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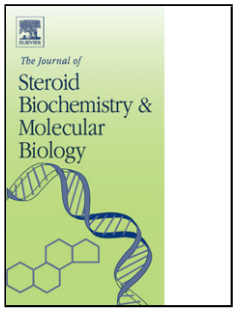

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Vitamin D metabolites in captivity? Should we measure free or total 25(OH)D to assess vitamin D status?

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Highlights

- Free 25(OH)D can be measured directly by a two step immunoassay, ultrafiltration or dialysis. All such assays need further validation.
- Free 25(OH)D can also be calculated based on measurements of total vitamin D metabolites, DBP, albumin, and the affinities between the metabolite and the binding proteins.
- The measurement of DBP needs validation and standardization, and the affinity for the polymorphic DBP needs reassessment.
- Some DBP assays are compromised by unequal affinity of the antibody employed for all known DBP isoforms, and the affinity of DBP for the vitamin D metabolites may vary under different physiologic conditions.
- Assays to evaluate functional differences in DBP genetic variants may shed light on the role of DBP in regulating free 25(OH)D levels
- Free 25(OH)D (measured or calculated with the best available methodology) is closely correlated with total 25(OH)D concentrations in healthy adults
- Calculated and measured free 25(OH)D (using the best presently available methods) is lower in African Americans than in US Caucasians. Africans living in The Gambia, in contrast to African Americans, have high total and high free 25(OH)D while having the same DBP/GC genotypes, suggesting that differences in free 25(OH)D are not driven by genetic factors.
- Free not total 25(OH)D or $1,25(OH)_2D$ regulates vitamin D actions in all cells tested so far
- Whether free 25(OH)D correlates better than total 25(OH)D to serum PTH, bone mass and bone markers or extra-skeletal endpoints is not yet well established
- The relative importance of free versus total 25(OH)D or $1,25(OH)_2D$ under different physiological and pathological conditions requires further studies.

Abstract

There is general consensus that serum 25(OH)D is the best biochemical marker for nutritional vitamin D status. Whether free 25(OH)D would be a better marker than total 25(OH)D is so far unclear. Free 25(OH)D can either be calculated based on the measurement of the serum concentrations of total 25(OH)D, vitamin D-binding protein (DBP), albumin, and the affinity between 25(OH)D and its binding proteins in physiological situations. Free 25(OH)D can also be measured directly by equilibrium dialysis, ultrafitration or immunoassays. During the vitamin D workshop held in Boston in March 2016, a debate was organized about the measurements and clinical value of free 25(OH)D, and this debate is summarized in the present manuscript. Overall there is consensus that most cells apart from the renal tubular cells are exposed to free rather than to total 25(OH)D. Therefore free 25(OH)D may be highly relevant for the local production and action of $1,25(OH)_2D$. During the debate it became clear that there is a need for standardization of measurements of serum DBP and of direct measurements of free 25(OH)D. There seems to be very limited genetic or racial differences in DBP concentrations or (probably) in the affinity of DBP for its major ligands. Therefore, free 25(OH)D is strongly correlated to total 25(OH)D in most normal populations. Appropriate studies are needed to define the clinical implications of free rather than total 25(OH)D in normal subjects and in disease states. Special attention is needed for such studies in cases of abnormal DBP concentrations or when one could expect changes in its affinity for its ligands.

Key words: free vitamin D metabolites, vitamin D binding protein, centrifugal ultrafiltration, liver disease, pregnancy, keratinocytes, genetic polymorphism,

Introduction

The free hormone hypothesis claims that only hormones which are not bound to high-affinity carrier proteins are free to enter cells and exert biological activity (1). This fundamental concept has been well established for other hormones, such as sex steroid and thyroid hormones (1-3). Similar to steroid and thyroid hormones, vitamin D is highly lipophilic and has protein carriers that help maintain circulating serum stores. The majority of circulating vitamin D (25(OH)D) is tightly bound to DBP (85-90%), an abundant circulating α-globulin produced by the liver. Approximately 10-15% of serum 25(OH)D is bound to albumin. The binding constants of DBP and albumin are ~1000-fold different, with albumin being the weaker carrier (4). Less than 0.1% of vitamin D circulates freely (4,5) and measurement of free 25(OH)D and 1,25$(OH)_2$D is technically difficult due to their low concentrations and physico-chemical behavior.

DBP, added to cultures of keratinocytes, monocytes or osteoblasts, or to kidney homogenates or bone tissue cultures substantially inhibits cellular uptake and actions of vitamin D metabolites (6-9). However, DBP-null mice maintain normal calcium homeostasis when fed a vitamin D-replete diet and have no evidence of bone disease despite having extraordinarily low levels of total circulating 25(OH)D and 1,25$(OH)_2$D, approximately 1% of wild type controls (8,10). In addition, in DBP-null mice, vitamin D is more rapidly taken up by the liver, and 1,25$(OH)_2$D had a more rapid effect on calbindin induction in the intestines. However, the half life of these metabolites was much shorter in the DBP null mice, urinary losses of 25(OH)D were greater, and when placed on a vitamin D deficient diet the mice developed evidence of vitamin D deficiency more rapidly (secondary hyperparathyroidism and characteristic bone changes) (10). These in vivo observations in DBP null mice largely supported the idea that free 25(OH)D and 1,25$(OH)_2$D concentrations are biologically more important than their total concentrations, with DBP providing a circulating storage function for these metabolites.

In some types of cells it has been shown that specific serum protein carriers for these hormones may bind to and be transported across the cell membrane, thereby potentially enabling uptake of

their hormone cargo (11,12). This allows such cells to have access to the total hormone (bound and free) concentration. The best example is the renal tubular cell which expresses megalin and cubilin, together creating a cell surface receptor complex which acts to internalize DBP and its bound ligands. This megalin-cubilin protein complex functions as a cargo receptor and transport system for a large number of proteins, including but not limited to DBP and the bound vitamin D metabolites. This mechanism allows the kidney to recover essential proteins and their ligands and is also crucial for the reabsorption of DBP and its bound vitamin D metabolites from the glomerular filtrate (13). This explains the greater urinary losses of the vitamin D metabolites in DBP null mice (10) or in many cases of nephrotic syndrome (14). Some other tissues (including the parathyroid gland) express megalin, although its expression is far from universal and is usually low outside the kidney (15,16). Whether or not the expression of megalin/cubilin is expected to distinguish which cells will be responsive to DBP-bound vitamin D metabolites versus those which are only capable of responding to the free metabolite remains for future investigation.

The *GC* gene encoding DBP has several genetic polymorphisms that may alter its vitamin D binding affinity and carrying capacity. The three most common variants of DBP (also known as GC globulin) are GC1F, GC1S, and GC2. Each variant is characterized by a different combination of two single nucleotide polymorphisms (rs4588 and rs7041) resulting in two amino acid substitutions and differing glycosylation patterns (17). (Table 1, Figure 1).

There is a general belief that serum (total) 25(OH)D is the best marker of the vitamin D status. A large number of people around the world have low serum 25(OH)D concentrations which may increase their risk for skeletal and possibly also for extra-skeletal diseases and risks (18,19). More recently there have been several studies suggesting that unbound (free or bioavailable[1]) 25(OH)D concentrations may be a better marker for several outcomes (bone, PTH or other extra-skeletal end points) than total 25(OH)D. This question attracted much attention when Powe et al (20) reported that African Americans had much lower DBP concentrations (more than 50% lower than US Caucasians). As a result, despite their much lower total 25(OH)D concentration, their calculated

[1]
- Free 25(OH)D = unbound to whatever serum protein
- Bioavailable 25(OH)D: not bound to high affinity binding protein (= free 25(OH)D + albumin bound 25(OH)D)

free or bioavailable 25(OH)D was equal or even slightly higher than in Caucasians. This raised the question whether vitamin D supplementation of African Americans (as recommended by IOM 2010 and other guidelines) was required. More importantly, these observations questioned whether total 25(OH)D levels were the best marker of vitamin D status across different races or ethnic groups. Although several groups confirmed low DBP levels in African Americans (21-24) using the monoclonal R&D assay, questions were rapidly raised as to whether the monoclonal R&D assay used in these studies could be relied upon to measure polymorphic DBP as present in the serum of subjects of different races or DBP/GC genotypes (25). The organizers of the vitamin D workshop 2016 (Boston, March 2016) invited Roger Bouillon to chair a debate with 3 experts discussing the question of how to reliably measure free 25(OH)D and DBP, summarize the existing literature with regard to 25(OH)D and racial/genetic differences and whether or not free 25(OH)D estimations are better measurements of vitamin D status than total 25(OH)D.The key questions to be answered by the group are presented in table 2. Moreover the group was asked to identify key questions to be addressed in future studies. The present manuscript summarizes this debate and its major conclusions.

Vitamin D Status in Captivity: To Free or Not to Free? (R Thadhani)

Although DBP serves as an excellent high affinity reservoir for serum vitamin D metabolites, the tight affinity also implies that bound vitamin is not available for diffusion into target tissues (unless DBP is endocytosed and degraded). The relatively low affinity for albumin, in contrast, makes albumin-bound vitamin D more dissociable, diffusible and theoretically more “bioavailable” to surrounding tissues.

Experimental studies in genetically modified mice have suggested that circulating DBP prolongs half-life of 25(OH)D levels (10). When circulating DBP levels are lower, such as in cirrhotic subjects, total 25(OH)D levels are also lower, but importantly free 25(OH)D (surrogate of bioavailable) levels are relatively normal (see Table 1 and Table 2, Bikle section), supporting the hypothesis that

measurements of free or bioavailable 250HD may be superior to total 25(OH)D in critically ill subjects to assess vitamin D status.

Clinical studies performed by our group and others have shown that estimated bioavailable 25(OH)D ($B_{av}D$, the fraction of 25(OH)D not bound by DBP) is more strongly associated with classical measures of vitamin D adequacy such as bone mineral density (BMD) (26), parathyroid hormone (PTH), serum calcium (27), and the presence of osteoporosis (28) than total 25(OH)D, consistent with the hypothesis that $B_{av}D$ is the fraction relevant to key vitamin D effects.

In our study conducted in the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) cohort, we analyzed the DBP genotypes of HANDLS subjects for nucleotide polymorphisms rs4588 and rs7041, thus determining the expression of Gc1F, Gc1S, and Gc2 protein variants in each subject. We found that the prevalence of the Gc1F allele in American blacks was ~80%, whereas 80% of American whites carried the Gc1S allele. Furthermore, the strong associations that we observed between these genetic traits and levels of DBP, total 25(OH)D, and $B_{av}D$ suggested that these genetic traits may be the explanation for the known racial differences in total 25(OH)D levels. In addition to observations from our group and others in black and white Americans, a number of other large genetic studies in various populations across the globe (including Europeans, West Africans, Arabs, Danes, Korean, and Chinese) have demonstrated consistent associations between the Gc1F and Gc2 DBP variants and lower concentrations of serum 25(OH)D, providing corroborating evidence that the DBP variants and not ethnicity or skin color are responsible for maintaining vitamin D equilibrium (20,29-34). Alterations in the gene coding for DBP have also been linked in epidemiologic studies with response to vitamin D supplementation, osteoporosis, and cancer (35-41).

Although potentially paradigm-shifting, we acknowledge the limitation that our findings relied upon calculated (not directly measured) values for $B_{av}D$, and measured levels of DBP, as was highlighted in a commentary about our manuscript in Clinical Chemistry (42). There are multiple challenges to assuming the accuracy of $B_{av}D$ levels when the estimates are based upon measurements of

25(OH)D and DBP, namely: the method depends upon the accurate measurement of both 25(OH)D and DBP concentrations, and presumes that the interaction between 25(OH)D and DBP variants *in vivo* matches the measured *in vitro* binding affinity binding constants. Importantly, assays used to determine DBP concentrations differ depending on the assay kit (ie, Alpco Diagnostics vs R&D Kit), a fact that we and others have acknowledged (20,42). Furthermore, past studies that measured affinity binding constants of several DBP variants under different conditions obtained widely discrepant results which depended upon the buffering agent used (43-45). Such findings make it difficult to choose any set of affinity binding constants for calculation of $B_{av}D$.

Because of these caveats and limitations, several authorities have called for a simple direct assay to measure $B_{av}D$, a position that we strongly endorse. Direct measurement of $B_{av}D$ is preferable as it would: (1) bypass the need to determine each individual's DBP phenotype and DBP levels; (2) eliminate reliance on literature estimates of binding affinity constants for the different DBP variants to calculate $B_{av}D$; (3) allow determination of $B_{av}D$ in heterozygotes [as now these calculations are only applicable to individuals homozygous for Gc1F/Gc1F and Gc1S/Gc1S]; (4) avoid errors in calculated $B_{av}D$ values caused by additional DBP variants with unknown binding affinities; (5) eliminate the increased imprecision inherent to assays that use multiple measured variables for calculating results. The development of a direct $B_{av}D$ assay would, therefore, go a long way toward settling the ongoing debate about how to evaluate vitamin D status so that future studies could focus more on the mechanisms that drive genetically-based differences in vitamin D handling. In the absence of reliable and validated BavD assay or free 25(OH)D assays, we propose that other measures of vitamin D deficiency including blood levels of PTH or calcium, with assessment of bone health, be used in conjunction with total 25(OH)D levels to determine who requires supplementation with exogenous vitamin D.

The free vitamin D metabolite hypothesis revisited: development of the assay and its clinical utility (Daniel D Bikle)

Our motivation for developing the first direct method for determining the free concentrations of the vitamin D metabolites was three fold. The first and most obvious is that in order to test the free hormone hypothesis a method to measure the free hormone levels, in this case free 25(OH)D and $1,25(OH)_2D$, had to be developed. The second was to determine whether the low vitamin D metabolite levels in patients with liver disease truly indicated a vitamin D deficient state. There was good reason to question this in that we and others had shown that patients with liver disease develop osteoporosis, not osteomalacia (46,47), and generally do not respond to vitamin D supplementation (48,49). The third reason was that both DBP and $1,25(OH)_2D$ 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 $1,25(OH)_2D$ levels were a direct result of the increased DBP levels, or were the free levels also increased. The latter was suggested by the increased intestinal calcium absorption during pregnancy (50), a well-known physiologic target for $1,25(OH)_2D$. The following describes the results of those early studies, and will further describe the results of a newer and easier to perform assay for free 25(OH)D that we have used to confirm our original data but also to further probe the relative advantages of free 25(OH)D measurements vs total 25(OH)D measurements as an assessment of vitamin D sufficiency.

Development of the centrifugal ultrafiltration assay

The centrifugal ultrafiltration assay by which we measured the free levels of $1,25(OH)_2D$ and 25(OH)D (4,51) was patterned after the method developed by Hammond et al. (52) 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°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. We used this

method to determine that in normal serum binding of both $1,25(OH)_2D$ and 25(OH)D fit a two binding site model. The high affinity site, proven to be DBP by depleting DBP with an actin affinity column and thus eliminating the high affinity binding site, was shown by Scatchard analysis to have a Ka for $1,25(OH)_2D$ of 3.7-4.2 x 10^7M^{-1} and a lower affinity site corresponding to albumin with a Ka for $1,25(OH)_2D$ of 5.4 x 10^4M^{-1} (53). The Ka values for 25(OH)D were found to be 7-9 x 10^8M^{-1} and 6 x 10^5M^{-1} for DBP and albumin, respectively (4). Knowing these affinity constants permitted a calculation of the free metabolite concentration by measuring the DBP and albumin concentrations using the following formula: $1/F=1+ n1Ka1(DBP)_f + n2Ka2(alb)_f$ where F is the free metabolite fraction, 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 DBP and albumin concentrations ($(DBP)_f$ and $(alb)_f$) are taken as equivalent to the total concentration as so little is bound to the vitamin D metabolites. Although this calculation provides a reasonable approximation of free metabolite concentrations in normal serum, it relies on accurate measurement of DBP and albumin and assumes an invariant binding of DBP and albumin to the vitamin D metabolite being assessed. Unfortunately neither of these assumptions has proven valid with the wide variation in DBP measurements extant with existing antibodies (25,54), differences in the affinity of the different DBP alleles for the vitamin D metabolites (55), and a clear change in binding affinities by DBP and albumin in different physiologic and pathologic states (51,56,57). In particular, subjects with liver disease have a higher % free vitamin D metabolite concentration than expected for their DBP concentration, whereas the reverse is true in the sera from pregnant women. The reasons for this are not known.

Testing the free hormone hypothesis.

To determine whether only the free vitamin D metabolite could enter cells we (58) used cultured human foreskin keratinocytes. These cells are one of the most robust cells for evaluating vitamin D metabolism and one of the most sensitive cells in response to $1,25(OH)_2D$ with respect to regulating vitamin D metabolism (59,60). The keratinocytes were incubated in media containing 0.1-10% human serum albumin or 0.1-10% human serum to which 10^{-11} to 10^{-8}M $1,25(OH)_2D$ was added for

4hr to assess 1,25$(OH)_2$D production or 16hr to assess 24,25$(OH)_2$D production at which point [^{3}H]-25(OH)D was added for an additional 1hr. Free 1,25$(OH)_2$D was assessed in all media at all total 1,25$(OH)_2$D concentrations. The results showed that the amount of exogenous 1,25$(OH)_2$D required to suppress 1,25$(OH)_2$D production and stimulate 24,25$(OH)_2$D production increased with increasing concentrations of albumin or serum, but the ED50 for free 1,25$(OH)_2$D with respect to either suppression of 1,25$(OH)_2$D production or stimulation of 24,25$(OH)_2$D was constant at 10^{-11}M. This indicates that only the free 1,25$(OH)_2$D level is seen by the keratinocytes with respect to vitamin D metabolism, direct proof of the free hormone hypothesis. That said keratinocytes are not known to express megalin/cubilin, and similar experiments have not been done with cells like renal tubules or parathyroid glands, which express megalin/cubilin, and which may give different results.

Determination of free 1,25$(OH)_2$D and 25(OH)D levels in normal subjects, subjects with liver disease, and pregnant women.

The results of our initial studies (51,56) evaluating the free 25(OH)D and 1,25$(OH)_2$D levels in normal subjects and subjects with liver disease or in the latter stages of pregnancy are shown in table 1 except that the measurements of free 25(OH)D during pregnancy are from a more recent study (57) using the immunoassay for free 25(OH)D that will be discussed later.

As demonstrated in this table the free 1,25$(OH)_2$D and 25(OH)D levels in subjects with liver disease are at least as high if not higher than the controls despite the markedly reduced levels of the total 25(OH)D and 1,25$(OH)_2$D. DBP and albumin levels were reduced resulting in a marked increase in % free in subjects with liver disease. The increased free levels may be due to the care providers of such individuals providing additional vitamin D supplementation in response to the low total vitamin D metabolite levels, although those records are not available to us. The levels of total 1,25$(OH)_2$D in the pregnant women were quite high, as were the DBP levels, but the free 1,25$(OH)_2$D levels were also elevated despite the reduction in % free. However, total levels of 25(OH)D were not particularly elevated, and the free 25(OH)D was lower than that of controls. This likely reflects increased metabolism of 25(OH)D to 1,25$(OH)_2$D during pregnancy by the placenta (61).

Comparison of centrifugal ultrafiltration and the newer immunoassay for free 25(OH)D measurements.

Centrifugal ultrafiltration is a labor intensive and expensive assay. Commercially available ultrafiltration devices proved unreliable as did commercially available equilibrium dialysis methods because of the tendency for the tracer to stick to the sides of the vessel and membrane resulting in an artefactual reduction of free metabolite levels (56). Therefore we made our own equipment that avoided this problem, but this required time and attention to detail. Moreover, this assay required freshly prepared (by HPLC) metabolite tracers to eliminate all degradation products that would produce falsely high % free values in that polar metabolites have reduced affinity for DBP. However an immunoassay has recently been developed (Future Diagnostics) that is much easier to do and comes in kit form. In this assay, an anti-25(OH)D antibody is coated on a microtiter plate. Serum samples and calibrators are pipetted into the wells of the microtiter plate. Free 25(OH)D is captured by the antibody during a first incubation. After washing, a biotin-labeled 25(OH)D analog is allowed to react with the unoccupied antibody binding sites in a second incubation. After a second washing step and incubation with a streptavidin-peroxidase conjugate, bound enzyme is quantitated using a colorimetric reaction. Intensity of the signal is inversely proportional to the level of free 25(OH)D in the sample. The major downside of this assay is that the anti-25(OH)D antibody has lower affinity for 25(OH)D_2 by 20-30%, and so may underestimate free 25(OH)D_2. We (57) used this method to measure free 25(OH)D in normal, liver disease and pregnant subjects. Although these were different populations than those in our original studies, and the assays were done approximately 30 years apart, the similarity of results is striking with respect to the free 25(OH)D measurements. That said the DBP results show much lower values using the monoclonal R&D Systems assay for these newer studies compared to the polyclonal assay used previously. Table 3 shows the data for free 25(OH)D in pregnant subjects, for which we have no comparable data using centrifugal ultrafiltration. Table 4 shows the results in normal subjects and those with liver disease comparing the two assays. The results for centrifugal ultrafiltration from table 1 are reproduced here to facilitate comparison.

Both assays show increased % free and free 25(OH)D in the subjects with liver disease, and despite being completely different populations, the results for % free and free 25(OH)D are comparable between the two assays. However, when we (57) compared the directly measured free 25(OH)D to the calculated free 25(OH)D the results were quite discrepant, in part due to the very different DBP measurements. This was particularly true for those of African American descent (2.9x higher calculated than directly measured free values) and to a lesser extent Asians (2.1x higher calculated than directly measured free values), but the disparity was also observed in the Caucasian population (1.5x higher calculated than directly measured free values).

Potential utility of the free 25(OH)D assay.

As demonstrated above, measuring the free 25(OH)D is likely a better measure of vitamin D sufficiency in liver disease than is the total 25(OH)D. Similarly in pregnancy, measuring the free 25(OH)D is likely to call more attention to the extent of vitamin D insufficiency in many of these individuals than is the total, which will be increased by the higher DBP levels. In addition to these earlier studies we (57,62-64) have compared the correlations between total and free 25(OH)D with several potential markers of vitamin D action. In one cross sectional study we (57) observed a significant negative correlation between free 25(OH)D and PTH, but this correlation was no stronger than that for total 25(OH)D. On the other hand in an older population in which the subjects were given different doses of vitamin D supplementation we (64) observed a significant negative correlation between free 25(OH)D and PTH that was not seen with total 25(OH)D. In a third study focused on cirrhotics we (63) found significant correlations between free 25(OH)D and markers of bone turnover as well as PTH, but again the correlations were no stronger than those for total 25(OH)D. In a fourth study in which vitamin D deficient subjects were given enough vitamin D (1000-3000 iu/day) to increase their 25(OH)D levels above 25ng/ml, only the free 25(OH)D significantly correlated with the reduction in triglycerides and LDL cholesterol at least when combined with statin therapy. Given that similar data were found for campesterol suggested an effect of vitamin D on cholesterol absorption that was more readily seen when cholesterol synthesis was inhibited by the statin (62). Thus these studies are suggestive that free vitamin D metabolite

measurements may confer an advantage to total vitamin D metabolite measurements, but with the exception of individuals with extreme variations in DBP levels, these early studies are far from conclusive.

Conclusions

Free vitamin D metabolite measurements have provided an essential test of the free hormone hypothesis. Although the universality of this hypothesis that only the free metabolites can enter the cells is plausible and widely accepted, it certainly is the case for a least one cell, the keratinocyte. Moreover, free metabolite measurements have proven useful in reconsidering whether low total 25(OH)D and $1,25(OH)_2D$ in subjects with liver disease truly indicates a vitamin D deficient state. Moreover, free 25(OH)D and $1,25(OH)_2D$ measurements have demonstrated the high levels of free $1,25(OH)_2D$ and lower levels of free 25(OH)D in pregnancy not totally reflected by the total D metabolite levels. Whether measurement of free 25(OH)D, now possible relatively easily, will better reflect vitamin D signaling than measurement of total 25(OH)D in individuals without extreme DBP levels remains for future investigation. We (Schwartz and Bikle) are currently gathering data from a number of investigators who have measured both total and free 25(OH)D levels in association with a wide variety of markers of bone and mineral metabolism in hopes of determining the value of free 25(OH)D measurements in a variety of conditions not associated with extreme levels of DBP.

Free 25 hydroxy vitamin D in multi-racial studies (Inez Schoenmakers)

Recently free 25(OH)D has received considerable interest, particularly in the investigation of racial disparities in the relationship of 25(OH)D with diverse health outcomes (Table 5). It has been suggested that serum free 25(OH)D may be a better measure of tissue availability and utilisation and may be a better predictor of functionality of vitamin D than total plasma 25(OH)D (55). However, there is conflicting evidence regarding racial differences in the relationship between total and measured or calculated free 25(OH)D (20,25-27,54,57,65-68).

Free 25 hydroxy vitamin D and vitamin D binding protein in multi-ethnic groups

Recently, we presented data that part of the findings on free 25(OH)D and the racial differences in its proportion to total 25(OH)D were confounded by methodological issues in one of the most commonly used DBP assays (25,54). Since calculated free 25(OH)D is derived from the concentration of DBP, the validity of methodology to measure DBP is critical. In a large cohort consisting of men of different ethnic origin and geographical diverse regions, we showed that there are no racial differences in the plasma DBP concentration (25,54), except when a monoclonal immune assay for DBP (R&D Systems, Minneapolis, MN, US) was used. This finding was confirmed in other multi-ethnic cohorts, consistently showing that racial disparities in DBP concentrations were not present with other methods. These included the measurement of DBP with 3 different polyclonal assays, SRM-based proteomic analyses of genotypically variant regions of DBP by LC-MS and DBP quantification by LC-MS (69-72). It has been suggested that the pronounced differences in DBP concentrations between races and GC-genotypes, as produced by the monoclonal immune assay for DBP are likely caused by selective affinity differences of the monoclonal ELISA kit antibody for specific genotypes of DBP, with lower affinity for the DBP isoform GC-1F and a higher affinity for GC-1S (25,69). This however remains to be experimentally shown, preferably by using genotype specific purified DBP.

Racial differences in the predominant DBP genotype explain the reported differences in DBP concentrations between white and African Americans when the monoclonal DBP assay is used. DBP is a polymorphic glycoprotein, the frequencies of which vary according to race. As a result of the differences in plasma concentrations of DBP by DBP genotype as derived from the monoclonal assay, the calculated free 25(OH)D concentration is artificially elevated in those subjects carrying a GC1F allele (i.e. due a to an apparent lower DBP concentration) and vice versa lower in those with a Gc1S allele. Accordingly, the calculated ratio between free and total 25(OH)D is higher with a GC1F1F and GC1F2 genotype, compared to those with other genotypes. These findings were in discordance with the values derived from polyclonal methods and the novel ELISA for the direct measurement of free 25(OH)D. Using these methods, there was no difference between DBP genotypes or race in the free 25(OH)D to total 25(OH)D ratio (25,54). Similarly, we demonstrated

that measured and calculated plasma free 25(OH)D are highly correlated in a healthy population, irrespective of race, DBP genotype or 25(OH)D concentration (25,54). Other reports have confirmed this finding (68,72), whereas one group found a higher free to total ratio in African Americans even when using polyclonal DBP methods and the ELISA for the direct measurement of free 25(OH)D (73). The underlying explanation for the discrepancies between these reports needs to be determined. The law of mass action predicts that with a lower 25(OH)D and a similar DBP concentration, also free 25(OH)D would be lower, proportionally to total 25(OH)D. A further factor complicating the comparisons between reports is the lack of standardization of DBP assays. Although values produced by different poly clonal DBP assays generally correlate well, Bland-Altman analyses showed considerable stepwise differences between assays (54,69).

The relationship of total and free 25(OH)D with markers of vitamin D and calcium metabolism and non-calciotropic health outcomes

Table 5 summarises relevant studies reporting statistical association between total and free 25(OH)D and markers of vitamin D and calcium metabolism and non-calciotropic health outcomes. Details on the methodology used to derive free 25(OH)D (directly measured or calculated and if calculated the DBP assay details are given), the selected outcome measure and population characteristics are described. The last column indicates whether free 25(OH)D had a stronger statistical relationship with the outcome compared to total 25(OH)D. This summary table shows that some reports, while others do not, demonstrate stronger statistical associations for free 25(OH)D compared to total 25(OH)D with parathyroid hormone (PTH), bone mineral density (BMD) and various non-skeletal or calcaemic outcomes, including the risk of various types of cancer and complications of renal disease. For any category of these outcomes findings were inconsistent, irrespectively of methodology and whether cohorts were racially homogenous or heterogeneous. The predominant method was calculated free 25(OH)D and utilized the monoclonal DBP assay (19 of 38 reports). Although some corrected for race, the findings in these reports are therefore likely to be confounded by the above described factors affecting DBP measures and the calculated free 25(OH)D values. Further, many of these studies concerned patient groups in which physiological

and pathological factors may have influenced plasma DBP concentrations and/or the measured free 25(OH)D concentration and thus findings may depend on the methodology used. The assessment of these associations for diverse health outcomes with methods not confounded by DBP genotype and/or direct measurement of free 25(OH)D is an important field for future research. This research should aim to elucidate possible racial differences in vitamin D function and also focus on potential differences between organ systems, including those with and without a functional megalin/cubilin internalization mechanism. Further, DBP concertation and genotype may have effects independent of vitamin D and should be considered in these studies.

Ethnic differences in calcium and bone metabolism and free 25(OH)D

The value of free 25(OH)D to understand racial differences in bone mineral density and the relationship between total 25(OH)D and PTH and their respective associations with bone health indices has been evaluated by few authors (20,68,72,73). It is well-known that there are pronounced racial differences in bone mass and metabolism, the prevalence of osteoporosis, and age-related fragility fractures (summarized in Cauley (74), Redmond (75) and Yan (76)). In summary, whereas a chronically elevated PTH concentration is a risk factor for bone loss and fracture in white populations, this association is absent or weaker in non-white populations. This is thought to be partly related to differences in the skeletal and renal response to calciotropic hormones. African Americans have been shown to have a higher plasma PTH concentration at any 25(OH)D concentration. However, African Americans were reported to exhibit a relative skeletal resistance to PTH compared to white counterparts. This was not found in older healthy Gambians with an elevated PTH and a good vitamin D status (76). Differences in plasma concentrations of $1,25(OH)_2D$ between ethnic groups appear to be less consistent (27,54,73,77). Further, renal calcium conservation at any level of calcium intake was shown to be proportionally higher in those of an African descent (African Americans as well as Gambians) (75,78). None of the few studies investigating the potential value of free 25(OH)D to explain these racial disparities found that using free 25(OH)D strengthened the relationship with BMD or PTH, except Powe's study which used the monoclonal ELISA for DBP to derive free 25(OH)D, a method recently shown to be flawed by

selective affinity differences for the DBP genotypes as described above (20,69-72). The role of vitamin D in these racial differences in bone and calcium metabolism therefore remains unresolved.

Conclusion

In healthy people the ratio between total and free 25(OH)D is similar for those of a European and African descent, and free and total 25(OH)D are highly correlated over a wide concentration range (Figure 2). This implies that free 25(OH)D strongly depends on total 25(OH)D. Therefore, in the majority of the healthy populations (i.e. in those without conditions modulating DBP) free 25(OH)D and total 25(OH)D can be uniformly used, irrespectively of race, vitamin D status or geographical region, provided appropriate DBP quantification is used. Research is limited whether or not free 25(OH)D is a better marker of 25(OH)D availability to tissues and more strongly linked to health outcomes, and there is very little evidence that free 25(OH)D may provide an explanation for racial differences in bone and calcium metabolism.

General discussion and Conclusions

All vitamin D metabolites circulate mostly bound to serum proteins. The major binding protein, DBP, combines a high affinity and high capacity for the D metabolites so that the free concentrations are very low (less than 1% of the total concentrations). The estimation of free metabolites is therefore technically more complex than for most other hormones. Calculation of free 25(OH)D or free $1,25(OH)_2D$ depend on the concentration of total D metabolites (the measurement of which are now better standardized through several external quality assurance schemes and the international harmonization initiative (79), total DBP (so far not standardized) and correct estimation of the affinity of DBP for the D metabolites in conditions similar to that of serum.

DPB is a highly polymorphic protein and there are two well characterized amino acid differences in the C domain of DBP depending on the 3 common genotypes (GC 1f, 1s and GC2). This polymorphism has possible implications for the accurate measurements of DBP (whether by immunoassays or by MS/MS). Recent data using a variety of polyclonal antibodies or MS/MS indicate that the mean concentrations of DBP are largely race and genotype independent (except

for a slightly lower [approximately 10%] concentration of DBP in GC2-2 carriers). The recently published data on much lower concentrations of DBP in people of African origin (more than 50% lower than in Caucasians) are due to a monoclonal DBP assay that provides DBP concentrations that are DBP genotype-dependent. The data generated by these studies should be reanalyzed. Moreover there are posttranslational modifications of this glycoprotein that are so far poorly characterized. Whether there are genotype-specific differences in affinity of DBP is controversial but the majority of independent studies revealed no significant differences in affinity (Table 6). Therefore there is a need for ***a better standardization of DBP measurements*** using methodology similar to the previous successful standardization program for 25(OH)D.

Direct measurements of free 25(OH)D by either dialysis or ultrafiltration is technically possible but hindered by many pitfalls. However the results obtained so far are generally confirming the free hormone hypothesis for vitamin D metabolites. A direct two-step immunoassay for free 25(OH)D is now commercially available and preliminary data show good correlations with calculated or directly measured (dialysis) values, at least in normal subjects. Further validation and standardization is needed in subjects with abnormal DBP or protein concentrations or major illnesses (including CRF and critical illness). The absolute concentrations of free 25(OH)D measured by direct assays are substantially lower than calculated values, again demonstrating ***the need for international standardization***.

The data summarized in this report (Table 5) on the relative strength of associations of free versus total 25(OH)D with skeletal or extra-skeletal outcomes are inconclusive. This is partly due to the widespread use of a DBP assay that incorrectly detects low DBP concentrations in carriers of GC1f and partly due to lack of standardization of the free assays. Many tissues outside the kidney express CYP27B1 and can thus potentially produce $1,25(OH)_2D$ for autocrine and paracrine functions. There is so far no definite proof, such as based on tissue specific knock out models of Cyp27b1, for an important role for this extra renal production, but this remain a plausible hypothesis. If the local (extrarenal) production of $1,25(OH)_2D$ is physiologically important, it is plausible that free 25(OH)D is the rate limiting step for cellular access of this precursor in many extra renal tissues that express CYP27B1. Further studies with in vitro and preclinical animal models and above all,

properly designed studies using well validated free 25(OH)D measurements are essential to address these questions. This research may have important implications for better understanding of the vitamin D endocrine system and may have important implications for the choice of methods to be used in clinical chemistry laboratories. Finally, much attention so far has been focused on free 25(OH)D but free 1,25$(OH)_2$D may be even more relevant than total 1,25$(OH)_2$D concentrations as free 1,25$(OH)_2$D concentrations are most likely to be strictly regulated through endocrine feed-back systems. In contrast, free 25(OH)D concentrations are more dependent on total 25(OH)D without clear endogenous feed-back control. The debate on free vitamin D metabolites presented at the vitamin D workshop in March 2016 gave an update on the state-of the art of the research arena but generated more research questions than final answers. This should not be a total surprise as it took more than two decades before free thyroid hormone measurements in routine clinical chemistry laboratories are finally beginning to be harmonized and standardized (80). There is a fairly large agreement on the physiologic and clinical implications of free thyroid or sex steroid hormones but even in such fields there remain a number of controversial visions. The vitamin D community should learn from the vitamin D assay standardization programs and from the other fields of endocrinology as to accelerate the **understanding of free 25(OH)D and 1,25$(OH)_2$D concentration for physiology and clinical situations.**

Conflicts of interest:

The authors have no conflicts of interest to declare.

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Figure 1. Variation of DBP allele frequencies by country and race (adapted from Kamboh and Ferrell, 1986).

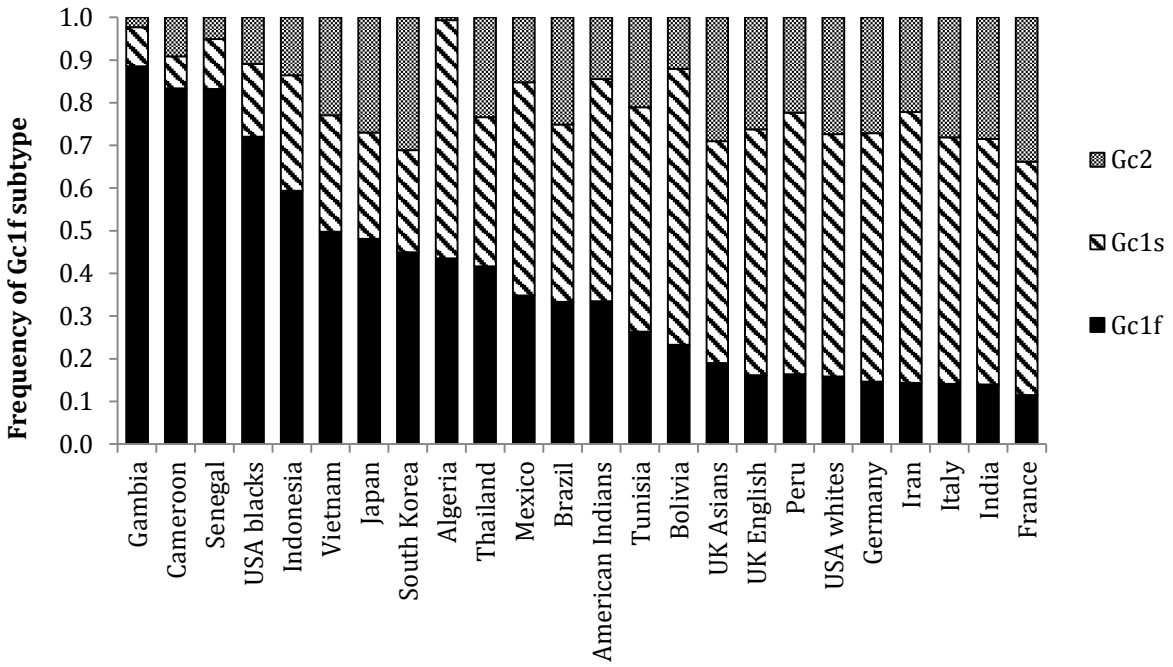

Figure 2. Relationship between total and calculated free 25(OH)D using a poly clonal RID (Top panel) or a monoclonal ELISA for the measurement of vitamin D binding protein. Values are given as standard deviation score and in absolute plasma concentrations. The mean ratio of free (f25(OH)D) total 25(OH)D (t25(OH)D) per race is given (adapted from Nielson et al., 2016).

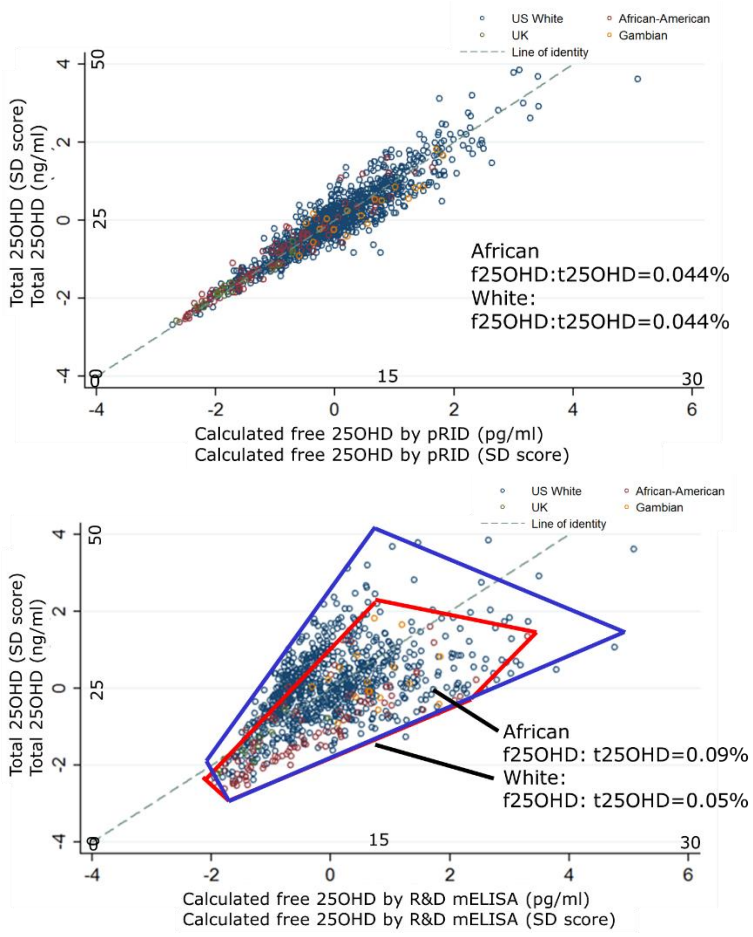

Table 1. DBP-GC genotype dependent protein structure

AA	**432(416)***	**436(420)***
Gc 1f	Asp	Thr**
Gc 1s	Glu	Thr**
Gc 2	Asp	Lys
Snp	rs 7041	rs 4588
	Asp → Glu	Thr → Lys

* renumbering by counting the pre-sequence

** Threonine of Gc1 is O-linked with NAcGalactosamine, Galactose (with or without additional sialic acid)

Co-dominant genetic transmission = 6 common genotypes

More than 120 variants known but mostly due to unknown protein modifications

Table 2. Debate on free vitamin D metabolites

Question 1: How do we presently evaluate free vitamin D metabolites?

1.1 Calculated free 25(OH)D (or 1,25$(OH)_2D_3$), according to the "law of mass action"

depends on accurate estimation of total 25(OH)D, DBP and affinity

1.1 Directly measured free 25(OH)D

- dialysis
- ultrafiltration
- (two step) immune assays

Question 2: Is free (= non-protein-bound) or bioavailable (= free + albumin-bound) the best choice?

Question 3: Do measurements of free or total concentrations of vitamin D metabolites provide better information on biological/clinical actions?

Table 3. Measurement of total and free $1,25(OH)_2D$ and 25(OH)D in normal subjects compared to those with liver disease or pregnancy

Measurement	Liver Disease	Pregnancy*	Controls
total $1,25(OH)_2D$ pg/ml	22.6 + 12.5	82 + 21	41.5 + 11.5
% free $1,25(OH)_2D$	1.098 + .50	0.359 + .07	0.424 + .07
free $1,25(OH)_2D$ fg/ml	209 + 91	294 + 98	174 + 46
DBP µg/ml	188 + 105	576 + 128	404 + 124
albumin g/dl	2.6 + .8	NM	4.4 + .2
total 25(OH)D ng/ml	10.9 + 9.5	26.7 + 10.0	19.2 + 6.7
% free 25(OH)D	0.068 + .029	0.02 + .00	0.030 + .007
free 25(OH)D pg/ml	6.61 + 4.61	4.0 + 1.1	5.88 + 2.27
DBP µg/ml	178 + 92	460.3 + 229.5	405 + 128
albumin g/dl	2.83 + .66	3.3 + .3	4.46 +.24

* The measurements of free 25(OH)D in pregnant subjects were made using an immunoassay (Future Diagnostics) and not by centrifugal ultrafiltration, although the results of the two assays are quite similar (even though done in different subjects). Likewise the assay for DBP in this group of subjects used a different method (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 our original analyses. Results are reported as mean + SD. NM=not measured. For further details and references: see text.

Table 4. Comparison Comparison of centrifugal ultrafiltration to immunoassay in the measurement of free 25(OH)D in normal subjects and those with liver disease

	Centrifugal Ultrafiltration		Immunoassay	
Measurement	**Liver Disease**	**Controls**	**Liver Disease**	**Controls**
total 25(OH)D ng/ml	10.9 + 9.5	19.2 + 6.7	14.0 + 7.3	26.2 + 11.4
% free 25(OH)D	0.068 + .029	0.030 + .007	0.05 + .02	0.02 + .01
free 25(OH)D pg/ml	6.61 + 4.61	5.88 + 2.27	6.3 + 3.2	4.5 + 1.6
DBP µg/ml	178 + 92	405 + 128	112.2 + 64	218 + 57
albumin g/dl	2.83 + .66	4.46 +.24	2.6 + .5	4.2 +.4

Results are reported as mean + SD. For further details and references: see text.

Table 5. Summary table of relevant studies reporting statistical association between total and free 25(OH)D and markers of vitamin D and calcium metabolism and non-calciotropic health outcomes.

Author	Method and assay used for free 25(OH)D;DBP	Outcome measure	Population	f25(OH)> t25(OH)D_3
Calciotropic (vitamin D and calcium metabolism, bone mineral density): observational cohort studies				
Powe (26)	Calculated; DBP mELISA[1]	PTH, LS-BMD	US; N= 49; 31 white, 18 non white; healthy men and women, 18-31y/a	Yes
Powe (20)	Calculated: DBP mELISA[1]	PTH, LS-BMD	US; N=2085; 904 white, 1181 AA; men and women, 30-64 y/a	Yes
Dastani (81)	Calculated; DBP p-immune assay (Dako)	PTH	Canada; N=2073; white Men and women	no
Jones (66)	Calculated; DBP pRID[6]	25(OH)D half-life	UK, The Gambia; N=36; 18 white; 18 african-gambians; healthy men, 25-40y/a	no
Johnson (82)	Calculated; DBP pRIA (in house)	PTH and BMD multiple sites	Norway; N=265; predominantly white, few sami; women with low BMD, 50-80y/	Yes for BMD
Pop (83)	Calculated; DBP pELISA[3]	PTH	US; N=165; 16 white; 149 non-white; healthy women, 26-75y/a	no
Jemielita (68)	Calculated; DBP mELISA[1] and pELISA[3]	BMD multiple sites	US; N=304; 146 white, 158 AA; men and women, 21-80 y/a	No
Goswani (84)	Calculated; DBP mELISA[1]	PTH	India; N=194 Indoor and outdoor workers; race not given; men.	no
Aloia (73)	Measured: f25(OH)D ELISA[2] Calculated; DBP mELISA[1] and pELISA[3]	PTH, BMD, CTX	US; N=164; 82 white; 82 AA; healthy women, 65 (IQR 62-68) y/a	No
Calciotropic (calcium metabolism, bone mineral density): Vitamin D intervention studies				
Aloia (77)	Measured: f25(OH)D ELISA[2]	Ca absorption, PTH, CTX, NTX	US; N=71; 53 white, 7 AA, 11 other; healthy women; 58.8 ± SD4.9 y/a	No
Alzaman (72)	Measured: f25(OH)D ELISA[2] Calculated; DBP: mELISA[1] and pELISA[5]	PTH, change in t25(OH)D, f25(OH)D	US; N=208; 151 white; 57 AA; DDM2 or pre-DDM2 overweight men and women; 59.1(Sd8.6)y/a	no
Shieh (85)	Measured: f25(OH)D: ELISA[2]	PTH, change in t25(OH)D, f25(OH)D	US; N=38: 11 white, 1 AA, 7 other; men and women, >18y/a	yes
Sollid (86)	Measured: f25(OH)D: ELISA[2] Calculated; DBP In house pRIA	PTH	Norway; N=472; predominantly white; prediabetic men and women; 21-80 y/a	No
Schwartz (64)	Measured: f25(OH)D ELISA[2]	PTH	US, N=81; 80 white, 1 other; men and women, 87.4 (SD8) y/a	Yes
Calciotropic (calcium metabolism, bone mineral density): case and case-control studies				
Reid (87)	Calculated; DBP pELISA[4]	PTH	UK; N=33 knee arthroplasty patients with osteoarthritis; race not given; men and women; 52-81y/a	No
Briggs (88)	Calculated; DBP mELISA[1]	PTH, FGF23	UK; N=28; patients with diaphyseal long bone fracture; race not given, men and women; 53.5(SD24.3)y/a	No
Aggarwal (89)	Calculated; DBP mELISA[1]	PTH, BMD hip and femoral neck	India, N=146: 106 nephrotic syndrome, 40 controls; no race given: men and women 18-70y/a	yes
Denburg	Calculated; DBP mELISA[1]	Indices of CKD,	US; N=148 CKD patients (stages 2-5); 104	Yes

(90)		PTH, FGF-23	white, 37 AA, 7 other; men and women; 5-21 y/a	
Denburg (65)	Calculated; DBP mELISA[1]	PTH, FGF23, Cortal BMD	US; N=171 CKD patients (stages 2-5); 117 white, 44 AA, 18 other; men and women; 5-21 y/a	No
Bhan (27)	Calculated; DBP mELISA[1]	PTH	US; Haemodialysis patients N= 47 and N=47 controls; both 23 white and 24 AA , men and women; 65(IQR50-74) y/a	Yes
Schwartz (57)	Measured f25(OH)D ELISA[2] Calculated; DBP mELISA[1]	PTH	US; N=151: 24 cirrhotic patients, 20 pregnant women (2^{nd}/3^{rd} trimester), 107 healthy controls; 61 white, 31 AA, 15 other; men and women, 16-90 y/a	No: calculated Yes: measured
Lai (91)	Measured: f25(OH)D ELISA[2]	PTH, CTX, BSAP, P1NP, OC	US, N=82 cirrhotic patients; 46 white, 36 other, men and women, 60 (54-63) y/a	Yes
Cancer risk: case control studies				
Anic (92)	25(OH)D:DBP ratio DBP mELISA[1]	RR colorectal cancer	Finland, N=416 colorectal cancer patients; 416 controls; No race given; men 50-69 y/a	No
Anic (93)	25(OH)D:DBP ratio DBP mELISA[1]	HR lung cancer death	Finland, N=428 cases of fatal lung cancer; 72 cases surviving lung cancer. No race given; men, 50-69 y/a	No
Mondul (94)	25(OH)D:DBP ratio DBP mELISA[1]	OR bladder cancer	Finland; N=250 bladder cancer cases; 250 controls; No race given; men, 50-69 y/a	Yes
Weinstein (95)	25(OH)D:DBP ratio DBP mELISA[1]	OR pancreatic cancer	Finland; N=234 pancreatic cancer patients; 234 controls; No race given; men, 50-69 y/a	Yes
Modul (96)	DBP mELISA[1]	OR renal cell carcinoma	Finland; N=262 cell carcinoma patients; 262 controls; No race given; men, 50-69 y/a	no
Ying (97)	25(OH)D:DBP ratio DBP mELISA[1]	OR colorectal cancer	China; N=212 colorectal cancer patients; 212 controls; no race given; men and women, 37-83 y/a	Yes
Weinstein (98)	25(OH)D:DBP ratio DBP mELISA[1]	OR colorectal cancer	US; N=476 colorectal cancer patients; 476 controls: both 412 white, 41 AA, 23 other; men and women, 55-74 y/a	no
Wang (99)	25(OH)D:DBP ratio DBP mELISA[1]	RR breast cancer	US; N= 584 breast cancer patients; 584 controls; 94% white; women, 25-42 y/a	No
Diverse other outcomes and designs				
Behrens (100)	Calculated; DBP mELISA[1]	RR Multiple Sclerosis	Germany, case-control study; N=76 MS patients, 76 controls; white; man and women, 19-56y/a	No
Ashraf (101)	Calculated; DBP mELISA[1]	Insulin resistance	US, N=47; 16 white, 31 AA; healthy post-menarchal women, 14-18y/a;	No
Ashraf (102)	Calculated; DBP mELISA[1]	Vascular health indices	US, N=47; 16 white, 31 AA; healthy post-menarchal women, 14-18y/a;	Yes
Berg (103)	Calculated; In house DBP ELISA (poly/mono clonal unknown)	Markers of COPD	US, N=498; 348 COPD patients, 150 controls; majority white, men and women, 40-75y/a	no
Leaf (104)	Calculated; DBP pELISA[3]	C-FGF23, RR mortality and sepsis	US, Case-control study: N=30 Acute Kidney Injury patients + 30 controls; both 13 white, 17 non-white; men and women, 18-70y/a	Yes
Srikanth (105)	Calculated; DBP mELISA[1] and pELISA[6] and pRID[7]	Inflammation markers	US, Observational cohort study N=679; N=679; 616 white, 22 AA, 41 other; older men	no
Rebholz (106)	Calculated; DBP LC-MS	OR ESRD	US, Case-control study; N=184 ESRD patients; 100 white, 84 AA, 251 controls; 146 white, 105 white; men and women, 45-65 y/a	yes
Kane (107)	Measured; f25(OH)D ELISA[2]	lipids, plant sterols, cholesterol synthesis	US, Vit D3 RCT ; N=26 supplemented; 19 white, 5 AA, 2 other; 23 placebo; 12 white, 6 AA, 1 other; men and women, 35-	Yes

85y/a

Abbreviation used: 25(OH)D: 25 hydroxy vitamin D; f25(OH)D: free 25(OH)D; t25(OH)D: total 25(OH)D; DBP: vitamin D binding protein; AA: African American; PTH: parathyroid hormone: BMD: bone mineral density; LS: lumbar spine; RID; radio immuno diffusion assay; CTX: C-terminal telopeptide of type 1 collagen; NTX: n-terminal telopeptide of type 1 collagen; FGF23: fibroblast growth factor 23; DDM" type 2 diabetes; CKD: chronic kidney disease; BSAP; bone specific alkaline phosphatase; P1NP: N-terminal propeptide of type 1 collagen; OC: osteocalcin: RR: realtive risk; HR: hazard ratio; OR: Odds ratio; MS: multiple Sclerosis; COPD: Chronic obstructive pulmonary disease: ESRD; end stage renal disease

Footnotes: [1]DBP monoclonal ELISA: R&D systems; [2] Free 25(OH)D: ELISA: Future Diagnostics; [3]DBP polyclonal ELISA: ALPCO; [4]DBP: Polyclonal ELISA: Biosupply UK Ltd; [5] DBP Polyclonal ELISA : BDRG Instruments; [6]DBP Polyclonal ELISA : Genway; [7]Poly clonal radial immune diffusion assay (in Jones et al[4])

Table 6. DBP genotype and 25(OH)D affinity *

		Ka (10^{-8}M)			
Authors	**sample**	**Gc1f**	**Gc1s**	**Gc2**	**Tracer**
Kawakami (108)	diluted serum n = 10	1.9	1.8	1.8	[^{3}H]25(OH)D
Bouillon (44)	diluted serum n = 9	3.6	3.0	4.9	[^{3}H]25(OH)D
Boutin (45)	purified homozygous GC (n=10)	2.7	2.7	2.8	[^{3}H]25(OH)D
Arnaud (43)	diluted serum n = 25	1.12	0.6	0.36	[^{3}H] vitamin D

* - All values reported as 10^{-8}M

- all methods use high specific activity [^{2}H]25(OH)D except Arnaud & Constans who used [^{3}]vitamin D_3 instead of 25(OH)D_3 (of low specific activity)