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Current Controversies: Are free vitamin metabolite levels a more accurate assessment of vitamin D status than total levels?

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Key Points

1. Vitamin D and its metabolites are tightly bound to serum proteins, of which the vitamin D binding protein (DBP) is the most important, such that less than 1% of the total concentration of vitamin D and its metabolites are free in the circulation.

2. For most tissues the vitamin D metabolites enter the cell as the free hormone presumably by diffusion (the free hormone hypothesis), although a few tissues such as the kidney express megalin/cubilin enabling by endocytosis vitamin D metabolites bound to DBP to enter the cell.

3. Measuring the free levels of the vitamin D metabolites may provide a better measure of the true vitamin D status than measuring the total levels.

4. Early methods to determine the free levels of 25(OH)D and $1,25(OH)_2D$ demonstrated that the free levels were normal in patients with liver disease despite low total levels and that free levels were elevated in pregnant women in the third trimester more that would be predicted based on total levels.

5. Newer methods for measuring free 25(OH)D have been developed that are easier to perform, and their wide spread application should help determine the clinical value of determining free 25(OH)D in addition to and/or instead of total 25(OH)D in the evaluation of vitamin D status.

Synopsis

The free hormone hypothesis postulates that only the non bound fraction (the free fraction) of hormones that otherwise circulate in blood bound to their carrier proteins is able to enter cells and exert their biologic effects. For the vitamin D metabolites less than 1% (0.4% for $1,25(OH)_2D$ and 0.03% for 25(OH)D) is free, with over 99% bound to the vitamin D binding protein (DBP) and albumin (approximately 85% and 15%, respectively). Assays to measure the free vitamin D metabolite levels have been developed, and initial studies indicated their value in subjects with altered DBP levels. With the recent development of an ELISA to directly measure free 25(OH)D, the question becomes whether it is better to use the free 25(OH)D measurement instead of the total 25(OH)D measurement to determine vitamin D status. This review will discuss the development of these assays, the initial results, and their potential role in assessing vitamin D status in a variety of clinical conditions.

Introduction

Circulating levels of 25-hydroxyvitamin D (25(OH)D) are the most commonly used marker for the assessment of vitamin D nutritional status. This is because its concentration in blood is higher than all other vitamin D metabolites, making it easier to measure, and because its conversion from vitamin D is substrate dependent with minimal regulation. The liver is the major source of this conversion, performed by a number of enzymes with 25-hydroxylase activity, the most specific of which is CYP2R1. However, 25OHD is not the most biologically active metabolite of vitamin D. Instead 25(OH)D must be further metabolized to 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) for vitamin D to achieve its full biologic potential. $1,25(OH)_2D$ is the ligand for a nuclear transcription factor, the vitamin D receptor (VDR), that mediates the genomic and at least some of the nongenomic actions of vitamin D within the cell. Nearly all if not all cells express the VDR at some stage in their development or activation. The kidney produces most of the circulating $1,25(OH)_2D$ through the enzyme CYP27B1, but many cells also

express CYP27B1, and so are able to form their own 1,25$(OH)_2$D. As the appreciation that vitamin D and its metabolites affect numerous physiologic processes and not just bone and mineral metabolism, and that these physiologic processes may have different requirements for these vitamin D metabolites [1], interest in determining optimal levels of the vitamin D metabolites to effect these different biologic processes has grown. Complicating this determination is the fact that all the vitamin D metabolites circulate in blood tightly bound to proteins, of which the vitamin D binding protein (DBP) plays the major role. For most cells these binding proteins limit the flux of the vitamin D metabolites from blood into the cell where they exert their biologic activity. This raises the issue then of what should we measure to determine vitamin D status: the total levels of these metabolites or the free levels. Before considering this subject directly, a brief review of vitamin D production and metabolism will be undertaken by way of introducing the key players in the vitamin D endocrine system.

Vitamin D production and metabolism

Vitamin D Production

Vitamin D_3 (D_3) (cholecalciferol) is produced from 7-dehydrocholesterol (7-DHC) in the skin through a two-step process in which the B ring is broken by ultraviolet light (UVB spectrum 280-320nm), forming pre- D_3 that isomerizes to D_3 in a thermo-sensitive but non catalytic process. Vitamin D is also obtained from the diet. Most foods with the exception of fatty fish contain little vitamin D unless fortified. The vitamin D in fish is D_3, whereas that used for fortification is often D_2 (ergocalciferol). D_2 is produced by UVB irradiation of ergosterol in plants and fungi (eg. mushrooms). It differs from D_3 in having a double bond between C22-C23 and a methyl group at C24 in the side chain. These differences from D_3 in the side chain lower its affinity for DBP resulting in a higher ratio of free to total vitamin D metabolite concentration as well as faster clearance from the circulation and altered catabolism by the 24-hydroxyase

(CYP24A1) [2-4]. Moreover, a number of immunoassays do not recognize the D_2 metabolites as well as the D_3 metabolites, a problem to which we will return. However, the biologic activity of D_2 and D_3 metabolites are comparable, and if no subscript is used, both forms are meant.

Vitamin D Metabolism

The three main steps in vitamin D metabolism, 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation are all performed by cytochrome P450 mixed function oxidases (CYPs) located either in the endoplasmic reticulum (ER) (eg. CYP2R1) or in the mitochondrion (eg.CYP27A1, CYP27B1 and CYP24A1).

25-hydroxylase. The liver is the major if not sole source of 25(OH)D production. There are multiple 25-hydroxyases, but the best studied are CYP27A1 and CYP2R1. CYP27A1 is the only mitochondrial 25-hydroxylase. It was initially identified as a sterol 27-hydroxylase involved in bile acid synthesis and so is not specific for vitamin D. Moreover, it preferentially hydroxylates D_3 vs D_2. It is widely distributed in the body, not just in the liver. Its relevance to vitamin D metabolism is unclear since its deletion in mice results in increased blood levels of 25(OH)D [5], and inactivating mutations in humans cause cerebrotendinous xanthomatosis not rickets [6]. CYP2R1 is in the microsomal fraction [7], with distribution primarily limited to the liver and testes. It 25-hydroxylates both D_2 and D_3 with comparable kinetics. Deletion of CYP2R1 reduces blood levels of 25OHD by 50%, but not to zero [8] suggesting compensation by other enzymes with 25-hydroxylase activity. However, inactivating mutations in CYP2R1 have been found in humans presenting with rickets [9]. Thus, CYP2R1 is considered the major albeit not the only 25-hydroxylase contributing to circulating levels of 25(OH)D. Regulation of vitamin D 25-hydroxylation is modest at best with production being primarily substrate dependent such that circulating levels of 25(OH)D are a useful marker of vitamin D nutrition.

25(OH)D 1α-hydroxylase (CYP27B1). CYP27B1 is the only known 25(OH)D 1-hydroxylase. Although the kidney is the main source of circulating $1,25(OH)_2D$, a number of

other tissues also express the enzyme. As we will discuss subsequently, most tissues expressing CYP27B1 rely on the free 25OHD level in blood for their available substrate, whereas the kidney tubule has a mechanism for taking up 25OHD still bound to its major binding protein, vitamin D binding protein (DBP). Moreover, regulation of the extrarenal CYP27B1 differs from that of the renal CYP27B1 (review in [10]). The renal 1α-hydroxylase is tightly regulated primarily by three hormones: parathyroid hormone (PTH) (stimulatory), FGF23 (inhibitory), and 1,25(OH_2D itself (inhibitory). CYP27B1 activity in extrarenal tissues is not regulated by PTH and FGF23, but at least in epidermal keratinocytes and cells of the immune system the regulation is by cytokines such as tumor necrosis factor-α (TNFa) and interferon-γ (IFNg) [11 12] [13]. In these tissues as well as in the kidney 1,25$(OH)_2$D induces CYP24A1, the 24-hydroxylase, that catabolizes both 25OHD and 1,25(OH_2D. This is the major mechanism controlling 1,25$(OH)_2$D levels in these cells. As for 25(OH)D, it is anticipated that it is the free concentration of 1,25$(OH)_2$D that enters most cells, the kidney proximal tubule, parathyroid gland, and placenta being likely exceptions as will be discussed.

24-hydroxylase (CYP24A1). CYP24A1 is the only established 24-hydroxylase involved with vitamin D metabolism. It is strongly induced by 1,25$(OH)_2$D. This enzyme has both 24-hydroxylase and 23-hydroxylase activity, the ratio of which is species dependent [14]; the human enzyme has both capabilities. The 24-hydroxylase pathway results in the biologically inactive calcitroic acid, although 1,24,25$(OH)_3$D, the first step in the pathway for 1,25$(OH)_2$D catabolism, has biological activity and 24,25(OH_2D, the first step in 25OHD catabolism, may be important for endochondral bone formation [15]. The 23-hydroxylase pathway produces the biologically active 25OHD-26,23-lactone and 1,25$(OH)_2$D-26,23 lactone. Moreover, unlike many other cancers, the expression of CYP24A1 in melanoma is inversely correlated with melanoma progression [16]. Thus, it is incorrect to consider the 24-hydroxylase as purely a catabolic enzyme for 25(OH)D and 1,25$(OH)_2$D. All steps are performed by one enzyme [17]. Regulation

of CYP24A1 is the reciprocal of that of CYP27B1 at least in the kidney in that PTH inhibits but FGF23 stimulates its expression.

The free hormone hypothesis (figure 1).

The hypothesis and its modification

The free hormone hypothesis postulates that only the non bound fraction (the free fraction) of hormones that otherwise circulate in blood bound to their carrier proteins is able to enter cells and exert their biologic effects. Examples include the vitamin D metabolites, about which we are concerned in this review, sex steroids, cortisol, and thyroid hormone. These are lipophilic hormones assumed to cross the plasma membrane by diffusion and not by an active transport mechanism. However, the free hormone concentration is not the only factor involved with the rate at which the hormone enters the cells. As articulated by Mendel [18], movement of hormone into the cell *in vivo* is dependent not only on the free concentration but also on the dissociation of hormone from its binding protein, the rate of blood flow, the rate of uptake into the cell, and the catabolism/sequestration of hormone within the cell. These are components of the transport process that Mendel calls the free hormone transport process. Thus, the total concentration of hormone, by affecting the total amount of free hormone available to the cell, does influence the extent of transport of hormone into the cell when the rate of transport is not rate limiting. That said, our knowledge of most of these variables is limited, so our focus in this review will be on the free hormone itself, its measurement, and what influences this fraction of the total hormone in circulation.

Development of the hypothesis.

One of the earliest articulations of the free hormone hypothesis was published by Recant and Riggs [19] when they examined thyroid function in patients with protein losing nephropathy. They noted that circulating thyroid hormone (measured as protein bound iodine or PBI) was quite low in these patients along with increased urinary losses but with relatively little evidence

for clinical hypothyroidism. They concluded that “thyroid function and the supply of hormone to the tissues in nephrosis may be normal, and that the low concentration of protein-bound iodine in the plasma is due to the change in concentration or binding capacity of the plasma proteins in nephrosis”. Subsequent studies have established the free hormone hypothesis for the thyroid and steroid hormones [20 21]. Similar conclusions regarding the importance of the free levels of vitamin D metabolites came initially from observations that the increase in $1,25(OH)_2D$ levels with administration of oral contraceptives or during the third trimester of pregnancy was not associated with changes in calcium metabolism, at least until the latter stages of pregnancy, but was accompanied by a parallel increase in DBP [22]. This role of DBP as carrier of the vitamin D metabolites was well demonstrated in mice in which the DBP gene was deleted. Although these mice lost substantial amounts of the vitamin D metabolites in the urine, and their circulating levels of 25(OH) D were very low, they did not develop evidence of rickets until put on a low vitamin D diet [23]. These observations parallel the much earlier observations by Recant and Riggs [19] in their nephrotic patients with low thyroid hormone levels in the absence of clinical hypothyroidism. Subsequently the interest in free 25(OH)D and free $1,25(OH)_2D$ levels has increased substantially because of their relevance to disease states in which the binding proteins are markedly altered such as liver disease and protein losing nephropathy, normal physiologic states such as pregnancy, and genetic variations in binding proteins that may affect their affinity for the hormone in question [24].

The bound, free and bioavailable fractions in serum.

In serum samples from normal individuals, ~85% of circulating vitamin D metabolites are bound to DBP, whereas albumin with its substantially lower binding affinity binds only ~15% of these metabolites despite its 10-fold higher concentration than DBP. Approximately 0.4% of total $1,25(OH)_2D$ and 0.03% of total 25OHD is free in serum from normal non pregnant individuals. The fraction of “bioavailable” vitamin D metabolites is comprised of the fraction of the free vitamin D and the fraction bound to albumin, thus measuring around 15% in normal

individuals. At this point there is little evidence that the albumin fraction is truly bioavailable, although because the albumin-hormone complexes generally dissociate rapidly this fraction may be more bioavailable in a dynamically perfused tissue [25]. That said our discussion will focus on the free fraction, although data examining the relationship of bioavailable 25(OH)D to clinical outcomes will also be considered.

The megalin/cubilin transport system.

As noted above, the free hormone hypothesis postulates that only the free hormone can cross the plasma membrane. For the vitamin D metabolites this is not completely accurate. The renal tubule differs from most other tissues in its mechanism for at least 25(OH)D uptake, and likely for all DBP-bound vitamin D metabolites. The DBP-25(OH)D complex is filtered in the glomerulus and reabsorbed in the proximal tubule through endocytosis ~~mediated~~ by the megalin/cubilin complex, thereby providing 25(OH)D for CYP27B1 1α-hydroxylation in the kidney tubule as well as for the rest of the body [26 27]. This complex is not specific for DBP, but when megalin is deleted, the major protein lost in the urine is DBP. In the mice that survive long enough bone growth is retarded and osteopenic [26]. The impact of cubilin deletion is similar but not as severe [27]. A similar mechanism may operate in the parathyroid gland and placenta, which like the renal tubule express megalin/cubilin [28], but at this point experiments to determine the impact of either megalin or cubilin deletion from the parathyroid or gland or placenta have not been reported.

Assays and methods for assessing free vitamin D metabolite levels

Centrifugal ultrafiltration.

To test the free hormone hypothesis with respect to vitamin D metabolites, centrifugal ultrafiltration was developed to measure the free fraction of 25(OH)D and 1,25$(OH)_2$D [29-32]. The original motivation was first to determine whether the low vitamin D metabolite levels in patients with liver disease truly indicated vitamin D deficiency. There was good reason to

question this in that we and others had shown that patients with liver disease develop osteoporosis, not osteomalacia [33 34], and generally do not respond to vitamin D supplementation [35 36] with respect to their bone disease. In addition, as mentioned earlier, both DBP and 1,25$(OH)_2$D were known to increase during the latter portions of pregnancy, although 25(OH)D levels typically did not, raising the question as to whether the increased 1,25$(OH)_2$D levels were a direct result of the increased DBP levels, or were the free levels also increased disproportionate to that of the total levels [22]. The latter was suggested by the increased intestinal calcium absorption during pregnancy [37], a well-known physiologic target for 1,25$(OH)_2$D (presumably free).

The centrifugal ultrafiltration assay for the vitamin D metabolites was patterned after the method developed by Hammond et al. [38] for the measurement of free sex steroid hormone levels. It consisted of an inner vial capped on one end with dialysis membrane resting on filter pads at the bottom of an outer vial. The serum sample, following incubation with freshly purified ^{3}H-labeled vitamin D metabolite and ^{14}C-labeled glucose as a marker of free water, was placed in the inner vial and centrifuged at 37^{o}C for 45minutes. The ratio of ^{3}H/^{14}C in the ultrafiltrate to that in the sample determined the % free. The free concentration was then calculated by multiplying the % free times the total metabolite concentration. Although the clinical applications for these measurements will be covered subsequently, the initial results demonstrated that indeed patients with liver disease and low DBP and albumin concentrations in parallel with reduced total 25(OH)D and 1,25$(OH)_2$D did have normal free concentrations of these metabolites. This supported the concept of using the ratio of total vitamin D metabolite to DBP levels as predictive of the free vitamin D metabolite level, and indeed vitamin D nutritional status. On the other hand, women in their third trimester of pregnancy with elevated DBP and 1,25$(OH)_2$D levels nevertheless had elevated free 1,25$(OH)_2$D disproportionate to the total 1,25$(OH)_2$D, so the ratio of total vitamin D metabolite to DBP levels was not predictive of the free level. Similarly, the directly measured free 25(OH)D (in this case by the ELISA method to

be described subsequently) was higher than would be expected from the ratio of total 25(OH)D to DBP [39]. Thus during pregnancy it appears that the body has altered the affinity of DBP for the vitamin D metabolites to increase the free fraction. The mechanism for this is unknown. However, as we will now discuss, this apparent change in affinity provides a challenge to the use of calculating the free concentration based on the assumption that the affinity constants do not change under physiologic conditions.

Calculating the free vitamin D metabolite levels.

Centrifugal ultrafiltration was used to determine the affinity constants for DBP and albumin binding to 25(OH)D and 1,25$(OH)_2$D. Scatchard analysis indicated that binding of both 1,25$(OH)_2$D and 25(OH)D in serum fit a two binding site model. The high affinity site, shown to be that of DBP, had an affinity constant (Ka) for 1,25$(OH)_2$D of 3.7-4.2 x 10^7M^{-1} and for 25OHD of 7-9 x 10^8M^{-1}. The lower affinity site, corresponding to albumin, was found to have a Ka for 1,25$(OH)_2$D of 5.4 x 10^4M^{-1} and for 25(OH)D of 6 x 10^5M^{-1} [30 31]. Although differences in the affinity constants for the different DBP alleles have been reported [24], results from other laboratories have not confirmed these differences [40].

These affinity constants could then be used in the following formula for calculating the free fraction of the vitamin D metabolite (25(OH)D or 1,25$(OH)_2$D):

$$\frac{1}{F} = 1 + n1Ka(DBP) * [DBP]_f + n2Ka(alb) * [alb]_f$$

F is the free vitamin D metabolite fraction. $[DBP]_f$ is the free DBP concentration, meaning the level of DBP not bound to the vitamin D metabolite, and $[alb]_f$ is the free albumin concentration. [DBP]f and [alb]f are essentially equivalent to the total concentrations of these proteins (DBP concentration is approximately 5 micromolar, albumin 600 micromolar in normal serum) because the level of saturation of these binding proteins by the vitamin D metabolites is negligible (approximately 1% for DBP and 0.1% for albumin for 25(OH)D, and 3-fold lower for 1,25$(OH)_2$D) under normal circulating levels of the vitamin D metabolites. However, in cases of

vitamin D toxicity, this assumption becomes more problematic [41]. n1 and n2 are the number of sites on DBP and albumin to which the D metabolite binds. n=1 for DBP, but n for albumin is unknown and has been incorporated into the Ka for albumin as a constant. The free fraction is then multiplied by the total levels of the vitamin D metabolite of interest to obtain the free metabolite concentration. The formula has been rearranged since it was first introduced [31] as follows:

$$free\ vitamin\ D\ metabolite = \frac{total\ vitamin\ D\ metabolite}{1 + (Ka_{alb} * albumin) + (Ka_{DBP} * DBP)}$$

In using this formula, one requires accurate measurement of DBP, albumin, and the vitamin D metabolite of interest. Moreover, the calculation depends on an assumption that the affinity constants are invariant. In the initial studies with serum from normal subjects, in which DBP levels were measured with a polyclonal assay, the calculated values agreed reasonably well with the directly measured values using the centrifugal ultrafiltration method [29 31]. In both cases measurement of the total levels of the vitamin D metabolite was required to go from measurement of the free fraction to that of the free level. However, with the commercial development of assays for DBP using monoclonal antibodies that appear to differ in their ability to detect the different DBP alleles compared to the polyclonal antibody assays [42 43], and the apparent change in DBP affinities for the vitamin D metabolites under different physiologic/pathologic conditions [29 32 39], the calculated values diverged substantially from the directly measured levels [39]. Thus a brief discussion of the DBP and vitamin D metabolite assays in use today is in order before considering the recent development of assays that directly measure the free vitamin D metabolite level and so are not dependent of measurements of total levels or the binding proteins.

DBP and its assays

DBP is the major binding and transport protein for vitamin D and its metabolites. DBP is a 51–58 kDa multifunctional serum glycoprotein synthesized by hepatic parenchymal cells. DBP is found in plasma, ascites fluid, cerebrospinal fluid, and on the surface of many cell types. DBP is encoded by the single copy *GC* gene located on chromosome 4q12-q13 [44] and is a member of a multigene family that includes albumin, α-fetoprotein, and α-albumin/afamin [45]. Initially, isoelectric focusing migration patterns identified phenotypic variants [46] termed Group-Specific Component (Gc)1f, Gcs and Gc2 that bound vitamin D [47]. Subsequently the responsible genetic polymorphisms have been identified. Two common missense point mutations in exon 11 of SNPs rs7041 (G/T single-nucleotide variation) and rs4588 (an A/C single-nucleotide variation) result in three common isoforms and different protein products at positions 416 and 420: Gc1F (Asp416, Thr420), Gc1S (Glu 416, Thr420), and Gc2 (Asp416, Lys420) [48]. The SNPs are in complete linkage disequilibrium, and only six haplotypes are observed with any significant frequency (Table 1). Gc2 is the least abundant and Gc1f the most abundant. Gc alleles show distinct racial distribution patterns. Black and Asian populations are more likely to carry the Gc1f form and the Gc2 form is rare, while Whites more frequently exhibit the Gc1s and the Gc2 form. Gc1f has been stated to have the highest affinity and Gc2 the lowest affinity for vitamin D and its metabolites [24], but these differences among alleles have not been found by others [40]. In the absence of disease or pregnancy, DBP levels are relatively constant over time in adults [49]. That said, various substances in the blood such as polyunsaturated fatty acids may alter the affinity of DBP for the vitamin D metabolites [50]. Moreover, as noted previously liver disease leads to reduced levels of DBP [32] as do protein losing nephropathy [51] and acute illness (DBP is an acute phase reactant) [52 53], whereas DBP levels are elevated during the latter stages of pregnancy and with oral contraceptive use [22 29].

DBP is generally measured by immunoassays with either monoclonal or polyclonal antibodies. In 2013 Powe et al. [54] reported that, compared to white Americans, black Americans had similar levels of calculated bioavailable 25OHD despite lower levels of total

25OHD. Free 25(OH)D levels were not determined. This seemed like a logical explanation for the observations that although black Americans have lower total 25(OH)D as a group, they do not have obvious evidence of vitamin D deficiency with respect to skeletal BMD, parathyroid hormone, serum calcium, and phosphate levels. Powe et al. employed a monoclonal antibody-based ELISA to measure DBP levels, and found that those individuals (primarily of African American descent) with the 1f allele had lower DBP levels thus increasing the bioavailable fraction. However, Nielson et al. [42 43] subsequently reported their results with measuring DBP levels with 4 different assays: (1) monoclonal antibody-based ELISA, (2) polyclonal antibody-based radial immunodiffusion assay, and (3) two different polyclonal antibody-based ELISAs. Moreover, they measured free 25(OH)D directly using ELISA methodology to be described subsequently. The monoclonal antibody-based assay resulted in a 54% lower concentration of DBP in black Americans, compared to white Americans and, therefore, a significantly higher calculated free 25(OH)D level. In contrast, there were minimal differences in DBP levels using the 3 polyclonal assays, and significantly lower mean concentrations of free 25(OH)D when calculating this parameter using DBP concentrations from the polyclonal antibody-based method or when directly measuring free 25(OH)D by ELISA. Similarly, mass spectrometry measurements of DBP by Hoofnagle et al. [55] have failed to show a difference in DBP levels with the various alleles. Thus, although it is not clear whether the different DBP alleles have different affinities for the vitamin D metabolites, the alleles do affect the results of immunoassays when a monoclonal antibody is used but not when polyclonal antibodies are employed.

Vitamin D metabolite assays.

The different means of measuring the vitamin D metabolite assays have been extensively reviewed [56]. There are 4 general types of assays currently in use today: competitive protein binding assay (CPBA), immunoassays, liquid chromatography (LC)-UV, and LC-tandem mass spectrometry (LC-MS/MS). LC-MS/MS is becoming the gold standard,

gradually replacing the CPBA and immunoassays [57], and is now used as the reference method for measuring 25(OH)D by the National Institute of Standards and Technology and the Centers for Disease Control and Prevention. However, immunoassays still remain the dominant method in use today for both 25(OH)D and 1,25$(OH)_2$D [58]. Each method has its advantages and disadvantages. Immunoassays require less sophisticated equipment and technical expertise to set up, and they are very sensitive. However, they tend to be more variable than LC-MS/MS as they rely on antibodies that may differ in their recognition of both the D_2 and D_3 metabolites and may be more affected by interfering substances within the sample than LC-MS/MS. On the other hand, LC-MS/MS is less sensitive than immunoassays, generally requiring concentration of the samples with affinity columns and/or derivatization for metabolites other than 25(OH)D. Moreover, unless high resolution liquid chromatography is employed, LC-MS/MS fails to detect the 3-epimer of the vitamin D metabolites and is susceptible to ion suppression by interfering substances (so called matrix effects) [59] and mass spectral overlaps with isobaric compounds with comparable m/z ratios (eg. 7α-hydroxy-4 cholestene-3-one) [60]. However, unlike immunoassays, LC-MS/MS can measure multiple vitamin D metabolites in the same sample.

Directly measured free 25(OH)D

ELISA method. A two-step ELISA that directly measures free 25(OH)D levels has been developed (Future Diagnostics Solutions B.V., Wijchen, The Netherlands based on patented monoclonal antibodies from DIAsource Immunoassays, Louvain-la-Neuve, Belgium). In the first incubation step, an anti-vitamin D monoclonal antibody immobilized on a microtiter plate binds the free 25(OH)D in the serum sample. After washing away excess serum, the second incubation step is to add biotinylated 25(OH)D in a known amount to react with the unoccupied binding sites on the monoclonal antibody attached to the plate. The non-bound biotinylated 25(OH) D is then removed by a second washing. Thereafter, streptavidin peroxidase conjugate is added followed by the substrate 3,3',5,5'-Tetramethylbenzidine (TMB). The bound

streptavidin peroxidase can be quantified by measuring the absorbance at 450 nm generated in the reaction spectrophotometrically. The intensity is inversely proportional to the level of free 25(OH)D. The limit of detection is 2.8 pg/ml. This assay is dependent on the quality of the antibody used to bind the free 25(OH)D. The antibody in the current assay does not recognize $25(OH)D_2$ as well as $25(OH)D_3$ (77% of the 25(OH) D_3 value) so underestimates the free $25(OH)D_2$,. However, under most situations where the predominant vitamin D metabolite is $25(OH)D_3$, the data compare quite well to those obtained from similar populations using the centrifugal ultrafiltration assay [40 61].

LC-MS/MS method. LC-MS/MS has been used to detect 25(OH)D in saliva, which is expected to be free of DBP and albumin and so represents free 25(OH)D [62]. In this method one ml of saliva is deproteinized with acetonitrile, purified using a Strata-X cartridge, derivatized with 4-phenyl-1,2,4-triazoline-3,5 dione (PTAD), ionized by electron spray ionization (ESI) and subjected to LC–MS/MS. The limit of detection was reported as 2 pg/ml, comparable to that of the ELISA method described above. The range of values obtained in normal controls was between 3-15 pg/ml, likewise consistent with the values obtained with the ELISA method [39] and centrifugal ultrafiltration [32].

Thus armed with the means to measure free vitamin D metabolite levels, we turn our attention to whether measuring free levels provides a better means of assessing vitamin D status clinically than measuring the total levels.

Clinical Applications

Clinical studies have investigated relationships between calculated and directly measured free or bioavailable 25(OH)D and parathyroid hormone levels (iPTH), bone density, calcium and calcium absorption, inflammatory measures, and the disease states of cirrhosis, nephrotic syndrome, primary hyperparathyroidism, pregnancy, and in relationship to estrogens and birth control pills or race [40]. Results from investigations of calculated free 25(OH)D and

directly measured free 25(OH)D have not been in complete agreement primarily due to the challenges and differences in DBP assays, total 25(OH)D assays, uncertainty regarding DBP association constants, use of static equations as discussed above, and likely genetic racial admixtures affecting DBP allele distributions in contrast to self-identified race. For this reason, after a brief review of differences of results using the two measurement methods (calculated and directly measured free 25(OH)D), the bulk of the following discussion will be limited to investigations in humans using directly measured free 25(OH)D.

Comparisons of directly measured free 25(OH)D *to calculated free* 25(OH)D.

Direct positive statistically significant correlations are found between results with the two methods, but the relationship accounts for only 13% of the variation, and calculated free 25(OH)D concentrations are consistently higher than directly measured concentrations in healthy humans of all races [39 42 43 63 64]. Most studies also find weak but statistically significant inverse relationships between iPTH and free 25(OH)D in normal subjects and prediabetics [39 49 61 65 66] . Areas of uncertainty include relationships between free 25(OH)D and race, birth control pills, pregnancy in the second and third trimester, and biomarkers of vitamin D effects on bone.

Findings from studies of directly measured free 25(OH)D *in healthy humans.*

Directly measured free 25(OH)D concentrations are strongly correlated with total 25(OH)D concentrations and have been reported to be between 0.02% to 0.09% of total 25(OH)D concentrations (Tables 2, 3 and Figure 2). Concentrations generally range from 1.2-7.9 pg/mL. PTH is negatively correlated with free 25(OH)D as well as total 25(OH)D. Serum C-terminal telopeptide of type I collagen has been reported to have a moderate positive correlation with total and free 25(OH)D [67]. With vitamin D supplementation, free 25(OH)D concentrations rise in concert with total 25(OH)D concentrations [[65 67] [64] [68], rising more steeply with D3

supplementation compared to D2 [66]. With high dose D supplementation the changes in iPTH were significantly related to changes in free 25(OH)D but not to changes in total 25(OH)D or changes in total 1,25$(OH)_2$D [66]. Others have reported that circulating 1,25$(OH)_2$D levels may not correlate with free 25(OH)D [67].

Effects of Race

There are differences in DBP levels and DBP alleles by race that might predict differences in free 25(OH)D. The results from studies that directly measured free 25(OH)D differ in conclusions regarding racial effects. Two support the existence of racial differences with measured free 25(OH)D concentrations being lower in blacks than in whites that are related to lower total 25(OH)D. DBP levels by polyclonal assay did not differ between blacks and whites in these studies. [42 43]. In contrast, Aloia et al found no difference in free 25(OH)D concentrations between black and white postmenopausal women [69]. Where DBP genotype and phenotype were determined, direct measurement of free 25(OH)D diminished differences between DBP phenotypes as compared to serum total 25(OH)D [64].

Effects of Obesity

Walsh, et al studied obese and overweight subjects compared to normal weight subjects in the fall and spring. BMI was negatively correlated with total 25(OH)D, free 25(OH)D, as well as total 1,25$(OH)_2$D. However, obesity altered the relationship between iPTH with similar iPTH levels in all groups, but lower bone turnover markers and higher bone density in obese subjects [70].

Effects of Sex, Female Hormones, and Pregnancy

Investigations have not found differences in free levels of 25(OH)D between men and women in those with prediabetes when corrected for total 25(OH)D [64]. Similarly, we have

found no sex-related differences in free 25(OH) D in normal subjects when corrected for total 25 (OH)D (unpublished data from refs [61 71]. One pharmacologic action of estrogen is to increase DBP and thus one might expect lower free 25(OH)D levels in women receiving estrogen therapy either in oral contraceptives or for postmenopausal hormone replacement. Data on women receiving birth control pills have not been published, and our experience is limited such that definitive statements cannot be made. We have performed an analysis of data from multiple investigations that included information on estrogen administered as hormone replacement therapy to white postmenopausal women and white women of similar age not receiving estrogen. The women receiving estrogen therapy were younger (67 ± 6 years (n=33) compared to 76.6 ± 13.5 (n=116), but they had similar BMI (29.6 ± 6.6 compared to 27.9 ± 6.1). Total 25(OH)D was lower in estrogen-treated women (24.2 ± 10.5 compared 28.1 ± 11 ng/mL), and free 25(OH)D was significantly lower in those on estrogen (4.3 ± 1.9 compared to 5.9 ±2.8 pg/ml, or 0.015 vs 0.021% of total 25(OH)D). When corrected for total 25(OH)D, the effects of estrogen were no longer significant. DBP was measured in a subgroup (n=31 estrogen-treated and 41 without estrogen), and no between group differences were detected. These data are cross-sectional, but the clinical interpretation is that estrogen in doses prescribed clinically for postmenopausal hormone replacement does not appear to significantly alter DBP or free 25(OH)D or alter the relationship between total 25(OH)D and free 25(OH)D.

However, directly measured free 25(OH)D tends to be higher and free $1,25(OH)_2D$ is substantially higher in pregnant women vs comparator groups of women [29 39] (Tables 2 and 3 and Figure 2). These results suggest that the affinity of DBP for the vitamin D metabolites appears to be decreased during pregnancy, perhaps compensating for increased DBP concentrations and the needs of both the mother and fetus for calcium. Whether this reflects the influence of changes in the hormonal milieu during pregnancy on DBP affinity is not known.

Effects of Liver Disease/ Cirrhosis

Directly measured free 25(OH)D and 1,25$(OH)_2$D are higher in outpatients with cirrhosis compared to other groups [32] [39] despite lower total vitamin D metabolite concentrations. (Tables 2 and 3). The relationship between free 25OHD and total 25(OH)D is both steeper and more variable in patients with liver disease than in healthy people (Figure 2). Findings within cirrhotics vary based on the severity of disease and whether or not there is a marked protein synthesis dysfunction as characterized by low albumin concentrations (below 3.5 mg/dL). Those with the most severe cirrhosis and protein synthesis dysfunction, have a higher percentage of free 25(OH)D compared to cirrhotics without protein synthesis dysfunction, but free 25(OH)D concentrations are similar due to the presence of both lower total 25(OH)D concentrations as well as lower DBP. Free 25(OH)D concentrations range between 4.5-8.1 pg/mL in cirrhotics with low albumin and from 6.4-10.6 pg/mL in those with normal albumin. The expected relationships between total or free 25(OH)D and iPTH are present in cirrhotics with normal albumin/serum protein concentrations, but no relationship with iPTH or bone markers is detected in cirrhotic patients with low albumin.

Effects of Multimorbidity

Nursing home residents are older, have more medical problems, receive more medications, and are more likely to have poorer nutrition than younger people or community-dwelling elderly. In vitamin D dose titration studies [68], free 25(OH)D levels rise in response to increases in total 25(OH)D, but responses appear to be steeper than those of normal subjects, younger outpatients, diabetics, or HIV-infected patients (Figure 2). It is likely that this reflects impaired protein synthesis and lower DBP and albumin, but this has not been established.

Summary/Conclusions

Direct measurement of free vitamin D metabolite levels avoids potential errors inherent

in calculating free or bioavailable metabolite levels and is thus the preferred method for free vitamin D metabolite determinations. Directly measured free 25(OH)D is highly correlated with total 25(OH)D in most subjects, and is inversely related to iPTH. However, in disease states such as cirrhosis and in the elderly with multiple co-morbid conditions in whom DBP and albumin levels are likely reduced, or during the latter portions of pregnancy when DBP levels are elevated, conclusions regarding D status based on free vitamin D metabolite measurements may differ from those based on measurement of total metabolite levels. Under these circumstance free vitamin D metabolite levels may represent a better index of vitamin D status. However, experience with direct measurements of the free levels is limited, and firm conclusions cannot be reached regarding the influence of racial differences and the impact of inflammatory or other disease states that may alter the relationship between total and free metabolite levels. Moreover, at present the relationships between free and total vitamin D metabolite levels on markers of vitamin D status such as bone markers and PTH levels do not clearly demonstrate the superiority of free to total measurements. We anticipate, however, that free vitamin D measurements will play an increasingly important role in our assessment of vitamin D status, and the availability of a high throughput assay that can be performed by most clinical laboratories to measure the free 25(OH)D should stimulate further evaluation of its role in clinical medicine as well as in the research laboratory.

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Figure Legends

Figure 1. The Free D Hypothesis.

Vitamin D hydroxy metabolites (blue circles) in the circulation are primarily bound to vitamin D Binding Protein (DBP) and to a lesser extent to albumin (ALB) and possibly to lipoproteins with only the unbound 25(OH)D and 1,25$(OH)_2$D freely crossing the cell membrane or through the nuclear pore to interact with the vitamin D receptor. Many tissues express the 1α-25(OH)D-hydroxylase that metabolizes 25(OH)D to the active 1,25 $(OH)_2$ D. In several tissues such as the kidney and potentially the parathyroid and placenta 25(OH)D and 1,25$(OH)_2$D bound to DBP may enter cell tissues by endocytosis via megalin/cubulin and are not limited to diffusion by the free hormone.

Figure 2. Relationships between Total 25(OH)D and free 25(OH)D in clinical populations.

Total 25(OH) vitamin D concentrations are plotted on the x axis and directly measured free 25 (OH)D levels are plotted on the y axis. Symbols are as follows: X (red) represents pregnant

women in the 2nd or 3rd trimester, squares (orange) represent patients with cirrhosis, open triangles indicate nursing home residents with multimorbidities, solid (purple) triangles are HIV-infected patients, and solid circles are normal subjects (green), outpatients (grey) and diabetics (blue). Relationships between total and free 25(OH)D were significantly different for cirrhotics and nursing home residents as compared to the other groups. No differences in relationships between total and free 25(OH)D were detected between normals, pregnant women, diabetics, outpatients, or HIV-infected subjects. Data from Schwartz JB, Lai J, Lizaola B, et al. A comparison of measured and calculated free 25(OH) vitamin D levels in clinical populations. The Journal of clinical endocrinology and metabolism 2014;**99**(5):1631-7 and Kane L, Moore K, Lütjohann D, Bikle D, Schwartz J. Vitamin D3 effects on lipids differ in statin and non-statin-treated humans: superiority of free 25-OH D levels in detecting relationships. The Journal of clinical endocrinology and metabolism 2013;**98**:4400-9.

Table 1. Vitamin D Binding Protein (DBP) Characteristics by Haplotype

DBP (Gc) Haplotype	Relative Frequency by Race	Sensitivity of Assay for Alleles	
		Monoclonal Antibody	Polyclonal Antibiotic or Proteomic
1f-1f	Black (50%) & Chinese > Hispanic (13%) >White (6%)^	Low	High
1f-1s	Unclear *	Low	High
1f-2	Unclear*	Low	High
1s-1s	White > Chinese> Black	High	High
1s-2	White > Black	High	High

2-2	Chinese> White > Black	High	High

^ Data from C. D. Engelman, T. E. Fingerlin, C. D. Langefeld et al., "Genetic and environmental determinants of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels in hispanic and African Americans," *The Journal of Clinical Endocrinology & Metabolism*, vol. 93, no. 9, pp. 3381–3388, 2008; Xu W, Sun J, Wang W, et al. Association of genetic variants of vit D binding protein (DBP/GC) and of the enzyme catalyzing its 25-hydroxylation (DCYP2R1) and serum vit D in postmenopausal women.Hormones 2014,13(3)345-352 and Yao P, Sun L, Lu L et al. Effects of Genetic and Non-genetic Factors on Total and Bioavailable 25(OH)D Responses to Vitamin D Supplementation. J Clin Endocrinol Metab. 102: 100–110, 2017)

*present in both self-reported Black, Chinese, Hispanic, and white

Table 2. Free 1,25 $(OH)_2$ D in Alcoholic Liver Disease and Pregnancy compared to Controls

Measurement	Alcoholic Liver Disease (n=25)	Controls (n=24)	Pregnancy (n= 17)
Total 1,25$(OH)_2$D (pg/ml) ^	22.6 ± 12.5	41.5 ± 11.5	82 ± 21
Free 1,25$(OH)_2$D (fg/ml)^	209 ± 91	174 ± 46	294 ± 98
% free 1,25$(OH)_2$D	1.098 ± .50	0.424 ± .07	0.359 ±.07
Total 25 (OH) D (ng/ml)~	9.7 ± 4.5	19.2 ± 6.6	27.8 ±8.8
DBP (µg/ml)#	188 ± 105	404 ± 124 (n=18)	576 ± 128

^ method was centrifugal ultrafiltration . # by rocket immunoelectrophoresis. ~ by competitive protein-binding radioassay.
Data from Lundgren S, Carling T, Hjalm G, et al. Tissue distribution of human gp330/megalin, a putative Ca(2+)-sensing protein. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 1997;**45**(3):383-92.

Table 3. Free 25(OH)D in normal subjects compared to patients with cirrhotic liver disease or pregnancy

Measurement	**Liver Disease * n= 42**	**Controls* n=22**	**Cirrhotics ~ N=82**	**Pregnancy^ (n=20)**	**Controls ^ (n=111)**
Total 25(OH)D (ng/ml)	10.9 ± 9.5	19.2 ± 6.7	15.5 † (10.2–23.8)	26.2 ± 11.4#	26.7 ± 10.0 #
% free 25(OH)D	0.068 ± .029	0.030 ± .007	0.043 (0.037–0.053)	0.016 ±0.004	0.02 ± 0.014
Free 25(OH)D (pg/ml)	6.61 ± 4.61	5.88 ± 2.27	6.8 (5.0–9.1)	4.5 ±1.6	4.0 ±1.1
DBP (µg/ml)	178 ± 92	405 ± 128	100.6 (63.3–157.1)	460.3 ±229.5	220.3 ±100

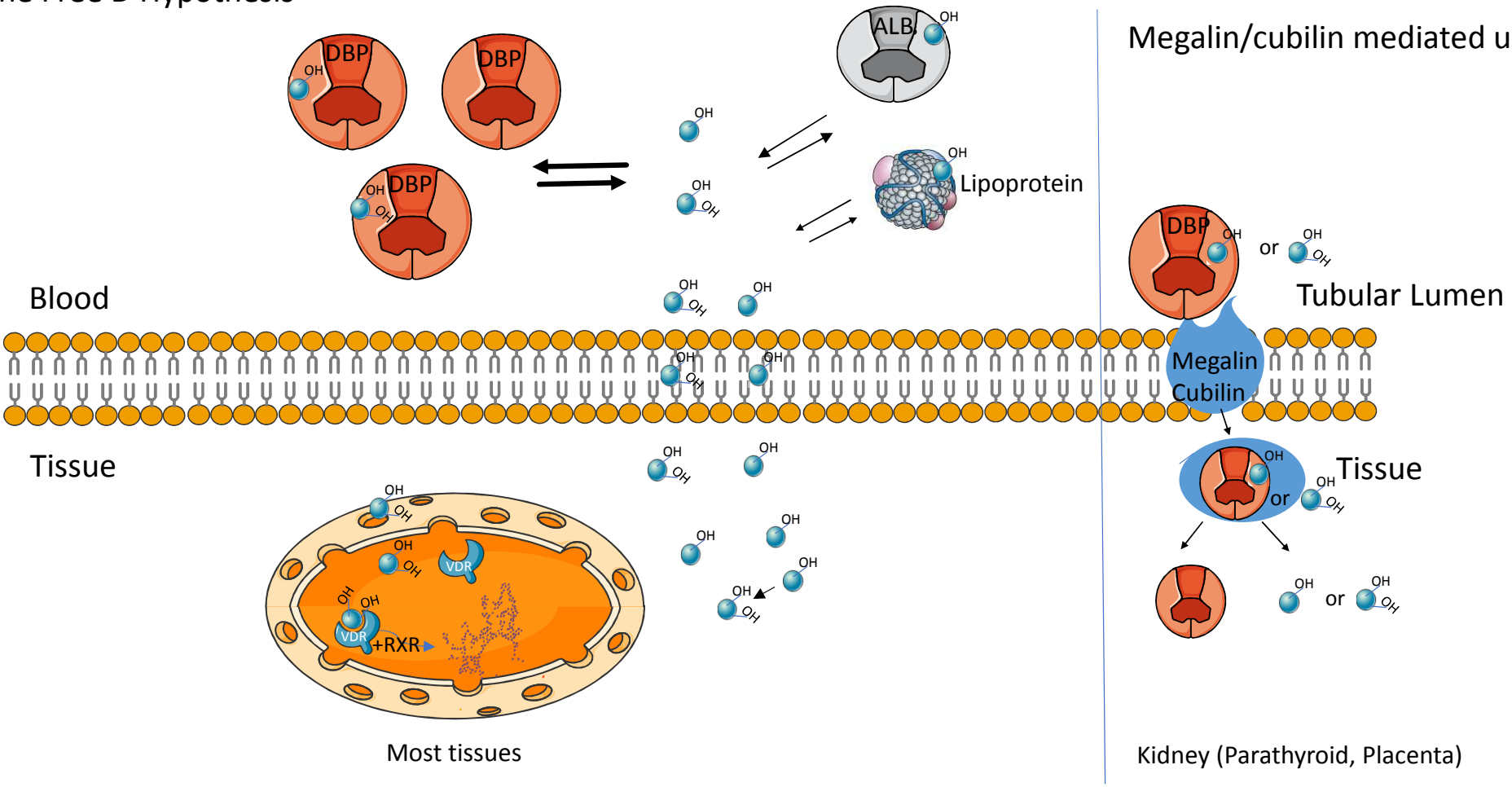
Albumin (g/dl)	2.83 ± .66	----------	3.2 (2.7–3.8)	3.3 ±0.3	4.2 ± 0.3

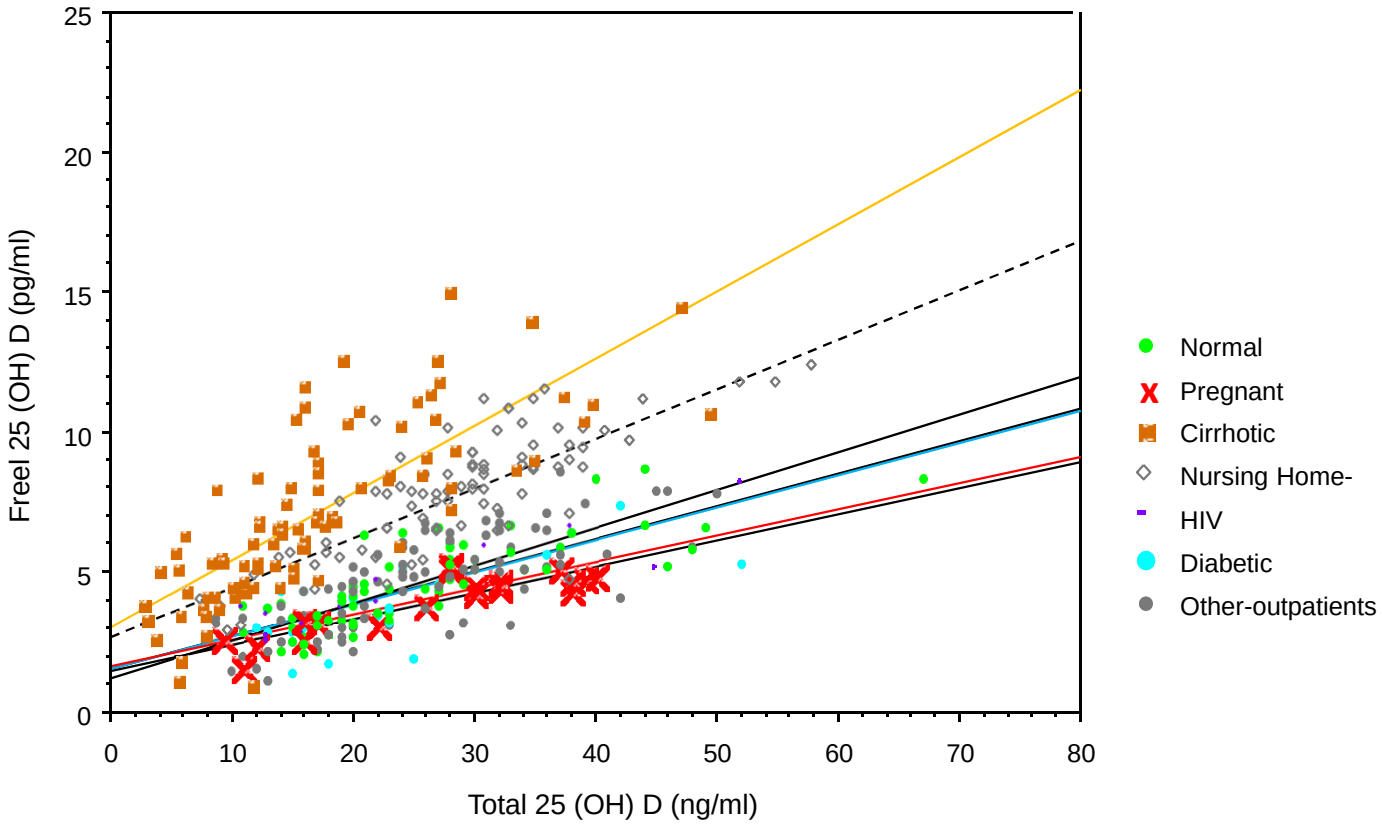
*Shaded area and asterisk indicate Data from Reference 30 with measurements of free 25(OH)D made by centrifugal ultrafiltration and DBP by rocket immune-electrophoreseis. ~ indicates data are from reference 69. ^ indicates Data are from Reference 60 – in both studies measurements of free 25(OH) D were by immunoassay (Future Diagnostics) and DBP was by monoclonal assay (Quantikine Human Vitamin D Binding Protein Immunoassay kit, R&D Systems, Inc) which results in lower DBP levels than the original polyclonal assay employed in Ref 30.

measured by MS/MS. ¶Measured by immunoassay (R& D). Data are reported as mean + SD except for cirrhotics indicated by † for data not normally distributed and median and range are presented.

Data from references 30, 60, 69.

The Free D Hypothesis




Freel 25 (OH) D (pg/ml)
Total 25 (OH) D (ng/ml)
0
5
10
15
20
25
0
10
20
30
40
50
60
70
80
Normal
Pregnant
Cirrhotic
Nursing Home-
HIV
Diabetic
Other-outpatients