatography tandem mass spectrometry (LC MS/MS) using National Institute of Standards and Technology (NIST) reference standard (U.S. sites participated in National Institutes of Health Office of Dietary Supplements funded quality assurance program for analysis of D metabolites in human serum; European sites participated in the external quality program DEQAS with the exception that two-thirds of samples from cirrhotics were by immunoassay (Diasorin (LIAISON), and the results converted to (LC MS/MS) equivalent by the manufacturer provided calibration factor.
3. DBP was measured by radial immunodiffusion (polyclonal) assay (KU Leuven, Belgium) for all groups except pregnant (monoclonal ELISA R&D Systems (Minneapolis, MN)).
4. Albumin, creatinine, calcium, were measured with autoanalysers in clinical laboratories. iPTH was measured by multiple immunoassays: two-site sandwich immunoassay using direct chemiluminometric technology (ADVIA Centaur, Siemens, Malvern, PA, for UCSF samples), Diasorin immunoradiometric assay (for Creighton University samples), automated clinical chemistry analyzer (Immulite 2000, Siemens Healthcare Diagnostics, Los Angeles, CA, USA for Tromso Norway and UK samples), and by Scantibodies immunoradiometric assay (Santee, CA) for MrOs samples. Assay method was coded.
5. DBP haplotyping (959 subjects). In 471 prediabetics from University of Tromso haplotyping was done by KBioscience (http://www.kbioscience.co.uk) using the KBioscience Competitive Allele-specific PCR genotyping system; in 205 young and older men and women from Sheffield England at Sheffield Children's Hospital, United Kingdom a pyrosequencing assay was developed with PSQ software (version 1.0.6; Qiagen) to detect rs4588 and rs7041 polymorphisms; in 254 older community outpatient men (multiple U.S. MrOS sites), two nonsynonymous *GC* single nucleotide polymorphisms were used to define *GC* haplotypes, rs4588 (Thr436Lys) and rs7041 (Asp432Glu), and in 29 young normals (MRC/Gambia) samples were analyzed at Vesalius Research Center (Katholieke Universiteit, Leuven, Belgium) by iPLEX technology on a MassARRAY compact analyzer (Sequenom Inc).

C. Data analysis.

Demographic, clinical characteristics, and assay results are presented as mean ± S. D. Analysis of variance for trends followed by post hoc analyses for between group comparisons using Bonferroni correction for multiple comparisons was used to test for differences in total, free, or per cent free 25(OH)D between clinical groups, DBP haplotypes or self-reported racial groups. Relationships between free and total 25(OH)D were examined using a mixed effects model incorporating clinical condition, DBP haplotypes with sex, race, eGFR, BMI and other biologically plausible covariates and interactions. Relationships between free or total 25(OH)D and iPTH were examined in the same manner including iPTH assay method as a covariate. Analyses were performed in R (*R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/) using the function lmer from the package lme4).* The fixed effect part of the model takes the form Free = a + (b +c COV)Total, where a is the intercept, b is the slope of the relationship Free vs Total, and c is a vector of parameters

quantifying the relationship of the slope with covariates. Slopes are assumed to be normally distributed across individuals. Model selection was conducted using standard procedures according to the Akaike Criterion (27) and visual inspection of diagnostic plots. After model selection, comparisons to the reference group were computed according to 2-sided t-test using the Satterthwaite approximation (R lmerTest). Exploratory analyses of effects of sex hormones in women were performed using linear regression.

3. RESULTS

A. Subject data.

Data were from 1661 subjects. Demographic characteristics by clinical group (normal, pre-diabetic, community-dwelling outpatient, cirrhotic, pregnant, nursing home (NH)) estimated glomerular filtration rate (eGFR), albumin, calcium, albumin-corrected calcium, DBP, total and free 25(OH)D, and iPTH are in Table 1. 25(OH)D_2 was identified in no pregnant, 10% of nursing home residents, 25% of cirrhotics, and 61% of normals and outpatients). Average 25(OH)D_2 was < 7% of total in normals and outpatients and 24% of total in cirrhotics. No relationship was detected between free 25(OH)D and 25(OH)D_2. Assays measuring C3-epimer of 25(OH)D were used in 498 samples and C3-epimer was detected in 296 (59%). C3-epimer concentrations over 1 ng/mL were not detected until total 25(OH)D exceeded 20 ng/mL; C3-epimer was < 2 ng/mL at total 25(OH)D up to 30 ng/mL.

B. Free 25(OH)D Distribution.

Distribution of free 25(OH)D concentrations by clinical group is shown in Figure 1. Data reflected steady-state conditions with and without D supplementation as part of clinical care (but not during dose titration studies). Free 25(OH)D levels from 0.5 to 8.1 pg/mL include 95% of healthy subjects and is similar to the 0.9- 8.1 pg/mL range encompassing 95% of the almost three times larger group of stable outpatients. Significant effects of clinical condition on free 25(OH)D, DBP, total 25(OH)D, and per cent free 25(OH)D were detected (ANOVA p<.0001; Table 1). The highest mean free 25(OH)D was in NH residents accompanied by higher total 25(OH)D and lower DBP than normals, outpatients, prediabetics and pregnant women, but higher DBP than in cirrhotics (p<.0033). The next highest mean free 25(OH)D was in cirrhotics (higher than healthy, pregnant, prediabetic, and outpatients (post hoc p<.0033 for all). Between group differences were detected for all comparisons (post hoc p<.0033) except normals vs. pregnant or outpatients, and for pregnant vs. outpatients. Both DBP and total 25(OH)D were lowest in cirrhotics. Pregnant women had the second highest total 25(OH)D levels and the highest DBP (post hoc p<.0033), despite measurement by a less sensitive assay. Albumin concentrations were not correlated with DBP (r^2= 0.0004, p=0.83) in the absence of pregnancy or cirrhosis. Per cent free 25(OH)D was higher in cirrhotics and nursing home residents compared to other clinical groups (post hoc p<.0033) and between group comparisons were significant for all but normals compared to pregnant or outpatients, and for pregnant vs. outpatients.

C. Effects of race and DBP haplotype.

Genotype data were available for 959 (outpatients, prediabetics and normals, Table 2). Ninety-eight were of self-reported black race, 860 white and 1 of self-reported other race. Differences in free 25(OH)D between whites and blacks were detected (4.9 ± 1.9 vs. 4.0 ±1.5 pg/mL, respectively, p<.0033). As expected, the 1f allele was more common in blacks and the 1s allele more common in whites. (Table 2). Gc 2/2 haplotype was present in 5.5% of whites and no blacks. DBP haplotype had significant effects on total 25(OH)D, free 25(OH)D, and DBP (ANOVA, p<.0001). The lowest total and free 25(OH)D were seen with the least frequent Gc 2/2

haplotype (4.2 ±2.2 pg/mL). Total and free 25(OH)D were higher in the presence of 1s alleles. Post hoc analyses detected lower free 25(OH)D levels in 2/2 haplotype compared to 1s/1s or 1s/1f haplotypes and in 1f/1f haplotypes compared to 1f/1s haplotypes (p<.0033). DBP haplotype also affected percent free 25(OH)D (p<.0001) (Figure 2). The lowest percent free was seen with the 1s/1s haplotype that was lower compared to 1s/1f, 1f/2, 1f/1f or 1s/2 haplotypes (p<.0033). Percent free was higher with 1f/1f haplotype compared to 1s/2, and 1f/2 and was higher with 1f/2 compared to the 1s/2 haplotype (p<.0033). Magnitude of differences, however, were less than observed between some clinical conditions. DBP haplotypes differed in DBP concentrations with the 2/2 haplotype having the lowest DBP, total, and free 25(OH)D (post hoc p<.0033) yet percent free 25(OH)D that was in the middle of observed means. The highest DBP was seen with the 1s/1s haplotype that had the highest total and free 25(OH)D but lowest percent free 25(OH)D. DBP levels were higher for the 1s/1s haplotype compared to any haplotype with at least one Gc2 allele (p<.0033) but not when compared to haplotypes 1s/1f or1f/1f. DBP levels were significantly lower for haplotype 2/2 compared to 1f/2,1f/1f; and 1s/1f (p<.0033). No differences were detected between haplotypes 1s/1s vs 1s/1f or 1f/1f; 1s/2 vs 1f/2; 1s/2 vs 1f/1f; or 1s/1f vs 1f/1f. Differences between haplotypes 1s/1f vs 1f/2 approached (p=.0045) but failed to reach p<.0033 post hoc criteria for significance).

D. Relationships between free and total 25(OH)D.

Individual data are plotted by clinical group and DBP haplotype in Figure 3. Linear mixed effects modelling identified significant contributors to the relationship as the clinical condition and BMI. (see Table 3). Rejected covariates included eGFR and race. Clinically normal subjects are associated with the baseline slope (b) of the model. The steepest slope (b+0.1577) was in cirrhotics with the lowest DBP, the second steepest slope was in NH subjects with the second lowest DBP levels, and the least steep slope was in pregnant women with the highest DBP. Excluding cirrhosis and pregnancy from the model, sex was selected for inclusion (male sex with coefficient estimate of 0.03 ±.004). DBP haplotype effects on the free vs. total 25(OH)D relationship were not detected in subjects (n=959) with these data.

E. Relationships between free and total 25(OH)D and iPTH.

Both total and free 25(OH)D concentrations were negatively related to iPTH levels, but the mixed effects model fits favored total 25(OH)D (coefficient estimate of -0.96 ±0.51). Covariates selected included BMI (continuous variable) with a small effect (0.02±.004) and iPTH assay method that varied within the sites precluding further clinical group analyses.

F. Exploratory analyses- female sex hormones.

Forty young non-pregnant and non-cirrhotic women reported taking oral contraceptives (OC). Total and free 25(OH)D were 21.0 ± 13.1 ng/mL and 3.4±2.2 pg/mL, respectively, not different from total or free 25(OH)D levels of 20.1±8.3 ng/mL and 3.6±1.5 pg/mL in 21 young non-pregnant non-cirrhotic women not taking oral contraceptives. Relationships between free and total 25(OH)D in oral contraceptive users had a slope of 0.150 (lower 95% confidence interval (C.I.) of 0.126 and upper 95% C.I. of 0.175) compared to slope of 0.125 (lower 95% C.I. of 0.066 and upper 95% C.I. of 0.185) in non-users (ns). Thirty-five postmenopausal women reported estrogen use for hormone replacement, and 82 age-health matched women reported no use. Total 25(OH)D concentrations were 24.8 ±11ng/mL in estrogen users vs. 26.1 ±10.2 in non-users. Free 25(OH)D was 4.4±2 in estrogen users and 4.6±2.2 pg/mL in non-users (ns), and the slope of relationships between free and total 25(OH)D did not differ (users: 0.164 (lower 95%

CI of 0.136 and upper 95% C.I. of 0.195 compared to slope of 0.158, lower 95% C.I. of 0.124 and upper 95% C.I. of 0.192 in non-users). DBP data were not available.

4. DISCUSSION

There is currently debate about the best serum measurement to determine vitamin D status. (4) Circulating levels of 25-hydroxyvitamin D (25(OH)D) are the most commonly used marker because its concentration in blood is higher than other D metabolites making it easier to measure, its conversion from vitamin D is substrate dependent with minimal regulation, and it has a relatively long circulating half-life. However, the free hormone hypothesis postulates that only non-bound or "free" fraction of hormones that circulates in blood can enter cells and exert biologic effects. This would suggest that the free fraction is key to the intracrine functions of vitamin D except in cells such as those in the kidney or parathyroid gland capable of megalin/cubilin-mediated internalization of DBP-bound 25(OH)D. (9)

Assays to directly measure free 25(OH)D are not currently applied in clinical care but have been utilized in research investigations. It has been demonstrated that directly measured free 25(OH)D concentrations differ from estimated (calculated) free 25(OH)D concentrations based on DBP assays using monoclonal or polyclonal antibodies and single or DBP haplotype estimated DBP dissociation constants. (2, 3, 6, 19, 21-23, 28) Directly measured free 25(OH)D has also been reported to correlate better than total 25(OH)D with some biologic measurements (2, 3, 6, 19, 21-23, 28), whereas other reports do not report a stronger relationship (summarized in (4-7)). Most investigations, however, have small sample sizes or selected populations such that the distribution of free 25(OH)D concentrations in many clinical populations is unknown. This paper is the compilation of data from an international Working Group of Vitamin D investigators in order to describe free 25(OH)D concentrations in a wide range of people with various clinical conditions. The data were from healthy young and older people, people with pre-diabetes, community-dwelling outpatients enrolled in longitudinal studies or vitamin D studies, pre- and post-menopausal women with low vitamin D status, pregnant women, cirrhotics, and nursing home residents with multiple morbidities enrolled in observational or vitamin D studies. A major strength is that our international data represent by far the largest and most diverse sample of adults studied to date and included patients with conditions that alter both free 25(OH)D levels and the relationship between free and total 25(OH)D, groups for whom these data have not been previously available. Importantly, 98% of DBP measures were performed with one polyclonal method at one laboratory, and 95.8% of 25(OH)D measures were performed by labs participating in quality standardization programs (National Institute of Standards and Technology (NIST) or Vitamin D External Quality Assessment Scheme (DEQAS)) and 100% of free 25(OH)D measurements were performed using the same method.

A strict definition of "normal" subjects was used to identify people with normal laboratory chemistry tests, no known chronic medical diseases, and no chronic oral medications excepting thyroid, hormone replacement therapy, oral contraceptives or dietary supplements. In these individuals, the mean concentration of free 25(OH)D was 4.3±1.9 pg/mL when mean total 25(OH)D concentration was 21.9 ±9.9 ng/mL. A range from 0.5 to 8.1 pg/mL included 95% of healthy normal subjects and was similar to the 0.9- 8.1 pg/mL range encompassing 95% of the nearly three times larger group of stable outpatients. Mean free and total 25(OH)D concentrations as well as percent free were slightly higher in prediabetics yet the upper bound of the 95% confidence interval was similar at 8.9 pg/mL. Free 25(OH)D measurements in pre-diabetics was performed using the same technique but at a different site than all other assays and

some assay variation may explain the small differences (as some diabetics were included in the outpatient samples and did not show either higher free or percent free 25(OH)D (data not shown). In our prior observations in pregnant women and a subset of the cirrhotics, DBP was measured using a monoclonal antibody DBP assay. (1-3, 23) In the current analyses a polyclonal antibody was used in the radial immunodiffusion assay performed at the same laboratory for all groups with the exception of the pregnant women. The data on the current larger group of cirrhotics are consistent with early reports of lower DBP with higher directly measured mean free 25(OH)D despite lower total 25 (OH)D levels. (26) The data from pregnant women mirror the almost two-fold higher DBP initially reported in pregnant women in the second and third trimester compared to non-pregnant women (29, 30) and with less variability in free 25(OH)D. Although the group of pregnant women was small, similar mean free 25(OH)D with lesser variability than in other groups has been reproduced using the same methods in a larger group of about 300 Caucasian women, despite somewhat higher DBP in the second and third trimesters when measured by ELISA with a polyclonal antibody.(31) We had limited data on women reporting oral contraceptive use or hormone replacement therapy with estrogen, but free 25(OH)D levels and relationships between total and free 25(OH)D did not appear to be significantly influenced by use of these agents at currently prescribed dosages and routes of administration.

An unexpected observation was that mean free 25(OH)D was higher in the nursing home residents with distribution of values shifted toward higher concentrations. Likely contributors were both the lower DBP levels and the higher total 25(OH)D in the nursing home residents compared to the normal subjects, prediabetics, community-dwelling and pregnant subjects. Mean albumin concentrations were slightly lower in the nursing home residents compared to normals, outpatients, or prediabietcs but as only 12-15% of 25(OH)D is bound to albumin it is unlikely to have been a major factor. Inflammation and/or elevated cytokines that accompany very old age (32) or multiple morbidities could also alter affinity of 25(OH)D to DBP. Whatever the underlying mechanisms, both percent free 25(OH)D concentrations and the relationship between free and total 25(OH)D differ in pregnant women, people with cirrhosis, and elderly people with multiple morbidities compared to normals or community-dwelling outpatients, and relationships are affected to a much smaller extent by BMI in all groups. It also appears that stable medical conditions such as hypertension, prediabetes, diabetes, osteoporosis, or mild renal disease do not appear to significantly alter relationships between free and total 25(OH)D.

Free 25(OH)D concentrations are related to total 25(OH)D concentrations as well as albumin and DBP and their binding affinities for 25(OH)D. (29) DBP is a highly polymorphic protein. (33) Our sample included whites and blacks and several Asians, and distribution of DBP haplotypes mirrored reported racial differences in that black (and Chinese) populations are more likely to carry the Gc1f allele and whites more likely to possess the Gc1s and the less frequent Gc2 allele. (34) DBP haplotype affected DBP and both total and free 25(OH)D concentrations. The Gc2/2 haplotypes and presence of 1f alleles were associated with lower total 25(OH)D concentrations as previously reported. (35) Gc1f has been reported to have the highest affinity and Gc2 the lowest affinity for vitamin D and its metabolites, but this has not been uniformly detected. (7, 33, 36, 37) In our sample, the highest percent free was seen with the 1f/1f haplotype and 1f/2 haplotypes and the lowest percent free was seen with 1s/1s despite similar DBP concentrations. Mean percent free 25(OH)D in people with the 2/2 haplotype was in the midpoint of the range and did not differ significantly from the 1s/1f or 1s/ 2 haplotypes. These data do not support the earlier report of Gc1f having the highest and Gc2 having the lowest

affinity for 25(OH)D. The maximum mean percent differences between haplotypes was on the order of about 19-24 percent. DBP concentrations differed between some haplotypes, and free 25(OH)D concentrations were in the expected relationship—i.e. higher free 25(OH)D concentrations with lower DBP, but the percent free 25(OH)D did not show the same relationship. In contrast to differences in percent free 25(OH)D by DBP haplotype, haplotype was not selected as a significant covariate in the linear mixed effects model of relationships between free and total 25(OH)D in these individuals. This suggests that haplotype does not have a marked effect on the relationship. We did not have DBP haplotype data on cirrhotics, nursing home residents or pregnant women to allow comparisons of clinical condition effects to haplotype effects in the same model. Nevertheless, the magnitude of differences seen between the clinical groups was greater than that seen between DBP haplotypes.

This study has limitations. Data were not from random population-wide samples and analyses of BMI, sex, race or other subgroup effects might not be representative of all populations. Samples were from medically stable individuals and may not apply to acute medical conditions. The only potential biomarker for vitamin D status analyzed was iPTH with differing methods in clinical laboratories limiting our analyses. However, the parathyroid gland has the megalin/cubilin mechanism for cellular uptake of DBP, so PTH levels are unlikely to discriminate between free and total 25(OH)D effects on biological function. Bone biomarkers were not assessed. Bone density has been reported to correlate better with measures of free than total 25(OH)D in the prediabetics included in the current analyses (19), but others have found similar relations between markers of bone metabolism and free or total 25(OH)D. (38) However, D and bone relationships are somewhat difficult to interpret as measures of vitamin D and its metabolites are often done only at a single timepoint while bone density is the result of cumulative time effects. As many of the subjects sampled received D supplementation, we could not address seasonal effects.

5. CONCLUSIONS.

Free 25(OH)D concentrations are affected by health conditions in addition to total 25(OH)D concentrations and DBP haplotype. Free 25(OH)D distributions were similar in normal individuals and stable community-dwelling outpatients with 95% within the range of 0.5 to 8.1 pg/mL and 0.9- 8.1 pg/mL, respectively. Per cent free 25(OH)D was affected by clinical condition (cirrhotics>nursing home residents, >outpatient, >normal>pregnant), self-reported race (black>white>Asian), and DBP haplotype (1f/1f +1f/2>1f/1s,2/2, 1s/2>1s/1s). Relationships between free and total 25(OH)D were influenced by BMI to a small extent and to a larger extent by health conditions with cirrhotics and nursing home residents having the steepest slopes and pregnant women the least steep without significant effects of DBP haplotype detected in mixed effects models. Clinical outcomes data other than PTH levels are needed to determine the role of free 25(OH)D measurements in clinical decision-making with the growing recognition of the role that vitamin D and its metabolites play in promoting optimal health beyond bone and calcium absorption metabolism. (39) Currently, most vitamin D intake recommendations are based on immunoassay-measured total 25(OH)D levels associated with lower risk of osteoporotic fractures in postmenopausal women. (40) Clinicaltrials.gov lists over 600 completed phase 2, 3, and 4 trials of vitamin D relationships to various health conditions, 59 active and not recruiting, 149 clinical trials currently recruiting and 36 in the planning stages. (https:\\clinical trials.gov accessed May 30, 2018). Results from two very large randomized double-blind trials investigating vitamin D supplementation effects on cancer, cardiovascular disease and mortality

(VITAL:NCT01169259, and VIDAL:ISRCTN46328341) will soon be available and will provide data on relationships with total 25(OH)D. However, to the extent that the free hormone hypothesis applies to cellular availability of vitamin D metabolites, total 25(OH)D measurements may be misleading in subjects with altered total to free relationships and analysis of free 25(OH)D could provide further insights.

6. ACKNOWLEDGMENTS

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Figure 1. Distribution of free 25(OH)D concentrations are shown for Normal subjects, stable community-dwelling Outpatients, Pregnant women, elderly Nursing Home residents, and Cirrhotics. Free 25(OH)D concentrations are on the horizontal axis, and the number of subjects is plotted on the vertical axis. The curved line represents the normal distribution. Data are only study entry (baseline) concentrations for any subjects enrolled in vitamin D supplementation or dose titration studies.

Figure 2. Per cent free 25(OH)D concentrations are presented by Clinical Subgroup in the left panel and by DBP haplotypes in the right panel (subset of n=974). The box plot shows the 10^{th}, 25^{th}, median, 75^{th} and 90^{th} percentile values. Individual points represent values above the 90^{th} and below the 10^{th} percentile. Both clinical subgroup and DBP genotype had significant effects on per cent free 25(OH)D (ANOVA, $p<.0001$). *Horizontal parentheses indicate statistically significant post hoc between group comparisons (meeting Bonferroni criteria of $p<.0033$). Post hoc between clinical group comparisons were significant for all but normals compared to pregnant or outpatients, or for pregnant compared to outpatients. For DBP haplotypes, smaller but significant differences were detected between the 1s/1s haplotype and 1s/1f, 1f/2, 1f/1f, and 1s/2 haplotypes; and between the 1s/2 and 1f/2 and 1f/1f haplotypes and between the 1s/1f and 1f/1f haplotypes.

Figure 3. Relationships between free and total 25(OH)D by clinical subgroup and DBP haplotype. Total 25(OH)D concentration is plotted on the x axis and free 25(OH)D concentration is plotted on the y axis. In the left panel, open circles represent data from community-dwelling outpatients, closed blue circles represent data from older nursing home (NH) residents, closed brown circles represent data from cirrhotics, pink x represent data from pregnant women, half- filled circles represent data from prediabetics, and closed green circles indicate data from normal/healthy subjects. Data include multiple measures in a subset of healthy normal and NH residents enrolled in vitamin D supplementation studies (n=243 samples). In the right panel, closed blue circles represent the 1s/1s DBP haplotype, half blue and half white circles represent 1s/2 haplotypes, solid green circles represent 1s/1f, solid diamonds represent 2/2, open cross hatched diamonds represent 1f/2, and solid red circles represent 1f/1f. DBP haplotype data were from normals, community-dwelling outpatients, and prediabetics. Linear mixed modelling detected significant effects of clinical groupings on the relationship between free and total 25(OH) D (*$p<.05$, ** $p<.0001$, *** $p<.000001$ for comparisons to

normal/healthy subjects). Significant effects of DBP haplotype on the relationship were not detected.

Table 1. Description of Populations Sampled and Serum Measurements by Clinical Subgroups

	Normal	Community-dwelling Outpatients	Prediabetics	Cirrhotics	Nursing Home	Pregnant
N (%)	279 (16.8)	714 (43)	479 (28.8)	90 (5.4)	79 (4.8)	20 (1.2)
Age	36.6±8.5	68.7±8.5	62±8.6	58.0±8.8	87.4± 8.0	30.7±6.9
Sex –Women n (%)	178 (63.8)	324 (45.4)	184 (38.4)	36 (40)	51 (64.6)	20 (100)
Men	90 (32.3)	390 54.6)	295 (61.6)	54 (60)	28 (35.4)	0 (0)
unknown	11 (3.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Race- White, black	187, 65 (67, 23.3)	518,191 (72.5, 26.8)	479, 0 (100,0)	69, 11 (76.7,12.2)	78, 0 (98.7)	15, 4 (75, 20)
Asian, other, Nat Amer, unknown	12, 1, 2, 12 (4.3,0.4,7.2,4.3)	3, 0,2, 0 0.4,0,0.3,0)	0,0,0,0	6, 0, 4 (6.7, 0,4.4)	1 (1.3)	1 (5)
Weight (kg)	78.5±18.6	83.7±16.7	88.4±16.6	85.5 ± 18.8	69.9 ± 16.4	81.1 ±20.9
BMI	28.0±6.2	29.4±6.0	29.9±4.3	29.1±5.8	27.3±5.8	32.1±7.4
eGFR (ml/min/1.73M^2)	107.1±15.3	79.6±18.1	93.4±12.2	N.A.	63.8 ± 19.4	81.6±25.6
Creatinine (mg/dL)	0.8±0.1	1.0±0.3	0.8±0.2	1.0 ±0.8	0.9 ±0.3	--
Albumin (mg/dL)	4.3±0.4	4.3±0.3	4.5±0.2	3.2±0.8	3.6±0.4	3.6±0.3
Calcium (mg/dL)	9.3±0.4	9.4±0.4	9.2±0.3	8.8±0.7	9.0±0.4	9.1±0.6
Corrected Calcium (mg/dL)	9.1±0.3	9.1±0.4	8.8±0.3	9.4±0.6	9.4±0.2	n.a.
iPTH (pg/mL)^	42.2 ±20.0	44.1±24.7	52.8±20.8	38.8 ±35.3	48.1±25.5	21.8 ±18.0
Free 25(OH)D (pg/mL) •~*$^{\lozenge}$	4.3±1.9$^{\lozenge}$	4.5±1.8$^{\lozenge}$	5.5±1.7$^{\lozenge}$	7.1 ±3.0$^{\lozenge}$	9.5±3.8$^{\lozenge}$	4.0 ±1.1$^{\lozenge}$
Total 25 (OH)D (ng/mL) #~*∞	21.9±9.9~∞	22.5±9.1~∞	24.4±8.7∞	18.7±10.6∞	34.9±12.8∞	26.7 ±10.0∞
Per Cent Free 25(OH) D*$^{\nabla}$	0.020±.006$^{\nabla}$	0.021±.008$^{\nabla}$	.023±.006$^{\nabla}$	.040±.020$^{\nabla}$	.028±.006$^{\nabla}$	.016±.006$^{\nabla}$
D Binding Protein (mcg/mL) `*$^{\vee}$	293 ±51.1$^{\vee}$ (n=159)	294.1±36.5$^{\vee}$ (n=495)	299.2±41.4$^{\vee}$ (n=476)	175.5±64.7$^{\vee}$ (n=58)	264.2±38$^{\vee}$ (n=78)	*529±49.5*$^{\vee}$ (n=20)

Data are mean ± S.D. unless otherwise noted. ^ measured in clinical laboratories by multiple methods. • Assays performed at Future Diagnostics, BV except prediabetics had assays using same method at the Investigator site. # Assays were by LC MS/MS except for 69 (of 90) cirrhotics by Diasorin (LIAISON) that were corrected by a calibration factor provided by the Manufacturer. ~ multiple samples of total and free 25(OH D from some individuals from dose titrations studies. ` D Binding Protein Measurements by radial immunodiffusion assay (Leuven)—with the exception of pregnant women determined by R&D assay (in italics).* Significant effect of clinical group (ANOVA, p<.0001), ◊ post hoc between group comparisons were significant at p<.0033 for all but normals vs. pregnant or outpatients, or for pregnant vs. outpatients. ∞ post hoc between group comparisons were significant at p<.0033 for all but normals compared to outpatients, or pregnant or cirrhotic, or for pregnant compared to outpatients or prediabetics. $^{\nabla}$ Post hoc between group comparisons were significant at p<.0033 for all but normals compared to pregnant or outpatients, or for pregnant vs. outpatients. $^{\vee}$ Post Hoc between group comparisons were significant at p<.0033 for all but normals compared to outpatients or prediabetics, or for prediabetics compared to outpatients.

Table 2. Free, Total, and Per cent Free 25(OH)D and D Binding Protein by DBP Haplotype

DBP Haplotype	Frequency (%)*			Free 25(OH)D (pg/mL)**	Total 25(OH)D (ng/mL)**	Per Cent Free 25(OH)D**	DBP (RID) (mcg/mL)**
	Whites (n=860)	Blacks (n=98)	Other (n=1)				
1s/1s	31.9	1	0	5.1±1.8	25.6 ± 10.0	.021±.006	308.6 ±40 n=209
1s/2	29	1	100	5.1±2.1	23.1 ±8.4	.023±.007	287.9 ±36.2 n=182
1s/1f	22.4	27	0	5.4±2.0	24.2 ±9.0	.023±.007	304.5 ±39.7 n=189
2/2	5.5	0	0	4.1 ±2.0	17.8 ±7.3	.023±.007	260.4 ±25.1 n=24
1f/2	8.3	18	0	4.7±1.8	19.6 ±7.7	.026±.010	289.3 ±34.1 n=73
1f/1f	3	51	0	4.4±1.6	18.2± 8.2	.026±.008	300.1 ±43.5 n=73

*Significant differences in frequencies of haplotypes between the races for all haplotypes except for Gc1s/1f were detected.
**Statistically significant effects of DBP haplotype were detected for Total, free and per cent free 25 (OH)D concentrations and DBP (ANOVA, p<.0001; see Fig 2 and text for individual between haplotype post hoc comparisons.)

Table 3. Linear mixed model analysis of Relationship between Free and Total 25(OH)D

Model: Linear mixed effects regression	Coefficient	S.E.	t value	p value
Model Selected Covariates				
a (Intercept)	1.291	.0781	16.521	<.000001
b (slope)	0.186	.0085	22.024	<.000001
Selected Covariates				
Clinical Class				
Community –dwelling/Outpatients	-.0094	.0046	-2.026	<.05
Prediabetics	0.0245	.0049	5.010	<.000001
Cirrhotics	0.1577	.0080	19.763	<.000001
Nursing Home Residents	0.0873	.0064	13.585	<.000001
Pregnant	-.0450	.0126	-0.357	<.0001
BMI	-.0013	.0003	-4.926	<.000001

The fixed effect model takes the form Free = a + (b +c COV)Total, where a is the intercept, b is the slope of the relationship Free vs Total 25(OH)D, and c is a vector of parameters quantifying the relationship of the slope with covariates. Variables tested but not selected included eGFR and race. Sex was not tested in this model. T and p values represent comparisons to the baseline slope of the model (normals).

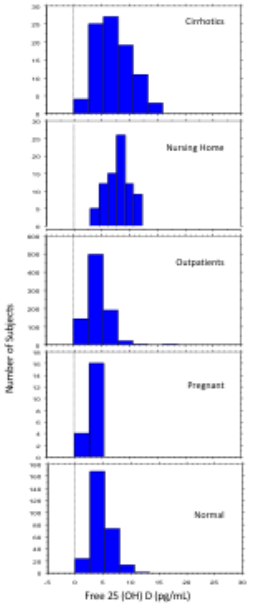

Figure 1

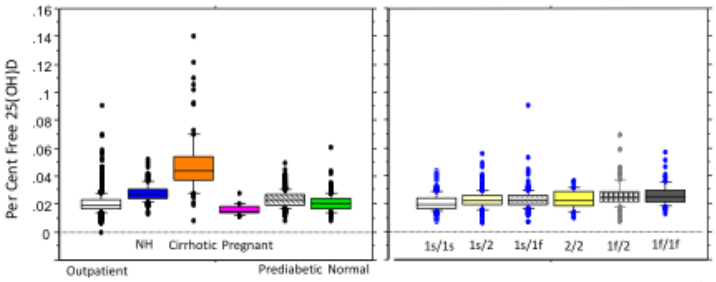

Fig. 2