VITAMIN D BINDING PROTEIN, TOTAL AND FREE VITAMIN D LEVELS IN DIFFERENT PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL CONDITIONS

EDITED BY: Daniel David Bikle, Zhongjian Xie and Xiangbing Wang
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VITAMIN D BINDING PROTEIN, TOTAL AND FREE VITAMIN D LEVELS IN DIFFERENT PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL CONDITIONS

Topic Editors:
Daniel David Bikle, University of California, San Francisco, United States
Zhongjian Xie, Central South University, China
Xiangbing Wang, Rutgers, The State University of New Jersey, United States

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EDITORIAL
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Editorial: Vitamin D Binding Protein, Total and Free Vitamin D Levels in Different Physiological and Pathophysiological Conditions

Zhongjian Xie[1]*, *Xiangbing Wang*[2] and *Daniel D. Bikle*[3]

[1] Hunan Provincial Key Laboratory of Metabolic Bone Diseases, National Clinical Research Center for Metabolic Diseases, Department of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, Changsha, China, [2] Division of Endocrinology, Metabolism and Nutrition, Rutgers University-Robert Wood Johnson Medical School, New Brunswick, NJ, United States, [3] Endocrine Unit, Veterans Affairs Medical Center, University of California, San Francisco, San Francisco, CA, United States

Keywords: vitamin D binding protein, total 25-hydroxyvitamin D, free 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, bioavailable 25-hydroxyvitamin D

Editorial on the Research Topic

Vitamin D Binding Protein, Total and Free Vitamin D Levels in Different Physiological and Pathophysiological Conditions

Vitamin D binding protein (DBP) is a major plasma carrier for vitamin D and its metabolites. In recent years, there has been growing interest in understanding the physiological functions and attributes of DBP. The current issue is comprised of five review articles and two original research papers concerning the physiology of DBP and its role in different disorders.

Poor vitamin D status is highly prevalent in many different countries (1–4), but the exact definition of vitamin D status is controversial. The plasma concentration of total 25-hydroxyvitamin D [25(OH)D] is currently used as an indicator of vitamin D status. In the past decades, however, there has been argument as to whether just measuring total 25(OH)D is appropriate for the assessment of vitamin D status in different physiological and pathophysiological conditions (5, 6). About 85% of the total circulating 25(OH)D is bound to DBP, and 15% is bound to albumin. About 0.03% of 25(OH)D circulates in free form. Since 25(OH)D is weakly bound to albumin and dissociates from it during tissue perfusion, the sum of the free and the albumin-bound 25(OH)D represents the bioavailable 25(OH)D, which may be readily available for metabolic function. In contrast, the DBP-bound vitamin D is relatively unavailable to target tissue, with the exception of a few tissues such as the kidney that express a megalin/cubilin transport system for DBP-bound 25(OH)D. The concept that it is the free hormone and not the DBP-bound hormone that enters cells is known as the free hormone hypothesis. In the review by Bikle and Schwartz it is highlighted that the DBP level is regulated by estrogen, glucocorticoids, and inflammatory cytokines but not by vitamin D itself, and therefore, these regulators would affect levels of total 25(OH)D. The review by Bikle and Schwartz focuses on the biological importance of DBP with emphasis on its regulation of total and free vitamin D metabolite levels in various clinical conditions. They also point out that attempts to calculate the free level using affinity constants generated in a normal individual along with measurement of DBP and total 25(OH)D have not accurately reflected directly measured free levels in a number of clinical conditions. The authors examine the impact of different clinical conditions as well as different DBP alleles on the relationship between total and free 25(OH)D, using only data in which the free 25(OH)D

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***Correspondence:**
Zhongjian Xie
zhongjian.xie@csu.edu.cn

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level was directly measured. Following their previous review (7), the review by Chun et al. discussed a number of important questions including the following. Is the total 25(OH)D (bound plus free) or the unbound free 25(OH)D the crucial determinant of the non-classical actions of vitamin D? While DBP-bound 25(OH)D is important for renal handling of 25(OH)D and endocrine synthesis of $1,25(OH)_2D$, how does DBP impact extra-renal synthesis of $1,25(OH)_2D$ and subsequent $1,25(OH)_2D$ actions? Are there pathophysiological contexts where total 25(OH)D and free 25(OH)D would diverge in value as a marker of vitamin D status? This review aims to introduce the concept of free 25(OH)D and the molecular biology and biochemistry of vitamin D and DBP, which provides the context for free 25(OH)D, and surveys *in vitro*, animal, and human studies taking free 25(OH)D into consideration.

Low DBP levels in patients with primary hyperparathyroidism (PHPT) were first reported in 2013 by Wang's group (8) and confirmed by Battista et al. (9). In the paper by Wang et al., the authors recruited 75 patients with PHPT and 75 healthy control subjects. In addition, 25 PHPT patients underwent parathyroidectomy and had a 3-month follow up visit. The results showed that serum DBP levels were lower in patients with PHPT but that parathyroidectomy restored DBP levels. Lower DBP levels may be one of the contributing factors of low total 25(OH)D level in PHPT patients, and the total 25(OH)D levels might not reflect true vitamin D status in patients with PHPT.

In the comprehensive review by Bouillon et al. it was noted that DBP was originally discovered as a highly polymorphic protein useful for population studies and originally called Group-specific Component (GC). It is now known that DBP and GC are the same protein and appeared early in the evolution of vertebrates. DBP is genetically the oldest member of the albuminoid family (which includes albumin, α-fetoprotein, and afamin, all involved in the transport of fatty acids or hormones). DBP has a single binding site for all vitamin D metabolites with a high affinity for 25(OH)D, thereby creating a large pool of circulating 25(OH)D, which prevents rapid vitamin D deficiency. The review also highlighted the roles of DBP in preventing the urinary loss of 25(OH)D and the formation of polymeric actin fibrils in the circulation after tissue damage. DBP also plays a minor role in transporting fatty acids. Based on the fact that the total concentrations of 25(OH)D and $1,25(OH)_2D$ in DBP null mice or humans are extremely low but calcium and bone homeostasis remain normal, the "free hormone hypothesis" appears to apply to the vitamin D hormones, 25(OH)D or $1,25(OH)_2D$, as it does to other steroid hormones and thyroid hormone.

Vitamin D is important for bone health but may also have extra-skeletal effects. Cunningham et al. examined vitamin D metabolites in serum samples from age- and weight-matched women with and without PCOS and reported results in their paper. The authors found that 25-hydroxy-3epi-Vitamin D_3, 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, and 24,25-dihydroxyvitamin D_3, but not 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], were associated with embryo parameters. The data suggest that vitamin D metabolites other than $1,25(OH)_2D_3$ are important in fertility. Kew, in his review assesses the fundamental role of DBP in neutrophilic inflammation and injury. As highlighted by Kew, DBP induces selective recruitment of neutrophils. DBP is also an extracellular scavenger for actin released from damaged/dead cells, and formation of DBP-actin complexes is an immediate host response to tissue injury. DBP bound to G-actin functions as an indirect but essential cofactor for neutrophil migration.

Vitamin D and DBP have immunological effects and may be important in the development of type 1 diabetes (T1DM). Moreover, low total 25(OH)D levels are associated with the development of type 2 diabetes (T2DM). However, there are no convincing data showing that vitamin D supplementation has an effect on the prevention of T2DM (10). The review by Jorde discusses the relations between DBP and total and free 25(OH)D in T1DM and T2DM.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Association of Vitamin D Metabolites With Embryo Development and Fertilization in Women With and Without PCOS Undergoing Subfertility Treatment

*Thomas Keith Cunningham[1,2], Victoria Allgar[3], Soha R. Dargham[4], Eric Kilpatrick[5], Thozhukat Sathyapalan[2], Stephen Maguiness[1], Haira R. Mokhtar Rudin[6], Nour M. Abdul Ghani[6], Aishah Latiff[6] and Stephen L. Atkin[4]**

[1] Hull IVF Unit, Women and Children's Hospital, Hull Royal Infirmary, Hull, United Kingdom, [2] Centre for Diabetes and Metabolic Research, Hull York Medical School, University of Hull, Hull, United Kingdom, [3] Department of Statistics, Hull York Medical School, University of Hull, Hull, United Kingdom, [4] Weill Cornell Medicine Qatar, Doha, Qatar, [5] Sidra Medical and Research Centre, Doha, Qatar, [6] Antidoping Laboratory Qatar, Doha, Qatar

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Edited by:
Daniel David Bikle,
University of California, San Francisco, United States

Reviewed by:
Carol L. Wagner,
Medical University of South Carolina, United States
Michael F. Holick,
Boston Medical Center, United States

***Correspondence:**
Stephen L. Atkin
sla2002@qatar-med.cornell.edu

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Objective: The relationship between fertilization rates and 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), 25-hydroxyvitamin D2 ($25(OH)D_2$), 25-hydroxyvitamin D3 ($25(OH)D_3$), 24,25-dihydroxyvitamin D ($24,25(OH)_2D_3$), and 25-hydroxy-3epi-Vitamin D3 ($3epi25(OH)D_3$) concentrations in age and weight matched women with and without PCOS was studied.

Methods: Fifty nine non-obese women, 29 with PCOS, and 30 non-PCOS undergoing IVF, matched for age and weight were included. Serum vitamin D metabolites were taken the menstrual cycle prior to commencing controlled ovarian hyperstimulation.

Results: Vitamin D metabolites did not differ between PCOS and controls; however, $25(OH)D_3$ correlated with embryo fertilization rates in PCOS patients alone ($p = 0.03$). For all subjects, $3epi25(OH)D_3$ correlated with fertilization rate ($p < 0.04$) and negatively with HOMA-IR ($p < 0.02$); $25(OH)D_2$ correlated with cleavage rate, G3D3 and blastocyst ($p < 0.05$; $p < 0.009$; $p < 0.002$, respectively). $24,25(OH)_2D_3$ correlated with AMH, antral follicle count, eggs retrieved and top quality embryos (G3D3) ($p < 0.03$; $p < 0.003$; $p < 0.009$; $p < 0.002$, respectively), and negatively with HOMA-IR ($p < 0.01$). $1,25(OH)_2D_3$ did not correlate with any of the metabolic or embryo parameters. In slim PCOS, $25(OH)D_3$ correlated with increased fertilization rates in PCOS, but other vitamin D parameters did not differ to matched controls.

Conclusion: $3epi25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$, but not $1,25(OH)_2D_3$, were associated with embryo parameters suggesting that vitamin D metabolites other than $1,25(OH)_2D_3$ are important in fertility.

Keywords: vitamin D, vitamin D epimers, vitamin D metabolites, fertilization rates, PCOS

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders amongst women of reproductive age affecting 9–21% of the female population and is the main cause of anovulatory infertility (1). It is associated with clinical and biochemical hyperandrogenism, and insulin resistance (IR) in PCOS is associated with obesity, type 2 diabetes, and hypercholestrolemia (2). Vitamin D levels are low in 67–85% of women with PCOS (3), which are suggested to exacerbate IR and the free androgen index (FAI) in PCOS (4, 5). IR itself is both independent of and exacerbated by obesity and is present in 65–80% of women with PCOS (6) and may be improved by vitamin D replacement (7). In a recent meta-analysis, it was shown that in weight matched PCOS women, vitamin D was negatively predicted by weight hip ratio, glucose and LH (8).

Vitamin D deficiency has become the most common nutritional deficiency throughout the world (9). Studies of subfertile women have demonstrated that vitamin D deficiency is present in between 58 and 91% of cases (9–12). Obesity can exacerbate vitamin D deficiency, as a result of decreased bioavailability from cutaneous and dietary sources because of deposition in the body fat compartments (13).

Vitamin D3 (cholecalciferol) is endogenously produced or taken as a dietary supplement, whist vitamin D2 (ergocalciferol) is derived from the diet (primarily from mushrooms and fungi), though both are hydroxylated to $25(OH)D_3$ or $25(OH)D_2$ by multiple 25-hydroxylases (14, 15) (**Figure 1**). 25(OH)D is transported to the kidney and converted to either the active 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) by 1 alpha hydroxylase, or to 24,25-dihydroxyvitamin D ($24,25(OH)_2D_3$), that is also active, by the 24 alpha hydroxylase, in the renal tubular and other cells widely in the body (**Figure 1**) (16). It has been recently reported that extrarenal tissues may also convert 25(OH)D to $1,25(OH)_2D$ (17). $1,25(OH)_2D$ binds to the vitamin D receptor (VDR) subsequently heterodimerizes with the retinoid X receptor for its action that may be effected in several hours (16); however, a more rapid action has been reported with binding membrane VDR or through the $1,25D_3$-membrane-associated, rapid response steroid-binding protein receptor with activation of protein kinases A and C (18). Vitamin D receptors have been located within structures of the female reproductive tract, including the ovary and endometrium (19, 20).

Vitamin D_2 is derived from the diet as ergocalciferol that has lower binding efficacy to VDR resulting in greater serum clearance, limiting the formation of $25(OH)D_2$, though $1,25(OH)_2D_2$ has a high an affinity for VDR as $1,25(OH)_2D_3$ (14, 16). In the United States and other countries vitamin D2 is available both as a supplement and as a pharmaceutical to treat vitamin D deficiency.

3- epimerase isomerizes the C-3 hydroxy group of the natural vitamin D from the α to the β orientation leading to $3epi25(OH)D_3$ (14, 21) that may be measured inadvertently whilst measuring $25(OH)D_3$ (22). $3epi25(OH)D_3$ is thought to be less potent physiologically as $25(OH)D_3$, and $1,25(OH)_2$-3-epi-D_3 has less affinity to VDR thus less biologically active; however, the 3-epimer may be as potent as $1,25(OH)_2D_3$ in other circumstances such as PTH suppression (14, 23) however, data is sparse on the biological potency of the C3 epimers.

Two IVF cohort studies have suggested that clinical pregnancy rates were significantly lower in women who were vitamin D deficient (24, 25), but no differences in the embryological data have been associated with $25(OH)D_3$ levels (25). However, it remains unknown if there is a relationship of baseline $1,25(0H)_2D_3$, $25(OH)D_3$, $25(OH)D_2$, $24,25(0H)_2D_3$, or its epimer $3epi25(OH)D_3$ to fertilization in non-obese PCOS subjects when age and weight are matched to control subjects, and therefore this study was undertaken.

MATERIALS AND METHODS

This prospective cohort study was performed within the Hull IVF Unit, UK following approval by the Yorkshire and The Humber NRES ethical committee, UK and all gave their written informed consent. The PCOS subjects were recruited using the revised 2003 criteria (26), namely any 2 out of 3 criteria were met; menstrual disturbance (oligo or amenorrhoea), clinical and/or biochemical signs of androgenism and polycystic ovaries on ultrasound, with the exclusion of other conditions. All women were on folic acid 400 mcg daily but no other medication. Exclusion criteria were patients with diabetes, renal or liver insufficiency, acute or chronic infections, systemic inflammatory diseases, age <20, age >45, known Immunological disease.

Sample Collection

A fasting blood sample was taken in the luteal phase of the cycle before commencing IVF treatment. The bloods were centrifuged at 3,500 g for 15 min and placed into aliquots and frozen at $-80°C$ until analysis. The bloods were analyzed for FSH (Architect analyser, Abbott laboratories, Maidenhead, United Kingdom), SHBG, insulin (DPC Immulite 200 analyser, Euro/DPC, Llanberis United Kingdom), and plasma glucose (Synchron LX20 analyser, Beckman-Coulter, High Wycombe, United Kingdom). Free androgen index (FAI) was calculated by dividing the total testosterone by SHBG, and then multiplying by one hundred. Insulin resistance (IR) was calculated using the homeostasis model assessment (HOMA-IR). Serum vitamin D levels and testosterone were quantified using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS). Vitamin D metabolites (1,25(OH)2D3), 25(OH)D2, 25(OH)D3, 24,25(OH)2D3 and 3epi25(OH)D3 and three labeled internal standards (d6-25(OH)D3, d6-1,25(OH)2D3 and d6-3-epi-25(OH)D3) were simultaneously extracted from 250 μL serum using supportive liquid-liquid extraction and Diels-Alder derivatization prior to LC-MS/MS analysis. Chromatographic separations were achieved using Hypersil Gold C18 column (150×2.1 mm; 1.9 μ) at flow rate 0.2 ml/min, operated in Electrospray Ionization (ESI) positive mode and analyzed by multiple reaction monitoring (MRM) method. The limit of quantification (LOQ) for 1,25(OH)2D were 10 pg/mL, 3-epi-25(OH)D, and 24,25(OH)2D were 50 pg/mL while 25(OH)D3 and 25(OH)D2 were 0.5 and 0.25 ng/mL, respectively. All methods employed were performed in accordance with the relevant guidelines and regulations.

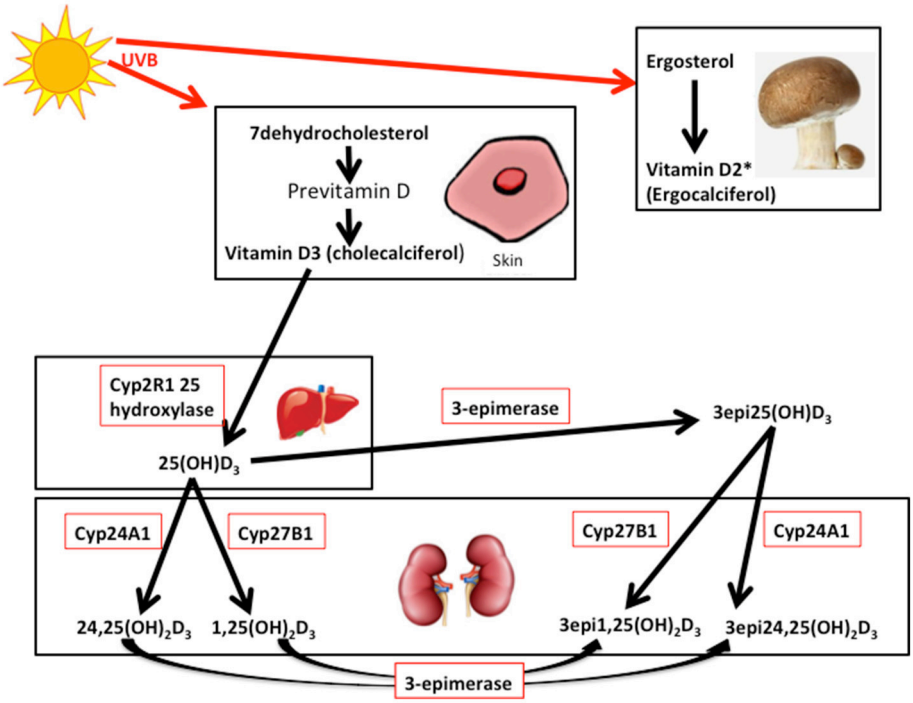

FIGURE 1 | 7-dehydrocholesterol in the skin is converted to previtamin D3 and then is thermally isomerize to vitamin D3. Transport of vitamin D_3 from the skin to the liver is via the Vitamin D binding protein (DBP) transports 25 hydroxy vitamin D ($25(OH)D_3$) to the kidney. $25(OH)D_3$/DBP is filtered by the glomeruli and $25(OH)D_3$ is taken up into the tubular cells, following DBP binding to megalin, a transmembrane protein. $25(OH)D_3$ undergoes a second hydroxylation step by the 1-alpha-hydroxylase Cyp27B1, converting to the active $1\alpha,25(OH)_2D_3$, whilst 24 hydroxylase Cyp27A1 converts to $24,25(OH)_2D_3$. Keratinocytes contribute to the 3-epimerase activity converting $25(OH)D_3$ to 3-epi-$25(OH)D_3$, and $1\alpha,25(OH)_2D_3$ to 3-epi-$1\alpha,25(OH)_2D_3$, but the exact sites of activity remain unknown. $3epi25(OH)D_3$ is equally converted by Cyp27A1 and Cyp27B1 as $25(OH)D_3$. *Vitamin D_2 derived from yeasts and fungi (mushrooms) is converted to $25(OH)D_2$ in the liver and to $1,25(OH)_2D_2$ and $24,25(OH)_2D_2$ in the kidney (adapted from 14, 16, and 21). It is unclear if the 3-epimers may be back converted.

All patients underwent a standard IVF antagonist protocol. The patients commenced their rFSH stimulation on day 2 of their menstrual cycle using either Merional (Pharmasure) or Gonal-F (Merck Serono). A GnRH antagonist (Cetrotide: Merck Serono) was used to prevent a premature LH surge.

The patients underwent ultrasound scans from day 7 to observe the ovarian response to stimulation and were repeated every 48 h. The scans were used to measure the diameters of the follicles thus observing response and follicle numbers. Final maturation was triggered when two or more leading follicles were $\geq$18 mm using human chorionic gonadotrophin [hCG, Pregnyl (Merck Sharp and Dohme)].

Transcervical embryo transfer was performed and embryos were classified using standard criteria (27) at the cleavage stage (day 2–3 after egg collection) and for blastocyst stage (day 5–6 after egg collection). Top Quality embryos on Day 3 as per Alpha Consensus (28). Embryo transfers were performed on either day 3 or ideally at day 5 (blastocyst) to give the best chance for implantation as this timing is similar compared to natural cycle embryos moving into the uterus.

Data Analysis and Statistics

Statistical analysis was performed using SPSS (v22, Chicago, Illinios). Descriptive data is presented as mean ± SD for continuous data and n (%) for categorical data. *t*-tests or Mann Whitney tests were used to compare means/medians where appropriate, and associations used Pearson's correlation or Spearman's correlation as appropriate. A $p < 0.05$ was considered to indicate statistical significance. There was no comparative study on which to base a formal power calculation; therefore, power and sample size for a pilot study was performed (29); therefore, to account for a minimum of 20 degrees-of-freedom to estimate effect size and variability a minimum of 25 patients per group were required to allow covariate adjustment.

RESULTS

Baseline characteristics of the 59 patients are shown in **Table 1** where is can be seen that patients were non-obese, age, and weight matched. There were significant differences in ovarian reserve parameters antral follicle count (AFC) and anti-Mullerian Hormone, (AMH), and androgen status between the groups, however there was no significant difference in fasting insulin, HOMA-IR or the vitamin D metabolites (**Table 1**).

There was a correlation between the levels of $25(OH)D_3$, and embryo fertilization rates in PCOS patients ($r = 0.44$; $p = 0.03$) that were not seen in the control group. However, between the PCOS and control groups there were no differences for any of the metabolic or embryo parameters for $25(OH)D_2$, $24R,25(OH)_2D_3$, $1,25(OH)_2D_3$, or $3epi25(OH)D_3$. When all of

TABLE 1 | Mean demographics and biochemical data.

	Control ($n = 30$)	PCOS ($n = 29$)	p-value
	Mean data (±S.D.)	Mean data (±S.D.)	
Age	32.6 ± 4.7	30.9 ± 4.8	0.14
BMI	25.5 ± 3.6	26.0 ± 3.8	0.56
Menarche	13.0 ± 2.0	13.0 ± 1.1	0.99
Anovulatory	5	25	0.0001***
Duration of subfertility	3.9 ± 1.8	3.4 ± 1.6	0.84
Total antral follicle count	17.2 ± 6.8	38.4 ± 17.8	0.0001***
Fasting insulin (mIU/ml)	7.68 ± 4.0	8.13 ± 4.7	0.69
Fasting glucose (mmol/L)	4.81 ± 0.4	4.62 ± 0.4	0.06
HOMA-IR	1.71 ± 1.0	1.72 ± 1.0	0.97
SHBG	110.9 ± 82.4	63.9 ± 49.8	0.01*
Testosterone (mmol/L)	0.8 ± 0.4	1.4 ± 0.8	0.0004***
Free androgen index	1.35 ± 0.6	4.21 ± 2.9	0.0001***
25-hydroxyvitamin D3 (ng/mL)	46.2 ± 23.5	54.0 ± 27.4	0.24
25-hydroxyvitamin D2 (ng/ml)	0.5 ± 0.3	0.6 ± 0.5	0.73
1,25-dihydroxyvitamin D3	0.03 ± 0.02	0.04 ± 0.2	0.63
24R,25-dihydroxyvitamin D3	0.8 ± 0.5	1.3 ± 0.6	0.003**
3-epi-25-hydroxyvitamin D3	0.4 ± 0.4	0.7 ± 1.2	0.89

*$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$.

the subjects were combined there was a correlation between the levels of $25(OH)D_2$ and cleavage rate ($r = 0.31$; $p = 0.05$), G3D3 ($r = 0.40$; $p = 0.009$) and blastocyst ($r = 0.40$; $p = 0.022$); there was a correlation between $3epi25(OH)D_3$ and fertility rate ($r = 0.33$; $p < 0.04$) and a negative correlation with HOMA-IR ($r = -0.33$; $p < 0.02$); $24R,25(0H)_2D_3$ correlated with AMH ($r = 0.1$; $p = 0.03$) antral follicle count ($r = 0.2$; $p = 0.003$), eggs retrieved ($r = 0.14$; $p = 0.009$) and G3D3 ($r = 0.22$; $p = 0.002$), and negatively with HOMA-IR ($r = -0.07$; $p < 0.01$). There was no correlation of the active $1,25(0H)_2D_3$ with any of the metabolic or embryo parameters.

There was a correlation between the levels of $25(OH)D_3$ with both $24R,25(OH)_2D_3$ and $3epi25(OH)D_3$ ($r = 0.91$, $p < 0.001$; $r = 0.35$, $p < 0.015$, respectively.

As a cohort, 25-hydroxyvitamin D levels were low did not differ between the controls and the PCOS group. The Endocrine Society defines vitamin D deficiency, insufficiency and replete as ($\leq$20 ng/mL, 20–30 ng/mL and $\geq$30 ng/mL, respectively (30) that was reflected in controls and PCOS as, deficient, 51 vs. 41%; insufficient, 33 vs. 35%; deficient, 10 vs. 24%.

IVF cycle characteristics are represented in **Table 2** showing that the PCOS group had significantly greater numbers of follicles aspirated and eggs retrieved compared to the controls, and the mean fertilization and cleavage rates were significantly higher for the PCOS group, though embryos quality did not differ.

There was a significantly negative correlation between SHBG and 25-hydroxyvitamin D3 in the PCOS subjects, however after adjusting for BMI, SHBG was not significantly associated with 25-hydroxyvitamin D3.

TABLE 2 | Mean outcome data for stimulated ovarian cycle for Control and PCOS groups. G3D3: Top Quality embryos on Day 3 as per Alpha Consensus (28).

	Control ($N = 30$)	PCOS ($N = 28$)	p-value
	Mean (±S.D.)	Mean (±S.D.)	
Endometrium at oocyte retrieval	10.31 ± 1.78	10.72 ± 2.06	0.42
Follicles aspirated	11.47 ± 5.11	15.96 ± 5.30	0.002**
Eggs retrieved	8.47 ± 5.08	11.29 ± 5.02	0.04*
Fertilization	4.82 ± 2.65	8.43 ± 3.87	0.0003***
Cleavage	4.68 ± 2.72	7.26 ± 4.40	0.01*
G3D3	3.00 ± 2.29	4.17 ± 3.47	0.16
Blastocyst	1.46 ± 1.77	2.91 ± 3.01	0.05
PDT	11	10	0.86
Clinical pregnancy	10	7	0.24

*$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$.

DISCUSSION

This study has shown that $25(OH)D_3$ was associated with higher fertility rates in PCOS compared to non-obese, age, and weight matched control subjects, but that this was not seen for the other vitamin D metabolites. This was surprising given that there was no difference in the $25(OH)D_3$ levels between the PCOS and control group; however, it is recognized that ova in PCOS may be at a less mature stage compared to normal and therefore there is a possibility that they may be more 25-hydroxyvitamin D responsive to allow those ovum within the stimulated follicles to reach a more mature stage prior to ovum retrieval, resulting in a greater capability to achieve fertilization. PCOS women typically produced more poor quality oocytes, with lower fertilization, cleavage and implantation rates (31, 32). The impaired oocyte maturation and resultant embryonic developmental competence in PCOS women is possibly due to the abnormal endocrine/paracrine functions and the environment within the follicle at the time of folliculogenesis (33, 34). No differences in the embryological data for $25(OH)D_3$ were found, in accord with others (25). It may have been speculated that the active $1,25(OH)_2D_3$ may have a greater influence on fertility at higher levels, but this was not seen in this study, with no correlation with fertilization or embryo data. Vitamin D is involved in the regulation of AMH and FSH gene expression (31, 35), and high dose $25(OH)D_3$ has been shown to increase serum AMH levels in vitamin D insufficiency (36). In this study only the metabolite $24R,25(OH)_2D_3$ correlated with AMH and antral follicle count; $24R,25(OH)_2D_3$ is an active metabolite [It can be converted to 1,24,25-trihydroxyvitamin D3 through the C24 oxidation pathway (37)] as it has been shown to induce non-genomic signaling pathways and suppresses Apo A-1 in hep G cells (38), may have a physiological role in the growth plate formation (14), therefore a direct effect on the ovary cannot be excluded. However, 24,25 dihydroxyvitamin D is associated with blood levels of 25-hydroxyvitamin D and given that both were very significantly associated it is unlikely that $24R,25(OH)_2D_3$ was having a unique biological effect and indeed was dependent on serum $25(OH)D_3$ levels.

There was a positive association for an increase in overall fertility rate with the 3epi25(OH)D_3 that was not seen for the other vitamin D metabolites. Little is known about the epimers of vitamin D, with the assumption that they are biologically less potent (21, 23), but whilst that is likely for bone metabolism it may not be the case for ovarian effects. Epimers are compounds that have identical structure (and therefore identical molecular weight) with the exception of a stereochemical difference at one site. Other than the measurement of 3epi25(OH)D_3 to enable the more accurate determination of 25(OH)D_3, in children where they have been shown to be higher, there has been little research done on the C3 epimers (39) in order to know the significance of this observation on fertility, but it is of interest to note that serum lipids were discrepant for 25(OH)D epimeric forms suggesting a differential effect (40). However, 3epi25(OH)D_3 is associated with blood levels of 25-hydroxyvitamin D and given that both were very significantly associated it is unlikely that 3epi25(OH)D_3 was having a unique biological effect and indeed was dependent on serum 25(OH)D_3 levels.

Whole group analysis for 25(OH)D_2, but not 25(OH)D_3 was positively associated with increased cleavage rate, G3D3 and blastocyst, though overall embryo quality did not differ. Whilst vitamin D3 supplements are better than vitamin D2 to raise vitamin D levels (41, 42), their biological effects may not be the same in different systems (43), and their differential effect in the ovary needs to be clarified. The role of vitamin D in fertilization remains controversial and the specific roles of 25-hydroxyvitamin D levels largely unknown. Observational studies have reported vitamin D levels within serum and follicular fluid to be highly correlated and that those with higher serum and follicular fluid levels of vitamin D had significantly higher clinical pregnancy rates (12). Other studies have found no correlation between serum and follicular fluid levels of vitamin D and IVF outcomes (10, 35, 44). Conversely, two cohort studies comparing serum vitamin D levels and pregnancy rates in women undergoing fresh IVF showed that clinical pregnancy rates were significantly lower in women who were vitamin D deficient (24, 25). Given the controversy, the implication of this data is that in slim PCOS women undergoing IVF that their vitamin D status should be determined, vitamin D replacement undertaken for those deficient prior to IVF may be of benefit, or at least do not harm, until future clarification becomes available.

These data suggest that vitamin D deficiency may not be a homogeneous entity but rather may depend on the different vitamin D metabolites present giving resultants effects, and therefore may account for the heterogeneity and controversy surrounding vitamin D deficiency effects and the response to replacement (42, 45). Whilst the effects of vitamin D and its metabolites on bone and calcium metabolism are well-known, vitamin D metabolite effects on other systems may not be directly equipotent for each metabolite or comparable. For example 1,25$(OH)_2$-3-epi-D_3 may be biologically less active than 1,25(OH)2D3, for increasing calcium, but may have greater PTH suppression (39). However, the kidneys produce 1,25-dihydroxyvitamin D for regulating calcium and bone metabolism (46). Therefore, measuring a blood level of 1,25-dihydroxyvitamin D may not reflect its other biologic functions, but rather local production of 1,25-dihydroxyvitamin D may have its major benefit (47). Our observation that higher blood levels of 25-hydroxyvitamin D are related to the outcome measures could also be due to the higher substrate availability for the local production of 1,25-dihydroxyvitamin D rather than 25-hydroxyvitamin D having a direct effect.

When weight and aged matched then there was no difference in vitamin D metabolite levels between PCOS and normal women. These results differ from previous studies that found Vitamin D deficiency to be more common in PCOS subjects (48); however, in this study the PCOS patients were specifically non-obese and it is well-recognized that vitamin D levels fall in obesity that may account for this observation (6). Negative correlations with HOMA-IR were seen for both 24R,25$(OH)_2D_3$, and 3epi25(OH)D_3, but no association was seen for either 25(OH)D_2 or 25(OH)D_3, suggesting that an association with insulin resistance may depend on the metabolites present. There were no correlations between testosterone or oocyte quality in the PCOS patients in this study These observations are in accord with some studies, though not with others (4, 49); however, in those reported studies patients were not intentionally weight matched. High dose 25(OH)D_3 replacement was not associated with an improvement in insulin sensitivity in PCOS subjects (50).

This study was specifically designed to look at the relationship of vitamin D levels with fertilization rates and therefore was not powered to look at pregnancy rates that would require a much larger sample population. Furthermore, the overall sample size is small and further work is needed, particularly to determine if these findings are true for the differing PCOS phenotypes within the Rotterdam criteria. However, the strengths of this study were that the patients were age and weight matched from the same ethnic background and that all of the vitamin D metabolites were measured by state of the art methods.

In conclusion, non-obese age and weight matched PCOS women showed that 25(OH)D_3 was associated with the fertilization rate, compared to controls; however, vitamin D metabolites were associated with embryology parameters and HOMA-IR, suggesting a possible relationship between differing vitamin D metabolites, oocyte maturation and insulin sensitivity in non-obese PCOS patients.

AUTHOR CONTRIBUTIONS

TC was involved in the study design, acquisition of data, analysis and interpretation of data, and paper drafting. HM, NA, and AL were involved in vitamin D analysis and drafting the manuscript. VA and SD were involved in analysis and interpretation of data, and paper drafting. SA, EK, SM, and TS were involved in the study design, supervision, paper drafting, and contributed to the interpretation of the data. All authors read and approved the final manuscript.

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REVIEW
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The Role of Vitamin D Binding Protein, Total and Free 25-Hydroxyvitamin D in Diabetes

Rolf Jorde [1,2]*

[1] Tromsø Endocrine Research Group, Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway, [2] Division of Internal Medicine, University Hospital of North Norway, Tromsø, Norway

Vitamin D is important for bone health, but may also have extra-skeletal effects. Vitamin D and its binding protein DBP have immunological effects and may therefore be important in the development of type 1 diabetes (T1DM), and low serum levels of 25-hydroxyvitamin D (25(OH)D) are associated with later development of type 2 diabetes (T2DM). However, it has so far been difficult to convincingly show an effect of vitamin D supplementation on prevention or treatment of diabetes. The serum level of 25(OH)D has traditionally been used as a marker of a subject's vitamin D status. This measurement includes both 25(OH)D bound to DBP and albumin as well as the free from of 25(OH)D. However, according to the free hormone hypothesis, the free form is the biologically active. Previously the free form of 25(OH)D had to be calculated based on measurements of 25(OH)D, DBP, and albumin, but recently a method for direct measurement of free 25(OH)D has become commercially available. This is important in clinical conditions where the amount of DBP is affected, and has caused a renewed interest in which vitamin D metabolite to measure in clinical situations. In the present review the relations between DBP, total and free 25(OH)D in T1DM and T2DM are described.

Keywords: diabetes, free vitamin D, vitamin D binding protein (DBP), single nucelotide polymorphisms, 25- hydroxy vitamin D

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Daniel David Bikle,
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Roger Bouillon,
Katholieke Universiteit Leuven, Belgium
Jan Josef Stepan,
Charles University, Czechia

***Correspondence:**
Rolf Jorde
rolf.jorde@unn.no

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INTRODUCTION

Vitamin D is produced in the skin upon UV-B exposure and is obtained through the diet where fatty fish is the main source. Regardless of how it is obtained, vitamin D has to be hydroxylated first in the liver to 25-hydroxyvitamin D (25(OH)D) and then in the kidneys to the active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (1). These hydroxylations may also occur in peripheral tissues (2).

In the circulation the major part of vitamin D, 25(OH)D and $1,25(OH)_2D$ are bound to the vitamin D binding protein (DBP), and to a lesser extent also to albumin. Only a small fraction circulates in the free form (3). To exert their action, the vitamin D metabolites have to cross the cell membrane into the cell [and for vitamin D and 25(OH)D also to be hydroxylated], where the active form $1,25(OH)_2D$ connects to the nuclear vitamin D receptor (VDR) (1).

The endocytic receptors megalin and cubulin are present in the renal tubuli and parathyroid cells (4), and at least in the kidney enable transportation of the DBP-vitamin D complexes into the cells (5). In other (and perhaps most) cell types, the vitamin D metabolites have to pass the cell membranes in their free un-bound form by passive diffusion (6).

The serum concentrations of vitamin D and 25(OH)D are >100 times that of $1,25(OH)_2D$, and the DBP binding coefficients as well as the potential for passive diffusion through cell membranes differ between these vitamin D metabolites (6). Accordingly, it is difficult to say which vitamin D metabolite, or vitamin D metabolite-DBP complex is quantitatively the most important for VDR activation and the one that should be measured for evaluation of a subject's vitamin D status (6, 7).

For this purpose, one has traditionally measured the serum 25(OH)D level, since this metabolite is abundant, easy to measure, and has a long half-life and therefore stable levels. Furthermore, the hydroxylation from vitamin D to 25(OH)D is substrate driven and the serum 25(OH)D level correlates strongly with sun exposure and vitamin D intake and also correlates with known vitamin D effects, like the suppression of the parathyroid hormone (PTH) secretion (1).

The serum 25(OH)D that is measured is the total 25(OH)D, which includes the DBP and albumin bound 25(OH)D as well as the free form. Since the major part of 25(OH)D is bound to DBP, the concentration of total 25(OH)D will depend on the serum DBP concentration. The DBP concentration is fairly stable throughout life, but increases with pregnancy and estrogen supplementation. DBP is synthesized in the liver and accordingly the serum DBP concentration is reduced in liver failure as well as in malnutrition (8, 9). Loss of proteins in the urine (like in some subjects with diabetes) may also cause low serum DBP levels (10, 11). Thus, in situations with high serum DBP levels like pregnancy, an even larger portion of the total 25(OH)D in plasma is bound to DBP and accordingly the free form is reduced. Conversely, in patients with liver cirrhosis where the serum level of DBP is low, the free fraction is increased. Although there is a strong correlation between total and free 25(OH)D (12), measurement of total 25(OH)D may therefore not always reflect the free form.

According to the free hormone hypothesis, it is the free form of the hormone, which easily diffuses through cell membranes, that is the biologically active, and the one to be measured (13). This is exemplified for thyroid hormones, where the serum concentration of tree thyroxine is regulated in a negative feedback manner by the secretion of thyroid stimulating hormone (TSH). In this system, changes in the concentration of thyroid hormone binding globulin (TBG) will be compensated by increased or decreased secretion of TSH keeping the free concentration of thyroxine stable (14). This demonstrates the utility of the free hormone concept for thyroid hormones.

This concept does not necessarily apply to the vitamin D system where the active hormone $1,25(OH)_2D$ can be transported into (at least some) cells in a DBP-complex, and also have its activating hydroxylations intracellularly. Furthermore, 25(OH)D is in essence a pro-hormone not regulated by negative feed-back control. Changes in DBP will not induce changes in the hydroxylation of vitamin D to 25(OH)D since this is a substrate driven process. Increased serum 25(OH)D concentrations may be accompanied by an increased level of FGF-23, increased *CYP24A1* expression and 24-hydroxylase activity, and accelerated degradation of 25(OH)D to $24,25(OH)_2D$ (15). However, this mechanism must for 25(OH)D be of minor importance since the increase in free 25(OH)D and total 25(OH)D after vitamin D supplementation is, at least until serum 25(OH)D levels of approximately 150 nmol/L, quite linear (12). Therefore, whether the total or the free form of 25(OH)D is the best vitamin D parameter cannot be decided on theoretical grounds only, but has to be tested in clinical situations as well (16, 17).

There are many single nucleotide polymorphisms (SNPs) in the *DBP* gene (*GC* gene, globulin–complex gene). Combinations of two of these (rs7041 and rs4588) result in three polymorphic alleles and six major phenotypes. These phenotypes may have different binding affinities for the vitamin D metabolites (18) and the serum 25(OH)D levels do differ between subjects with different DBP phenotypes (12). The distribution of the six variants also differs between races (19).

In addition to the skeleton vitamin D deficiency has been associated with a number of diseases, like mortality, cancer, immunological diseases, cardio-vascular diseases, and diabetes (20). Most of these relations are based on observational studies only, where 25(OH)D has been measured in old serum samples and subsequent diseases recorded. For these studies, measurement of total serum 25(OH)D has been employed, whereas there has been little focus on DBP [where the major part of the circulating 25(OH)D is bound] or the free form which potentially may be the most important.

The serum level of free 25(OH)D has traditionally been calculated based on measurements of total 25(OH)D, DBP, and albumin concentrations (21–23). However, measurement of DBP depends on type of antibody employed (monoclonal or polyclonal) (19), and it has usually been assumed that the vitamin D binding-coefficient for each of the six prevalent DBP phenotypes are equal. The validity of the free 25(OH)D calculations have therefore been questioned (24). Lately, kits for direct measurement free 25(OH)D has become commercially available which has caused a renewed interest in the relation between free serum 25(OH)D, as well as DBP, and disease states (25). However, further validation and standardization of this assay is still needed in subjects with major illnesses or with abnormal DBP or protein concentrations (16).

In the present review these relations will be summarized for the metabolic disorders type 1 and type 2 diabetes (T1DM and T2DM), presented separately.

T2DM

Serum 25(OH)D and T2DM

There are many reasons for why vitamin D could influence the development of T2DM. Thus, the vitamin D activating hydroxylases and the VDR are found in the pancreatic beta-cells (26, 27), $1,25(OH)_2D$ may induce insulin secretion (28), and

vitamin D may have an anti-inflammatory effect that may prevent insulin resistance (29).

In line with this, there are a number of observational studies on the relation between serum 25(OH)D concentration and incident diabetes, and practically all confirm an association (30). Thus, in a study by Afzal et al. on 31,040 subjects with measurement of serum 25(OH)D followed for up to 34 years, participants who had a 20 nmol/L reduction in 25(OH)D had a 16% increased risk of T2DM (31). Similarly, Ye et al. combined 22 studies in a meta-analysis that included 8,492 cases and 89,698 controls and found a 21% increased risk of T2DM per 25 nmol/L lower 25(OH)D concentration (32).

However, for vitamin D there is a strong possibility of revers causation and other methods than observational studies are needed for confirmation, as recently reviewed by Angelotti and Pittas (30).

There are a few RCTs with vitamin D specifically designed for prevention of T2DM in subjects at risk. Thus, Davidson et al. included 109 subjects with prediabetes and randomized them to high dose vitamin D (mean weekly dose 88,865 IU) vs. placebo. However, no significant effects on insulin secretion, insulin sensitivity or development of diabetes were found after 1 year (33). Similarly, in a study from Tromsø, Norway, Jorde et al. randomized 511 subjects with reduced glucose tolerance to 20,000 IU vitamin D per week vs. placebo for a maximum of 5 years, but found no difference between the groups in development of T2DM (34). However, both studies were underpowered for detection of minor effects. And finally, the effect of giving vitamin D to subjects with established T2DM do at best show a marginal effect on HbA_{1c} with a reduction of 0.32% in HbA_{1c} as compared with placebo according to a review by Lee et al. that included nine trial with 3,324 participants (35).

Another approach to the vitamin D—T2DM question is the Mendelian randomization. Several SNPs are associated with serum 25(OH)D level; SNPs in the *DHCR7* gene related to vitamin D synthesis, the *CYP2R1* gene related to 25-hydroxylation, and the *CYP24A1* gene related to 24-hydroxylation and degradation (36). When these SNPs are combined to a genetic score, the highest vs. the lowest scores result in 5–20% difference in serum 25(OH)D levels. However, in the most recent and largest meta-analysis including five studies with 28,144 cases and 76,344 non-cases, no significant association with T2DM was found, neither for the individual SNPs tested, nor when combined to a genetic score (32).

There are, however, many shortcomings of the Mendelian randomization approach. So far it only predicts differences in serum 25(OH)D concentration and not the free fraction, and the alleles tested only explain a small part of the variance in serum 25(OH)D level.

One may therefore conclude that although a low serum 25(OH)D level do predict development of T2DM, this is most likely due to confounding or reverse causality, although minor effects cannot be excluded. Hopefully the ongoing D2d study that has included 2,423 participants with prediabets randomized to 4000 IU vitamin D daily vs. placebo may settle this question (37).

Free 25(OH)D and T2DM

There are several reports where the directly measured free fraction of 25(OH)D has been compared with total 25(OH)D regarding biological effects of vitamin D. Thus, Johnsen et al. found a better correlation for free than for total 25(OH)D regarding bone density (24), whereas that was not found in study by Michaelsson et al. (38). For PTH similar relations have been found for free and total 25(OH)D in most studies (24, 39–41), whereas Lopez-Molina et al. in healthy children found better correlation with markers of phosphocalcic metabolism for free than for total 25(OH)D (42). Shieh et al. found in the early phase (first 4 weeks) of vitamin D treatment the free 25(OH)D, but not the total 25(OH)D, to be associated with a decrease in serum PTH (43). In inflammatory diseases the results are also mixed with free 25(OH)D correlating better to disease activity in ulcerative colitis (44), whereas total 25(OH)D correlates best to activity in systemic lupus erythematosus (45). For markers of inflammation (IL-6) in older men free and total 25(OH)D appear to correlate equally (46). And finally and most important, in a study by Yu et al. the free but not total 25(OH)D was associated with risk of mortality in patients with coronary artery disease (47). The study included 1,387 patients followed for a median time of 6.7 years, during which period 205 patients died. The all-cause mortality was 64% higher in the lowest free 25(OH)D quartile vs. the highest free 25(OH)D quartile, whereas the corresponding analysis using 25(OH)D did not show a significant difference or trend across the quartiles.

So far, there are no studies where the free 25(OH)D has been compared with total 25(OH)D as predictor for development of T2DM. However, there is a publication by Lee et al. that included 1,189 non-diabetic subjects where the free and total form of 25(OH)D were measured and related to acute insulin response and glucose disposition index based on intravenous glucose tolerance tests (48). Both free and total 25(OH)D were positively associated with these measures, but after adjustment for BMI, only free 25(OH)D was significant related to insulin secretion.

Based on the above papers, one cannot conclude that measurements of free vs. total serum 25(OH)D has any advantage regarding vitamin D responses. This is also difficult to decide, as comparisons of *P*-values and correlation coefficients give indications only.

DBP and T2DM

In addition to being the carrier protein for vitamin D and its metabolites, DBP has a number of other effects. It acts as a carrier for free fatty acids (49), it binds actin and may prevent actin polymerization during tissue damage (50, 51), may act as a macrophage activator and play a part in the inflammation process by influencing the T-cell response (52). These immunological effects may differ between the phenotypes (53), and the level of DBP as well as the different DBP phenotypes might therefore at least theoretically affect the development of not only T1DM (see later) but also T2DM.

However, in a case-cohort study design with 958 cases and 3,489 controls Jorde et al. found no association between DBP phenotypes (based on genotyping of rs4588 and rs7041) and incident T2DM (54). Furthermore, there were no relations

between the DBP phenotypes and lipids and blood pressure, but a slight relation to hip circumference.

Prior to our study Wang et al. made a meta-analysis on *DBP* SNPs and T2DM that included six studies (three Caucasian and three Asian cohorts) with 1,191 cases and 882 controls. No overall association between the *DBP* SNPs rs4588 and rs7041 and T2DM was found. However, when analyzing the Asian cohorts separately, there were significant associations with T2DM for both rs7041 and rs4588 (55).

Also after the meta-analysis by Wang et al., Ye at al. meta-analyzed the *DBP* SNP rs4588 regarding T2DM in European cohorts including 28,144 cases and 76,344 controls. A strong relation between rs4588 and serum 25(OH)D was found, but not with T2DM (OR 1.00 (CI, 0.97 −1.03) (32). Accordingly, at least in Caucasians there appears to be no relation between DBP phenotypes and development of diabetes.

To the author's knowledge, there are no longitudinal studies regarding serum levels of DBP and T2DM. However, there is one cross-sectional study by Leong et al. on 2,122 adult subjects that included 201 with diabetes (56). The effect estimate per 50 mg/L DBP increase was 0.79 (95% CI 0.65–0.96) for diabetes, and there was a marginal relation between higher DBP and lower fasting blood glucose levels. However, as a cross-sectional study it could not examine the impact of biological variability of DBP over time.

T1DM

Serum 25(OH)D and T1DM

The 1α-hydroxylase, necessary for activation of vitamin D, is expressed in immune cells like the B- and T-cells and the antigen presenting cells (2). These cells may therefore synthesize active vitamin D locally. Vitamin D has immune-modulatory effects (57), and since T1DM is an autoimmune disorder, a role for vitamin D in pathogenesis as well as treatment thus possible (58).

However, in a study by Thorsen et al. using a case-cohort design that included 459 children with T1DM and a control group of 1,561, no association between maternal serum 25(OH)D levels sampled repeatedly during pregnancy and subsequent T1DM in the offsprings was found (59). Furthermore, in two large Danish populations, one case-cohort study with 912 cases and 2,866 controls followed for a maximum of 31 years and a case-control study with 527 matched pairs followed for a maximum of 23 years, Jacobsen et al. found no relation between neonatal vitamin D status and later risk of T1DM (60). On the other hand, there might be a link between intake of vitamin D in childhood and development of T1DM as reported by Hyppönen et al. in a birth-cohort study with 12,055 pregnant women in northern Finland (61). This was also the conclusion in a meta-analysis by Dong et al. from 2013 that included eight studies (six case-control and two cohort studies) with vitamin D intake during early life where the pooled OR for T1DM was 0.71 (95% CI, 0.51–0.98) (62).

Furthermore, the serum levels of 25(OH)D are lower in subjects with newly diagnosed T1DM (63) as well as later in the course of disease compared to similarly aged subjects (64). There may also be a beneficial effect by vitamin D supplementation in newly diagnosed T1DM. This was reviewed by Gregoriou et al. (65) who found a positive effects on the daily insulin dose, fasting, and stimulated C-peptide response by vitamin D in two studies. However, only 67 patients were randomized and the effect was marginal.

To the author's knowledge there are no studies reporting free 25(OH)D levels in T1DM.

DBP and T1DM

Since the immunological effects of DBP may differ between the DBP phenotypes (53), relations between the *DBP* SNPs rs4588 and rs7041 and T1DM are of interest. This was reviewed by Penna-Martinez and Badenhoop who found that in the majority of the studies there was no relation between these SNPs and T1DM (66). As an example, Cooper et al. who included 720 cases and 2,610 controls and used a Mendelian randomization approach, found no relation between rs4588 and T1DM (67), whereas in the two studies that did find an association with rs7041 the total number of cases was only 154 (68, 69).

There are a few cross-sectional reports on serum DBP levels in patients with T1DM. In a study by Blanton et al. that included 203 subjects with T1DM and 153 controls, the serum DBP levels were ∼10% lower in the T1DM patients (70). A similar result was found by Thraikill et al. but they could for a large part ascribe this to increased urinary loss of DBP in the urine (11). Low serum DBP levels have also been described in diabetic BB rats together with low serum $1,25(OH)_2D$ levels accompanied with reduced duodenal calcium absorption, indicating the possible physiological importance of urinary DBP loss (71).

CONCLUSIONS

For preventing or treating diabetes, the majority of clinical studies do not indicate a major role for vitamin D supplementation, with a possible exception for T1DM in children. As for many other presumed extra-skeletal effects of vitamin D, the effect on glucose metabolism must be small (if present at all) and accordingly difficult to demonstrate. In most of the vitamin D RCTs the results are also hampered by the inclusion of subjects who are not truly vitamin D deficient (72). However, since such subjects (and in particular young children) need vitamin D for bone health, there are many ethical problems in including vitamin D deficient subjects in long lasting RCTs. The "perfect" vitamin D RCT will therefore probably not be performed.

However, regarding vitamin D and health, the two crucial questions are how much vitamin D we need for skeletal health (which everyone agrees is vitamin D dependent), and if supplementation above that will give any additional health benefits.

So far, there are too few studies on the relative importance of measuring total or free 25(OH)D in diabetes and glucose

metabolism, and too few studies on the importance of DBP concentration on development and progression of diabetes, to draw firm conclusion. However, since it is difficult to show an effect of vitamin D supplementation regarding diabetes, it follows that finding the right form or metabolite of vitamin D to measure (7), may for diabetes simply be a search for another biomarker (73). In disease states with clearly altered DBP levels, like pregnancy and liver cirrhosis, the situation obviously is different (9).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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ORIGINAL RESEARCH
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25-Hydroxyvitamin D and Vitamin D Binding Protein Levels in Patients With Primary Hyperparathyroidism Before and After Parathyroidectomy

Xiangbing Wang[1], Zhifeng Sheng[2], Lingqiong Meng[3], Chi Su[1], Stanley Trooskin[4] and Sue A. Shapses[3]*

[1] Divisions of Endocrinology, Metabolism, and Nutrition, Departments of Medicine and Surgery, Rutgers-Robert Wood Johnson Medical School, New Brunswick, NJ, United States, [2] Institution of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, Changsha, China, [3] Department of Nutritional Sciences, Rutgers University, New Brunswick, NJ, United States, [4] Divisions of General Surgery, Departments of Medicine and Surgery, Rutgers-Robert Wood Johnson Medical School, New Brunswick, NJ, United States

Objective: To evaluate vitamin D binding protein and free 25-hydroxyvitamin D [25(OH)D] levels in healthy controls compared to primary hyperparathyroidism (PHPT) patients, and to examine PHPT before and after surgery.

Methods: Seventy-five PHPT patients and 75 healthy age, gender, and body mass index (BMI) -matched control subjects were examined. In addition, 25 PHPT patients underwent parathyroidectomy and had a 3-month follow up visit. Levels of total and free 25(OH)D, DBP, and intact parathyroid hormone (iPTH) were determined before and 3 months after surgery.

Results: There was no significant difference in age and BMI between PHPT patients and controls. Levels of 25(OH)D and DBP were lower in PHPT patients compared to controls ($p < 0.01$). There was no significant difference in calculated free and bioavailable 25(OH)D levels between PHPT patients and controls. Calcium and iPTH levels decreased to normal but DBP and DBP-bound-25(OH)D increased ($P < 0.001$) after parathyroidectomy. Levels of DBP were inversely correlated with iPTH ($r = -0.406$, $P < 0.001$) and calcium levels ($r = -0.423$, $P < 0.001$).

Conclusion: Serum DBP levels were lower in patients with PHPT and parathyroidectomy restored DBP levels. We suggest that lower DBP levels is one of contributing mechanisms of low total 25(OH)D in PTHP patients and the total 25(OH)D levels might not reflect true vitamin D status in PHPT patients.

Keywords: vitamin D binding protein, vitamin D deficiency, parathyroid hormone, calcium metabolism, hyperparathyroidism, parathyroidectomy

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Jan Josef Stepan,
Charles University, Czechia
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University Hospitals Leuven, Belgium

****Correspondence:***
Xiangbing Wang
wangx9@rwjms.rutgers.edu

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INTRODUCTION

Total 25(OH)D level has been recognized as an optimal indicator of vitamin D nutrition status, and lower 25(OH)D concentration is usually considered as vitamin D deficiency or insufficiency in clinical practice. Low total 25(OH)D concentration, which is common in PHPT patients, is associated with the severity of the disease and high parathyroid adenoma weight (1–3). Low

25(OH)D levels also exist in many chronic conditions such as end-stage liver disease and nephrotic syndrome, and in critical illness where intact parathyroid hormone levels are not elevated (4, 5). The majority of circulating 25(OH)D tightly bound to DBP, with a smaller amount (10–15%) bound to albumin. Less than 1% of circulating vitamin D metabolites exists in a free, unbound form (5). The variations in the 25(OH)D levels in these conditions result from variations in the binding of 25(OH)D to DBP (4).

Previous studies showed that DBP are lower in PHPT patients compared with age, BMI, and gender matched (6–8) or genetic background matched controlled subjects (7). It is unclear how DBP is regulated in and if the elevated iPTH plays a role in PHPT, or if DBP simply a biomarker of circulating 25(OH)D. Since the majority (>85%) of circulating 25(OH)D is bound to DBP, we suggest that decreased DBP might be one of mechanisms of low total 25(OH)D levels in PHPT patient (8). The causes of lower DBP concentration in the serum of PHPT patients remained unknown. We hypothesize that the elevated iPTH or calcium levels inhibit DBP production in the liver of PHPT patient. In our current study, we investigated the effects of lower calcium and iPTH levels by parathyroidectomy on DPB in PHPT patients. We also compared levels of 25(OH)D, DBP, and calculated free and bioavailable 25(OH)D in patients with PHPT with normal controls. The aim of this study is to investigate the effects of parathyroidectomy on DBP and DBP-bound 25(OH)D levels in PHPT patients.

METHODS

Study Subjects

Seventy-five PHPT patients (61 Caucasians, eight African American, four Asians, and two Hispanic Americans) were seen at the Endocrinology and General Surgery clinics of Robert Wood Johnson University Hospital from January 2010 to December 2016 at prior to treatment. Most of the patients were relatively asymptomatic with less severe PHPT profile (9). The inclusion criteria were: (1) a serum calcium level >10.6 mg/dL (8.6–10.4 mg/dl) and intact PTH (iPTH) >66 pg/mL (15–65 pg/mL), (2) age 20–80 years, and (3) 24-h urine calcium >100 mg (100–300 mg/24 h) with fraction excretion of calcium >0.01. The exclusion criteria were: (1) hormone replacement therapy or contraceptive pills, (2) hepatic dysfunction, or (3) renal dysfunction, and (4) BMI >40 (kg/m^2). Seventy-five age, gender, and BMI-matched healthy volunteers (62 Caucasians, eight African Americans, four Asians, and one Hispanic American) (10) from the community were included as controls after a multistep screening process and did not take contraceptive pills. The healthy controls took 400 IU vitamin D supplement. Supplemental vitamin D intake in patients before surgery is not known. Twenty-five PHPT patients (seven males and 18 females) underwent parathyroidectomy (PTX) monitored by intro-operative iPHT levels and were examined at 3 months during their follow-up visit after surgery. All minimally invasive PTX were done by one surgeon and all patients were advised to take 0.25 mcg calcitriol for 1–2 weeks and 1,000–2,000 IU vitamin D for 1–3 months after PTX to prevent hypocalcemia as standard post-operative clinical care (11). All subjects and patients signed an informed consent and the use of human subjects in this study was approved by the IRB at Rutgers University.

Sample Collections and Assays

Venous blood samples were collected from patients and controlled subjects after a 12-h overnight fast. Twenty-five PHPT patients had parathyroid surgery and finished 3 months' post-surgery follow up visit. Serum was separated and stored at −70 C for measurement of 25(OH)D and DBP levels. Intact-PTH, serum calcium, and albumin were determined by commercial laboratories. The laboratory uses both internal and external standards, and also participated in the international Vitamin D External Quality Assessment Scheme to ensure the quality and accuracy of the 25(OH)D analysis and serum 25(OH)D levels (radioimmunoassay; DiaSorin) CV <12.5%). DBP levels in serum were determined using a commercial polyclonal ELISA kit (ALPCO, Salem, NH). The intra- and inter-assay coefficients of variation are 5.0 and 12.7%, respectively. Free, bioavailable, albumin-bound and DBP-bound 25(OH)D concentrations were calculated using equations adapted from Bikle et al. (12).

Statistical Analyses

Results are expressed as mean ± SD. Shapiro-Wilk test was used to check normality. Two-tailed Student's *t*-test and Wilcoxon Rank Sum test were used to compare values between groups with normally and non-normally distribution, respectively. Changes before and after parathyroidectomy were compared with a paired Student's *t*-test. Correlation coefficients and linear regression were used to assess relationships between variables. A $P < 0.05$ was defined as the level of significance. Statistical analysis was performed with SAS v9.4.

TABLE 1 | Subject characteristics and serum concentrations.

Variable	Control	PHPT	*P*-value
	n = 75	*n* = 75	
Age (years)	58.0 ± 8.1	59.3 ± 12.3	0.314
BMI (kg/m^2)	29.9 ± 2.1	30.6 ± 4.8	0.373
Calcium (mg/dL)	9.4 ± 0.5	11.1 ± 0.6	< 0.001
iPTH (pg/mL)	37.9 ± 17.3	140.4 ± 70.5	< 0.001
25(OH)D (ng/mL)	28.3 ± 5.4	23.6 ± 8.3	< 0.001
DBP (mg/dL)	42.1 ± 7.0	35.2 ± 7.9	< 0.001
Albumin (g/dL)	4.5 ± 0.2	4.3 ± 0.3	< 0.001
DBP-bound 25(OH)D (ng/mL)	26.4 ± 5.1	21.8 ± 7.7	< 0.001
Albumin-bound 25(OH)D (ng/mL)	1.9 ± 0.5	1.8 ± 0.8	0.082
Bioavailable 25(OH)D (ng/mL)	1.9 ± 0.5	1.8 ± 0.8	0.083
Free 25(OH)D (pg/mL)	4.7 ± 1.1	4.7 ± 2.0	0.573

BMI, body mass index; iPTH, intact parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; DBP, vitamin D binding protein. Data shows as mean ± standard deviation.

RESULTS

Seventy-five PHPT patient (23 men, 11 premenopausal women, and 41 postmenopausal women) and 75 control subjects (19 men, 11 premenopausal women, and 45 postmenopausal women) were included in this study. The mean concentrations of calcium, albumin, 25(OH)D, iPTH, and DBP determined in the serum samples from control subjects and PHPT patients are shown in (**Table 1**). Both total 25(OH)D and DBP levels were about 17% lower in PHPT patients compared to control subjects ($P < 0.001$). There was no significant difference in albumin-bound 25(OH)D, but DBP-bound 25(OH)D was also 17% lower in PTHP patients compared to control subjects ($P < 0.001$). In addition, albumin levels were significantly lower in PHPT patients than in control subjects ($p < 0.001$). There were no significant differences between bioavailable and free 25(OH)D between healthy controls and PHPT patients (**Table 1**). Comparison of individual 25(OH), DBP, free 25(OH)D, and bioavailable 25(OH) D between healthy controls and PHPT patients were showed in **Figure 1**.

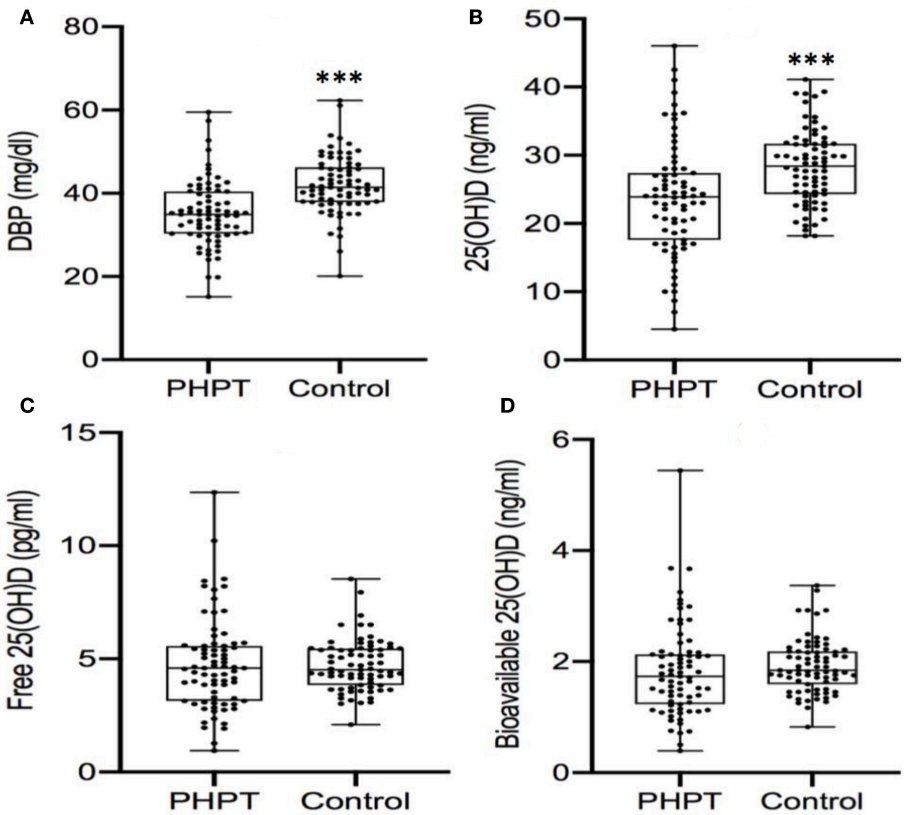

FIGURE 1 | (A-D) Comparison of serum levels of DBP and 25 (OH) D between PHPT patients and control subjects. DBP, vitamin D binding protein; 25OHD, 25-hydroxyvitamin D. ***$P < 0.001$.

TABLE 2 | Spearman correlation coefficients between DBP and other variables ($n = 150$).

	Age	BMI	Calcium	iPTH	25(OH)D	Albumin	Free 25(OH)D	Bioavailable 25(OH)D
DBP	**−0.210[a]**	**−0.192[a]**	**−0.423[c]**	**−0.406[c]**	**0.253[b]**	**0.139[a]**	**−0.344[c]**	**−0.295[c]**
Age		0.113	0.076	0.055	0.132	−0.074	**0.301[c]**	**0.278[c]**
BMI			0.038	0.074	**−0.272[c]**	−0.141	−0.121	−0.145
Calcium				**0.751[c]**	**−0.292[c]**	**−0.218[b]**	−0.057	−0.114
iPTH					**−0.418[c]**	**−0.312[c]**	−0.153	**−0.233[b]**
25(OH)D						**0.225[b]**	**0.767[c]**	**0.786[c]**
Albumin							0.092	**0.284[c]**
Free 25(OH)D								**0.972[c]**

BMI, body mass index; iPTH, intact parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; DBP, vitamin D binding protein. Bold means significan. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

Levels of DPB ($n = 150$) were positively correlated with total 25(OH)D ($r = 0.253$, $P < 0.01$) but inversely correlated with iPTH ($r = -0.406$, $P < 0.001$) and calcium ($r = -0.423$, $P < 0.001$; **Table 2**). Levels of iPTH inversely correlated with total 25(OH)D ($r = -0.418$, $P < 0.001$) and bioavailable 25(OH)D ($r = -0.233$, $P < 0.01$; **Table 2**).

In PHPT patients who underwent parathyroidectomy, serum iPTH, and calcium decreased to normal but DBP levels increased by 15% ($P < 0.01$, **Table 3**). Serum total 25(OH) D were increased by 43 % ($p < 0.001$) but DBP-bound 25(OH)D also increased by 43% ($P = 0.001$). As a result, there was an attenuated rise in bioavailable (23%, $P = 0.024$) and free 25(OH)D (21%, $P = 0.021$, **Table 3**). Comparison of individual 25(OH), DBP, free 25(OH)D, and bioavailable 25(OH) D before and after PTX was showed in **Figure 2**. Multiple regression showed that none of the variables (Ca, PTH or 25(OH)D) predicted the change in DBP after parathyroidectomy (not shown). In addition, there were no predictors for the rise in 25(OH)D due to surgery. Multiple Regression indicated that only the change in albumin predicted change in bioavailable 25(OH)D ($p = 0.027$), but not free 25(OH)D ($p = 0.122$) after parathyroidectomy.

DISCUSSION

The results of our current study demonstrate that PHPT patients have lower serum levels of DBP and total serum 25(OH)D consistent with our previous study (8) and study by Battista et al. (7). In addition, our data confirmed our previous studies that the calculated free or bioavailable 25(OH)D remained unchanged compared with normal control subjects (8). We also showed that PTX increases DBP and DBP-bound 25(OH)D levels. Thus, based on our findings and because supplemental vitamin D raises 25(OH)D, but not DBP (13–15), we suggest that the increased DBP level after PTX might be due to the decreased iPTH or lowered calcium levels after surgery. This supports the hypothesis that DBP is not simply a biomarker of 25(OH)D. Our current results also support the concept that the lower DBP levels in PHPT compared to healthy matched controls may be one of the factors contributing to the low total 25(OH)D levels in PHPT patients. Another possible factor leading to the low total 25(OH)D levels in PHPT patients include the conversion to 1,25 or 24,25 (OH)2D due to elevated iPTH or FGF-23 (6, 16).

TABLE 3 | PHPT profile changes before and after parathyroidectomy.

	Before	After	*P*-value
	n = 25	*n*= 25	
BMI (kg/ m^2)	31.0 ± 5.6	29.0 ± 4.3	0.127
Calcium (mg/ dl)	11.0 ± 0.6	9.7 ± 0.4	<0.001
iPTH (pg/ ml)	121.5 ± 40.5	44.7 ± 8.2	<0.001
25(OH)D (ng/ ml)	26.4 ± 7.8	37.7 ± 12.1	<0.001
DBP (mg/ dl)	38.9 ± 9.8	44.7 ± 8.2	0.001
Albumin (g/ dl)	4.3 ± 0.9	4.5 ± 0.2	0.046
DBP-bound 25(OH)D (ng/ mL)	24.6 ± 7.3	35.4 ± 11.1	<0.001
Albumin-bound 25(OH)D (ng/ mL)	2.0 ± 0.5	2.36 ± 0.75	0.024
Bioavailable 25(OH)D (ng/mL)	1.94 ± 0.94	2.36 ± 0.74	0.024
Free25(OH)D (pg/mL)	4.89 ± 2.05	5.90 ± 1.9	0.021

BMI, body mass index; DBP, vitamin D binding protein; iPTH, intact parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D. Data are means ± standard deviation (Paired t-test).

Aloia et al. found that black Americans have lower levels of total 25(OH)D but the free 25(OH)D remains relative unchanged by direct measurement of free 25(OH)D (17). Pre-menopausal women have higher serum DBP, estradiol, and 25(OH)D levels than postmenopausal women (18). The calculated free 25OHD was also lower in postmenopausal women than that of control subjects, but to a much lesser degree than total 25OHD (18). In a recent study, Pilz et al. found that women taking estrogen containing contraceptive measures have higher total 25(OH)D but unaltered free 25(OH)D levels by direct measurement (19). The results suggest that total 25(OH)D levels might not be an accurate marker of bioactive vitamin D status in at least a few situations, including black Americans or women taking hormonal contraceptive pill or other clinical situations (5, 20) Bioavailable 25(OH)D may be a better measure of vitamin D status with respect to bone mineral density (BMD) and mineral metabolism, as has been shown in nephrotic syndrome patients (21). Lai et al. found that cirrhosis patients with low albumin had lower DBP, total 25(OH)D, and free 25(OH)D levels and suggest that total 25(OH)D is not accurate marker for vitamin D status in these patients (22). Yu et al. reported that it is bioavailable and free 25(OH)D levels, not total 25(OH), associated with the risk of mortality in Chinese patients with coronary artery disease (23). Our results suggest that the total 25(OH)D levels in PHPT patients may not be a good indicator of vitamin D status before or after surgery since there is a much lower rise in both bioavailable and free 25(OH)D concentrations.

The appropriate management of asymptomatic PHPT still require more evidence from clinical studies (1, 24) despite the guidelines for PHPT have been revised recently (25). There are controversies about vitamin D supplementation in the PTHP patient with low 25(OH) levels. Marcocci et al. reviewed three studies; two demonstrated that vitamin D supplementation had no significant influence on serum and urinary calcium levels, and one study showed no clinical benefit, while in the third study of 27 PHPT patients, 12 patients developed either increased serum calcium levels or increased urine calcium excretion (24). Our data show here that there are no significant differences in free and bioavailable 25(OH)D levels between PHPT patients and control subjects. Given the pre-existing high serum Ca in PHPT patients, we suggest that clinicians should be aware of this treating PHPT patients with vitamin D, especially when using a loading dose of vitamin D supplementation (26).

The DBP concentration is relatively stable throughout life but is altered by gender, menopausal status (10), and genetic backgrounds (27–29). In the current study, we matched PHPT patients and control subjects for these factors. The underlying mechanism for lower DBP concentration in PHPT patients, at least in part, may be explained by the higher iPTH levels inhibition of liver-derived DBP in PHPT patient, a finding that has been reported previously (8) and PTH/PTH-related

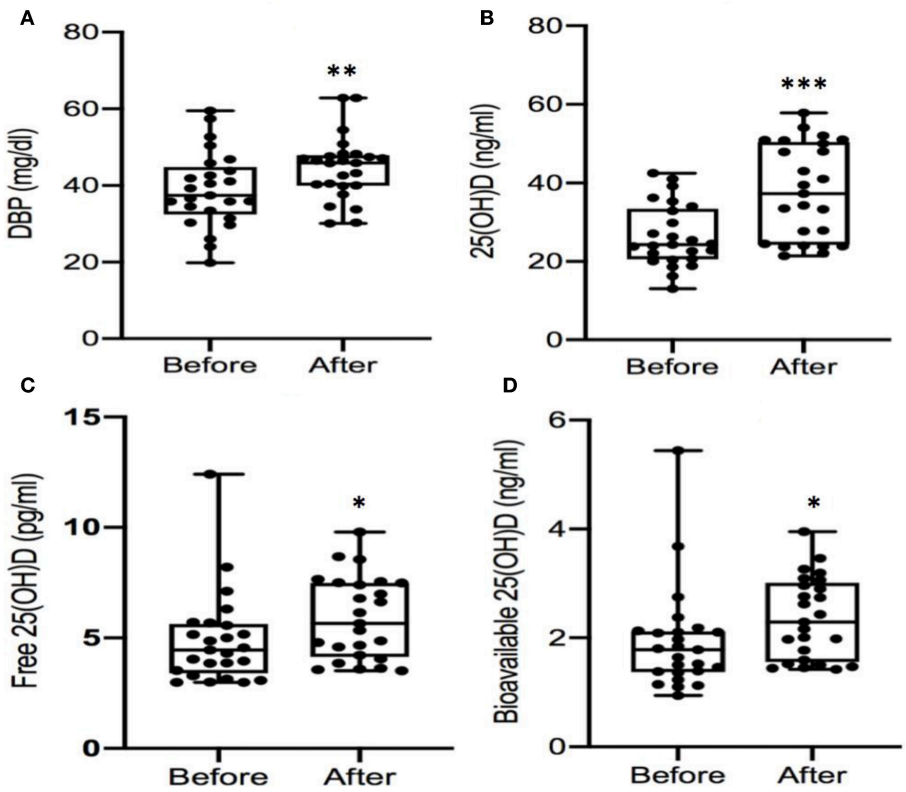

FIGURE 2 | (A-D) Comparison of serum levels of DBP and 25(OH)D before and after parathyroidectomy. DBP, vitamin D binding protein; 25OHD, 25-hydroxyvitamin D. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

protein receptor is highly expressed in the liver (30). Conditions such as malnutrition and liver failure might affect DBP, albumin, and other liver-specific protein status (22). Serum DBP concentrations were inversely correlated with iPTH and calcium levels and DBP increased after decreasing iPTH and calcium by parathyroid surgery. It is also possible that a reduced DPB is a compensatory mechanism in PHPT to ensure that under conditions of low total 25(OH)D, there is adequate circulating free or bioavailable 25(OH)D. Also, the mechanism regulating the rise in DBP after parathyroidectomy remains unclear, but it is suggested that studying this population may help to better understand the binding protein and its regulation of normal vitamin D metabolism.

The limitations of the study are the relatively small sample size, including only 25 PHPT patients had 3 months' post-surgery data. Total 25OHD levels were measured by RIA, and not by mass spectrometry which is considered more accurate, but we used internal and external controls to increase accuracy. Another limitation is that this study does not include serum phosphate or FGF-23 levels and the study design cannot confirm a mechanism of low total 25(OH)D or DBP in PHPT. All patients took calcitriol for 1–2 weeks after surgery and advised to take a vitamin D supplement, as standard post-operative clinical care (11) to prevent risk of low serum calcium levels. As a result, this may be another reason for the increase in serum total 25(OH)D at 3 months after parathyroidectomy. Moreover, calculated free 25(OH)D utilize equations that use average binding coefficients for DBP and albumin may not be as accurate as direct measurements (27).

In conclusion, total 25(OH)D and DBP levels are lower in PHPT patients but calculated free 25(OH) remained relatively unchanged. Parathyroidectomy increased DBP and DBP-bound 25(OH)D levels. Further research is required to investigate whether free 25OHD is the better marker of vitamin D status in the PHPT patient.

AUTHOR CONTRIBUTIONS

ZS, LM, and CS contributed to recruiting patients, data collection and analysis, and manuscript preparation. ST contributed to Parathyroidectomy and manuscript preparation. SS contributed to experimental design, recruiting control subjects, data analysis and manuscript preparation. XW contributed to experimental design, data analysis and manuscript preparation.

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REVIEW
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Vitamin D Binding Protein, Total and Free Vitamin D Levels in Different Physiological and Pathophysiological Conditions

Daniel David Bikle[1,2]* *and Janice Schwartz*[1]

[1] Department of Medicine, University of California, San Francisco, San Francisco, CA, United States, [2] Endocrine Research Unit, San Francisco Veterans Affairs Medical Center, San Francisco, CA, United States

This review focuses on the biologic importance of the vitamin D binding protein (DBP) with emphasis on its regulation of total and free vitamin D metabolite levels in various clinical conditions. Nearly all DBP is produced in the liver, where its regulation is influenced by estrogen, glucocorticoids and inflammatory cytokines but not by vitamin D itself. DBP is the most polymorphic protein known, and different DBP alleles can have substantial impact on its biologic functions. The three most common alleles—Gc1f, Gc1s, Gc2—differ in their affinity with the vitamin D metabolites and have been variably associated with a number of clinical conditions. Although DBP has a number of biologic functions independent of vitamin D, its major biologic function is that of regulating circulating free and total levels of vitamin D metabolites. 25 hydroxyvitamin D (25(OH)D) is the best studied form of vitamin D as it provides the best measure of vitamin D status. In a normal non-pregnant individual, approximately 0.03% of 25(OH)D is free; 85% is bound to DBP, 15% is bound to albumin. The free hormone hypothesis postulates that only free 25(OH)D can enter cells. This hypothesis is supported by the observation that mice lacking DBP, and therefore with essentially undetectable 25(OH)D levels, do not show signs of vitamin D deficiency unless put on a vitamin D deficient diet. Similar observations have recently been described in a family with a DBP mutation. This hypothesis also applies to other protein bound lipophilic hormones including glucocorticoids, sex steroids, and thyroid hormone. However, tissues expressing the megalin/cubilin complex, such as the kidney, have the capability of taking up 25(OH)D still bound to DBP, but most tissues rely on the free level. Attempts to calculate the free level using affinity constants generated in a normal individual along with measurement of DBP and total 25(OH)D have not accurately reflected directly measured free levels in a number of clinical conditions. In this review, we examine the impact of different clinical conditions as well as different DBP alleles on the relationship between total and free 25(OH)D, using only data in which the free 25(OH)D level was directly measured. The major conclusion is that a number of clinical conditions alter this relationship, raising the question whether measuring just total 25(OH)D might be misleading regarding the assessment of vitamin D status, and such assessment might be improved by measuring free 25(OH)D instead of or in addition to total 25(OH)D.

Keywords: vitamin D binding protein, vitamin D, free 25(OH)D, free hormone hypothesis, megalin, polymorphisms, liver cirrhosis, pregnancy

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***Correspondence:**
Daniel David Bikle
daniel.bikle@ucsf.edu

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INTRODUCTION

Vitamin D enters the body either from its production in the skin or absorption from the intestine. In either case, vitamin D must be transported to tissues such as the liver where it is metabolized to its major circulating form, 25(OH)D, by a variety of enzymes with 25-hydroxylase activity, the major one being CYP2R1. 25(OH)D is then transported to tissues such as the kidney where it gets further metabolized to its biologically active metabolite 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) by the mitochondrial based CYP27B1. CYP24A1, found in most tissues, is the major enzyme catabolizing 1,25(OH)2D, thus controlling its impact on a cell specific basis. Vitamin D binding protein (DBP) is the key transport protein which, along with albumin, binds over 99% of the circulating vitamin D metabolites. For most cells it is the unbound 25(OH)D that enters cells (free hormone hypothesis), but at least in some cells such as in the kidney, and likely in the parathyroid gland and placenta, DBP participates in the transport of the 25(OH)D into the cell via a megalin/cubilin complex. Although our focus will be on the transport function of DBP and how that relates to the total and free vitamin D levels in different physiologic and pathophysiologic conditions, DBP has a number of functions independent of its role as a vitamin D transport protein. These functions will be briefly reviewed as they do contribute to the role DBP plays in health and sickness independent of its role in vitamin D transport. DBP is a highly polymorphic protein with at least 120 isoforms distinguished by electrophoresis. Of these, three major isoforms have received the most interest—Gc1f, Gc1s, and Gc2. Their structural differences affect DBP function in ways that have an impact on a number of clinical conditions that will be reviewed.

VITAMIN D BINDING PROTEIN

Genomic Regulation

The human DBP gene is located on chromosome 4q12-q13. It is 35 kb in length and comprised of 13 exons encoding 474 amino acids including a 16 amino acid leader sequence, which is cleaved before release. Numerous tissues express DBP, but the liver is the major source (1). The expression of DBP is increased by estrogen (2) as appreciated with the rise in DBP during pregnancy (3, 4) and with oral contraceptive administration (5). However, the exact mechanism for this induction is not clear as a response element for the estrogen receptor in the DBP promoter has not been identified. Androgens, on the other hand, do not appear to affect DBP expression (2). Dexamethasone and certain cytokines such as IL-6 also increase DBP production, whereas TGFβ is inhibitory (6). As for estrogen, the mechanism underlying such regulation is unclear. However, these cytokines and glucocorticoids are likely to play a role in the increase in DBP production following trauma (after an initial decrease in levels due to actin clearance, see below) (7) and acute liver failure (8), which we will discuss subsequently. Primary hyperparathyroidism, on the other hand, is associated with a reduction in DBP levels, likely contributing to the lower 25(OH)D levels in these patients as the free 25(OH)D is not reduced (9). Vitamin D itself or any of its metabolites do not regulate DBP production (10).

Structure and Polymorphisms

The mature human DBP is approximately 58 kD in size, although differences in glycosylation of the protein for different alleles alter the actual size. DBP is the most polymorphic gene known. Before the appreciation of its role as a carrier of the vitamin D metabolites these polymorphisms in DBP were used by population geneticists to track different populations, referring to the protein as Gc globulin. Over 120 variants have been described based on electrophoretic properties (11) as noted above with 1,242 polymorphisms currently listed in the NCBI database (12). Of these variants, the Gc1f and Gc1s (rs7041 locus) and Gc2 (rs4588 locus) are the most common (**Figure 1**). Gc1f and Gc1s involve two polymorphisms, one at aa 432 (416 in the mature DBP) and one at 436 (420 in the mature DBP). The 1f allele encodes the sequence of aa between 432 and 436 as **D**ATPT, the 1s allele encodes the sequence **E**ATPT. This subtle difference in charge makes Gcf run faster (fast) than the Gcs (slow) during electrophoresis. The Gc2 allele encodes DATP**K** which runs slower still. Glycosylation further distinguishes the Gc1 variants from the Gc2 variant. The threonine (T) in Gc1 binds N-acetylgalactosamine to which galactose and sialic acid bind in tandem. The lysine (K) in comparable position in Gc2 is not glycosylated (13, 14). This affects the conversion of DBP to DBP-MAF (macrophage activating factor), which involves a partial deglycosylation removing the galactose and sialic acid by the sequential action of sialidase and β-galactosidase by T and B cells (15). The significance of this for the biologic function is described below.

DBP is comprised of 3 structurally similar domains. The first domain is the binding site for the vitamin D metabolites (aa 35–49). Fatty acid binding utilizes a single high affinity site for both palmitic acid and arachidonic acid, but only arachidonic acid competes with 25(OH)D for binding (16, 17). The actin binding site is located at aa 373–403, spanning parts of domains 2 and 3, but part of domain 1 is also involved (18, 19). The C5a/C5a des Arg binding site is located at aa 130–149 (20). DBP serves as a cochemotactic factor for C51/C5a des Arg in its regulation of neutrophil functions (21). Membrane binding sites have been identified in aa 150–172 and 379–402 (22).

Biologic Function

Binding to and Transport of Vitamin D Metabolites

DBP was discovered by Hirschfeld in 1959 (23), and originally called group specific component (Gc-globulin), but it was not until 1975 that its function as a vitamin D transport protein was appreciated (24). In normal individuals, ∼85% of circulating vitamin D metabolites are bound to DBP. Albumin binds ∼15% of these metabolites and does so with much lower affinity. Approximately 0.4% of total $1,25(OH)_2D_3$ and 0.03% of total $25OHD_3$ are free in serum from normal non-pregnant individuals. The affinity of DBP for the vitamin D_2 metabolites is somewhat less than that for the vitamin D_3 metabolites (25). The designation of "bioavailable" vitamin D metabolite is the sum of the free vitamin D metabolite and that bound to albumin,

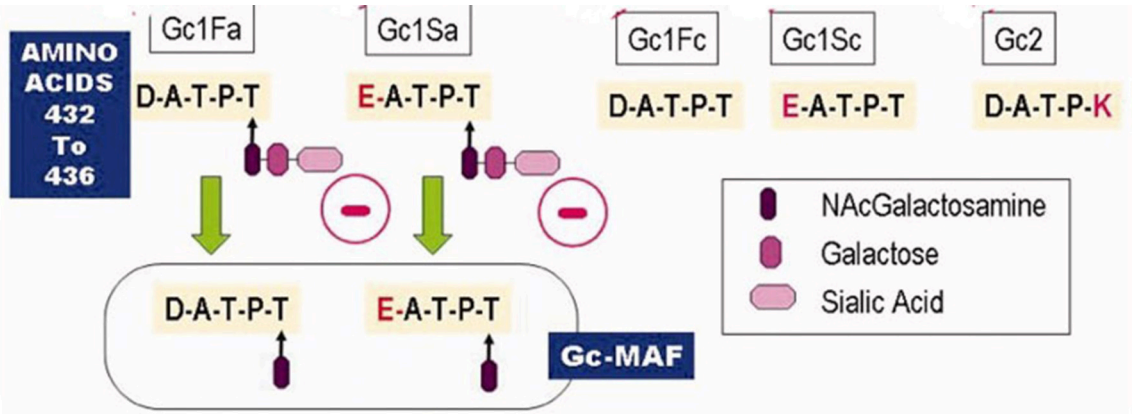

FIGURE 1 | The major DBP alleles. The amino acid differences between the three major DBP alleles are depicted. These differences affect not only their electrophoretic properties but also their glycosylation pattern. In particular Gc2 is not glycosylated, which prevents it from forming the DBP-macrophage activating factor (DBP-MAF). Other biologic differences are discussed in the text.

thus measuring around 15% in normal individuals [review in (26)]. However, the degree to which the albumin fraction is truly bioavailable is not clear (27). 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. However, at least for some tissues, a transport system has been identified that takes up the 25(OH)D (and presumably other vitamin D metabolites) attached to DBP. That system involves megalin/cubilin.

The role of megalin for vitamin D metabolism was discovered by Nykjaer et al. (28), who found extensive loss of DBP in the megalin knockout mouse and 25(OH)D in its urine. These mice have very poor survival rates. More recently, a kidney specific knockout of megalin was developed with a good survival rate, enabling longer term studies that demonstrated reduced circulating levels of the vitamin D metabolites, hypocalcemia, and osteomalacia (29). Cubilin, together with megalin, forms part of the complex facilitating this transport mechanism [review in (30)]. Other tissues express the megalin/cubilin complex including the parathyroid gland and placenta, but its role outside the kidney has received little interest (30). Moreover, activated monocytes may be able to accumulate DBP by a megalin independent process, although this too needs further study (31, 32).

The physiologic role of DBP is well-illustrated in the DBP knockout mouse. In these mice the vitamin D metabolites are presumably all free and/or bioavailable as albumin levels are normal. Unlike the megalin knockout mice, mice lacking DBP do not show evidence of vitamin D deficiency unless placed on a vitamin D deficient diet despite having very low levels of serum 25(OH)D and $1,25(OH)_2D$ and increased loss of these metabolites in the urine (33). Tissue levels of $1,25(OH)_2D$ were normal in the DBP knockout mice, and markers of vitamin D function such as expression of intestinal TRPV6, calbindin 9k, PMCA1b, and renal TRPV5 were maintained. Moreover, injection of $1,25(OH)_2D$ into these DBP knockouts showed a more rapid increase in the expression of Cyp24A1, TRPV5, and TRPV6 than in DBP intact controls (34). However, on a vitamin D deficient diet they quickly developed vitamin D deficiency. More recently, a family has been described to have a mutation in the DBP gene deleting it from the homozygous patient and decreasing its concentration to 50% of normal in a heterozygous sibling (35). The homozygous patient had nearly undetectable levels of total 25(OH)D, although the free concentration measured directly was comparable to that of the normal sibling, as was that of the heterozygote sibling. Parathyroid hormone, calcium, and phosphate were all normal. Thus, DBP does not appear necessary for getting the vitamin D metabolites into cells, supporting the free hormone hypothesis, but DBP clearly serves as a critical reservoir for the vitamin D metabolites, reducing the risk of vitamin D deficiency when intake or epidermal production is limited.

The DBP alleles have been reported to differ in their affinity to 25(OH)D. Gc1f was initially reported as having the highest affinity and Gc2 the lowest among the common alleles (36), but results from other laboratories have not confirmed these differences, and the results from later studies themselves are inconsistent (37, 38). In one such study evaluating the half life of 25(OH)D in serum, subjects homozygous for the Gc1f allele were found to have the shortest half life indicating a reduced affinity (39). On the other hand, serum containing the Gc1f variant of DBP reduced the ability of 25(OH)D and $1,25(OH)_2D$ to induce cathelicidin in monocytes more than that of serum with the Gc2 allele, suggesting the opposite order of affinity (31). Schwartz et al. (40) recently reported that DBP haplotype had significant effects on total 25(OH)D, free 25(OH)D, and DBP levels. The lowest total and free levels of 25(OH)D were seen with the Gc 2/2 haplotype which also tends to have the lowest DBP levels. Other studies have also found lower total 25(OH)D levels in subjects with the Gc2 allele (41–45). The reason the Gc2 allele is associated with lower DBP levels is unknown. DBP haplotype also affected percent free 25(OH)D. The lowest free percentage was seen with the 1s/1s haplotype and the highest one with the

1f/1f haplotype, suggesting that in this survey the Gc1s allele had a higher affinity for 25(OH)D than the Gc1f allele, with the Gc2 allele in between. Furthermore, the different Gc alleles affect the response to vitamin D supplementation. Individuals with the Gc2 variant have been shown to respond to vitamin D supplementation with a more robust increase in 25(OH)D (46). Moreover, within the Gc2 polymorphic region (rs4588), individuals in an Iranian population with an AA genotype within this polymorphic region showed a greater increase in 25(OH)D levels following vitamin D supplementation than those with the GG genotype did (47). Similar results were found with a different polymorphism at rs2282679 in Caucasian women (48). Rs2282679, an intronic polymorphism in the DBP gene that does not alter DBP structure, was previously shown in GWAS studies to be associated with lower 25(OH)D and DBP levels in several different populations (49–51). The clinical significance of these allelic differences is unclear. Differences in these alleles were not found to contribute to a difference in fracture rate in a large study including African Americans and Caucasians (52) or other calcemic and cardiometabolic diseases in the Canadian Multicentre Osteoporosis Study (50). However, as reviewed by Malik et al. (13) and Speeckaert et al. (53), a large number of chronic diseases including type 1 and 2 diabetes (54–56), osteoporosis (57–59), chronic obstructive lung disease (60), endometriosis (61), inflammatory bowel disease (62), some cancers (63–66) [although see (66–68)], and tuberculosis (69) have been associated with DBP variants. Other SNPs at rs4588 have been associated with susceptibility to the metabolic syndrome (70). At the Gc1 locus (rs7041) the G allele is associated with increased susceptibility to hepatitis C viral infection (71). Karras et al. (72) has summarized a number of studies showing the impact of DBP and DBP polymorphisms on various outcomes of pregnancy. These studies demonstrate the recent interest in the impact of polymorphisms on DBP function, but it remains to be seen whether these initial results will be generalized across different populations.

Actin Scavenging

A major function of DBP that has received considerably less interest than that of vitamin D metabolite binding is its role in actin scavenging. Following trauma (7), sepsis (73–75), liver trauma (8, 76, 77), acute lung injury (78), preeclampsia (79), surgery (80, 81), and burn injuries (82), large amounts of actin are released from the damaged cells forming polymerized filamentous F-actin that, in combination with coagulation factor Va, can lead to disseminated intravascular coagulation and multiorgan failure unless cleared (83). The actin scavenging system consists of gelsolin and DBP. Gelsolin depolymerizes F-actin to G (globular) actin. DBP, with its high affinity for G-actin (Kd = 10 nM), prevents the repolymerization and clears it from the blood (84, 85). No clear difference among the major DBP variants has been observed regarding binding to G-actin (53). The DBP-actin complexes are rapidly cleared (half life in blood approximately 30 min) (81), primarily by the liver, lungs and spleen. These tissues have receptors for the DBP-actin complexes (86). The acute conditions result in a fall in DBP levels, potentially decreasing the bioavailability of the vitamin D metabolites (8, 87, 88), with a rise in the DBP-actin complexes (7, 73, 77, 78). The ability of the organism to respond to the insult by increasing DBP production is correlated to survival (7, 8, 89), and has led to the consideration of the use of DBP therapeutically (90, 91).

Neutrophil Recruitment and Migration With Complement 5a (C5a) Binding

Neutrophil activation during inflammation increases their binding sites for DBP (92), and DBP binding to these sites facilitates C5a induced chemotaxis (21) as well as other chemoattractants such as CXCL1 during inflammation (93).The interaction with C5a involves residues 130–149 of DBP, a region which is common to all major DBP alleles (20), and no difference in these alleles has been found with respect to their promotion of C5a mediated chemotaxis (21). Binding of $1,25(OH)_2D$ but not 25(OH)D blocks the promotion by DBP of C5a activity (94).

Fatty Acid Binding

DBP binds fatty acids but with lower affinity ($Ka = 10^5$-$10^6 M^{-1}$) than albumin and via a single binding site (16, 95). Most of the fatty acids binding to DBP are mono-unsaturated or saturated, with only 5% poly-unsaturated. However, only poly-unsaturated fatty acids such as arachidonic acid and linoleic acid compete with vitamin D metabolites for DBP binding (17, 96). This suggests that the different fatty acids alter the configuration of DBP affecting the binding of the vitamin D metabolites rather than directly competing with the vitamin D metabolites for their binding site. The role of DBP in fatty acid transport appears limited.

Formation of the DBP-Macrophage Activating Factor (DBP-MAF) and its Functions

As described above, DBP-MAF is formed from certain alleles (Gc1s and 1f) of DBP following deglycoslyation during inflammatory processes (97). These deglycosylation steps are required for the role of DBP in macrophage activation (15), but further removal of the N-acetyl-galactosamine (NaGal) reduces this activity (98). DBP-MAF is able to activate osteoclasts (99) independent of its 25(OH)D binding function, and it has been shown to stimulate bone resorption in the osteopetrosis (OP) and the incisor absent (IA) rat (100). DBP-MAF has also shown efficacy in a number of tumor models (101–103). Removal of NaGal by α-NaGalase blocks DBP-MAF formation contributing to the loss of immunosuppression in cancer patients (104). α-NaGalase is produced in the liver, and appears to be directly related to tumor burden (105). Preparations of DBP-MAF may have therapeutic potential (14).

FREE HORMONE HYPOTHESIS

As previously noted, the free hormone hypothesis postulates that only the non-bound fraction (the free fraction) of hormones that otherwise circulates in blood bound to their carrier proteins is able to enter cells and exert their biologic effects (**Figure 2**). Examples include the vitamin D metabolites, which we are discussing in this review, sex steroids, cortisol, and thyroid hormone. These are lipophilic hormones assumed to cross

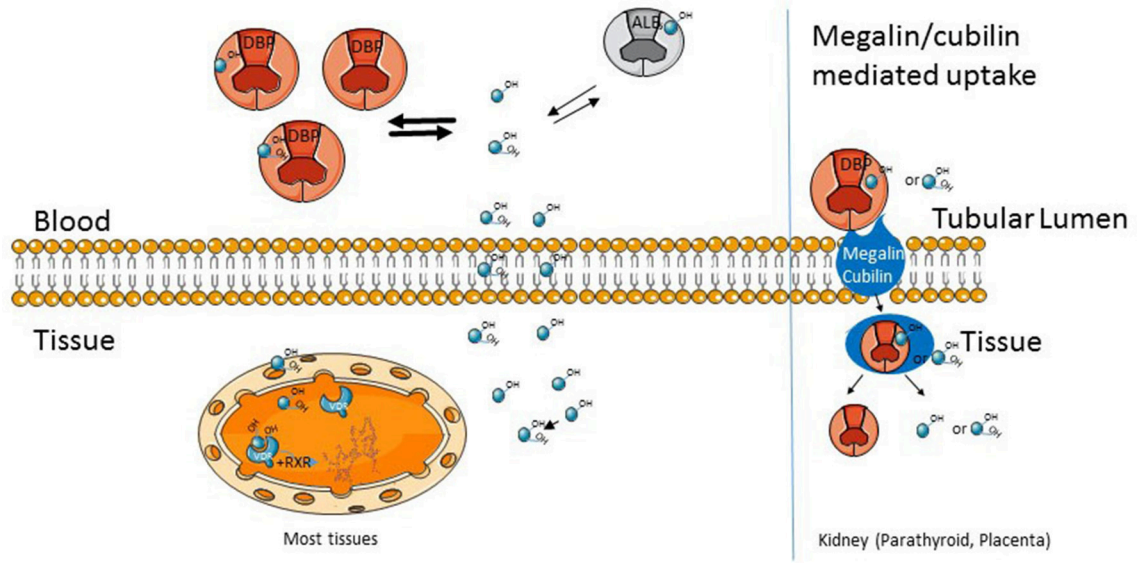

FIGURE 2 | The Free Vitamin D hypothesis. As noted in the text, vitamin D (OH) metabolites are bound to D Binding Protein (DBP) and to a lesser extent albumin in the circulation. These cross the cell membrane as the free (unbound) metabolite in most tissue. However, In the kidney, parathyroid gland, and placenta, the megalin/cubilin complex can transport bound D (OH) metabolites into cells.

the plasma membrane by diffusion and not by an active transport mechanism. One of the earliest clinical examples leading to the formulation of the free hormone hypothesis came from observations by Recant and Riggs (106) that patients with protein losing nephropathy developed quite low levels of thyroid hormone (PBI) along with increased urinary losses but without evidence of hypothyroidism. Subsequent studies have established the free hormone hypothesis for the thyroid and steroid hormones (107, 108), and measurements of the free concentrations of thyroid hormone, estrogen, and testosterone are standard practice. As will be discussed subsequently, this is likely to become the case for free 25(OH)D. As noted earlier, mice lacking DBP lost substantial amounts of the vitamin D metabolites in the urine with marked reductions in their circulating levels of 25(OH) D, but they did not develop evidence of rickets until put on a low vitamin D diet. Such results indicate the importance of the free fraction of 25(OH)D for biologic functions and the role of DBP as a circulating reservoir (33).

To address the clinical relevance of the free hormone hypothesis for vitamin D metabolites, a method to measure the free concentration needed to be developed. This was originally performed by centrifugal ultrafiltration to directly determine the free levels of 25(OH)D and $1,25(OH)_2D$ (109, 110) in various clinical situations. However, this method is labor intensive and has recently been replaced at least for free 25(OH)D by a two-step ELISA that directly measures free 25(OH)D (Future Diagnostics Solutions B.V., Wijchen, Netherlands) using monoclonal antibodies from DIAsource Immunoassays (Louvain-la-Neuve, Belgium). 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 (111, 112). The initial studies with the centrifugal ultrafiltration method established affinity constants for DBP and albumin binding to 25(OH)D and $1,25(OH)_2D$ in a healthy young adult (DD Bikle) and may not be generalizable to a broad range of individuals from different ethnic backgrounds or in different clinical conditions. However, prior to the development of a high throughput ELISA assay to measure the free concentration directly, these affinity constants proved useful in calculating the free concentrations (113, 114) from measurements of DBP, albumin and the total vitamin D metabolite of interest according to the formula:

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

As noted previously, the affinity of 25(OH)D for albumin is much less that than for DBP, leading some to consider albumin-bound 25(OHD) to be essentially "free" or "available" and define "bioavailable 25(OH)D" as free 25(OH)D plus albumin-bound 25(OH)D. Given that the albumin bound 25(OH)D (15%) is considerably higher than the free level (0.03%), this would imply that approximately 500 times as much 25(OH)D is available to cells than if only the free fractions were available. There is little evidence to support albumin bound 25(OH)D as being readily available to cells.

In sera from normal healthy younger individuals, the calculated values of free 25(OH)D and 1,25$(OH)_2$D using DBP measured with polyclonal antibodies correlate reasonably well with the directly measured free levels using centrifugal ultrafiltration for both metabolites or the ELISA assay for 25(OH)D. However, when applied to clinical populations with altered DBP levels either during physiologic (e.g., pregnancy) or pathologic (eg. liver disease) conditions, the calculated values no longer are consistent with those measured directly by either centrifugal ultrafiltration or the newly developed ELISA (115). Part of this is due to the disparity between assays for both the vitamin D metabolite (e.g., 25(OH)D) and DBP, each of which have generally relied on immunoassays. However, mass spectroscopy is becoming the gold standard for measurement of the vitamin D metabolites (116, 117) and is being developed for the measurement of DBP and its various isoforms as well (42, 118). The adoption of mass spectroscopy should reduce the variation in these measurements from different laboratories. But a major problem in attempting to calculate the free fraction of vitamin D metabolites is the assumption that all DBP alleles have the same affinity for the vitamin D metabolites, and that this is invariant under varying clinical conditions. As noted previously, the rank order of affinity of the different alleles for the vitamin D metabolites remains controversial, but differences have been found. Regardless, these potential differences in measured affinity do not begin to explain the large differences between the calculated and directly measured free metabolite levels in various disease states (40). Although there are statistically significant correlations between calculated and directly measured free 25(OH)D, the relationship accounts for only 13% of the variation. Calculated free 25(OH)D concentrations are consistently higher than directly measured concentrations in a variety of studies, such as those performed during the third trimester of pregnancy and in patients with liver disease or cystic fibrosis (115, 119–122). These studies suggest changes in the affinity of 25(OH)D to DBP independent of allelic variations in at least some of these clinical conditions.

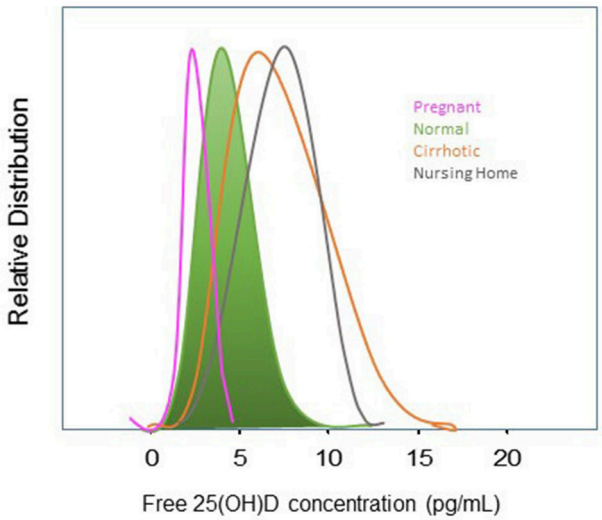

FIGURE 3 | Distribution of free 25(OH)D in Adults and Selected Patient Groups. Distribution of directly-measured free 25(OH)D in normal adults (in green), pregnant women (pink), cirrhotics (orange), and nursing home residents (gray). Distributions are shifted leftward toward lower free 25(OH)D concentrations in pregnant women in the 2nd and 3rd trimesters concordant with increased DBP while decreased synthetic function and DBP in cirrhotics shifts free 25(OH) concentrations to the right toward higher levels. The mechanism for higher free 25(OH) concentrations in Nursing home residents is likely related to D supplementation, somewhat lower, albumin, and the pro-inflammatory state of frailty. Figure generated form data in Schwartz et al. (40).

CLINICAL STUDIES

Healthy Populations

Determinations of free 25(OH)D concentrations in healthy populations show highly significant correlations with total 25(OH)D concentrations whether measured directly or indirectly. Assays to directly measure free 25(OH)D are not currently available for use in clinical care but have been used in research investigations. As noted above, calculated 25(OH)D values are usually higher than when measured directly, which is based on multiple unsubstantiated assumptions such that results obtained with the two methods can differ markedly in different clinical conditions. For these reasons only results from studies with directly measured free 25(OH)D will be discussed. When measured with the direct immunoassay, free 25(OH)D levels have been reported to be between 0.02 and 0.09% of total 25(OH)D concentrations and generally range from 0.5 to 8.1 pg/mL in 95% of healthy adults (**Figure 3**). However, clinical conditions that alter either DBP, the affinity of DBP for 25(OH)D metabolites or albumin, or disposition of vitamin D, may alter free 25(OH)D concentrations or relationships between free and total 25(OH)D concentrations. In this regard, a number of medications, hormones, and smoking have been shown to affect DBP levels (123). Thus, as shown in **Figure 3**, the free concentration of 25(OH)D varies among different clinical conditions. DBP haplotypes have also been hypothesized to alter the affinity between total 25(OH)D and free 25(OH)D, although, as shown in **Figure 4**, the variation in percent free 25(OH)D levels is less affected by DBP haplotype than clinical condition.

Free 25(OH) D in Conditions That Alter DBP

Pregnancy

As pregnancy progresses there are time dependent changes in DBP with almost two-fold increases between the second and third trimesters. Despite these marked DBP changes, mean free 25(OH)D may be the same as or only slightly lower than in non-pregnant women but with less variability than in other groups (40, 124). The slope of the free 25(OH)D vs. total 25(OH) D relationship, however, is significantly less steep than in healthy individuals. The same conclusion was drawn from earlier studies with measurements of free 1,25$(OH)_2$D (109). These results suggest that the affinity of DBP for vitamin D metabolites is decreased during pregnancy, perhaps compensating for increased DBP

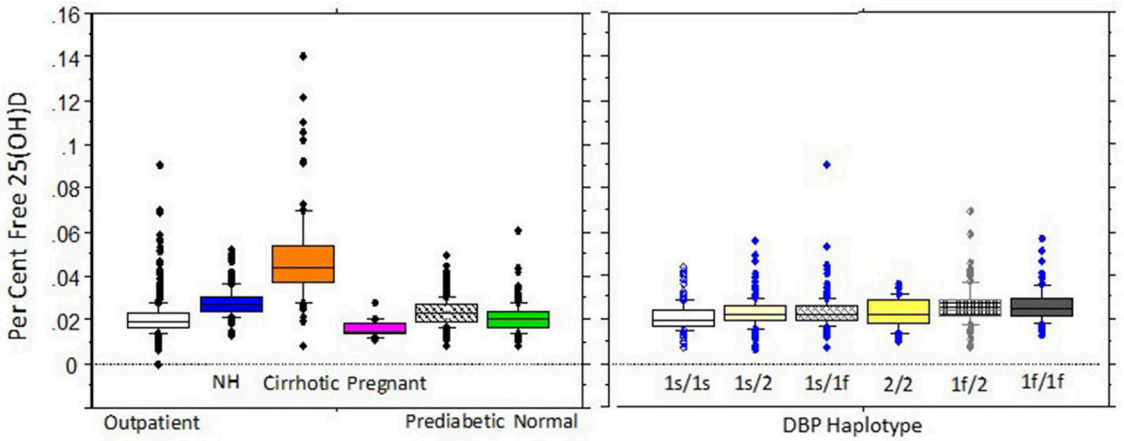

FIGURE 4 | Percent free 25(OH)D in adults by clinical condition or DBP Haplotype. Percent free 25(OH)D concentrations for selected clinical groups on the left panel (community outpatients, NH=nursing home patients, cirrhotics, pregnant women, prediabetics, and normal individuals) and by DBP haplotype on the right . Boxplots show 10th, 25th, median, 75th, and 90th percentile values. Individual points represent values above the 90th and below the 10th percentiles. Both clinical subgroup and DBP genotype significantly effect percentage free 25(OH)D. Between group comparisons for clinical conditions were significant for all but healthy persons compared with pregnant women or outpatients, or for pregnant women compared with outpatients. For DBP haplotypes, smaller but significant differences were detected between the 1s/1s haplotype and the 1s/1f,1f/2, 1f/1f, and 1s/2 haplotypes and between the 1s/2 and 1f/2 and 1f/1f haplotypes and between the 1s/1f and 1f/1f haplotypes. Data are reproduced with permission from Schwartz et al. (40).

concentrations and the needs of both the mother and fetus for calcium.

Liver Disease

Liver diseases that are associated with impaired protein synthetic function such as cirrhosis and acute liver failure result in reductions in DBP and albumin. In addition, the relationship between free 25(OH)D and total 25(OH)D is significantly steeper in patients with cirrhosis than in healthy people indicating altered affinity of DBP for 25(OH)D (40) (**Figure 4**). The net result is that directly measured free 25(OH)D is higher and shows greater variability in patients with cirrhosis compared to healthy individuals and stable outpatients with other chronic conditions (40, 110, 115) despite lower total 25(OH) D concentrations. Results regarding the effects of cirrhosis or acute liver failure on the relationship of total to free 25(OH)D are consistent, creating a strong argument for assessment of free 25(OH)D to assess vitamin D status in the presence of liver pathology as total 25(OH)D measurements may be misleading.

Renal Disease

Nephrotic syndrome, acute renal failure, acute tubular necrosis, or chronic kidney disease associated with renal tubular necrosis may have decreased transport capacity for DBP from the glomerular filtrate into the renal tubules. Heavy proteinuria can lead to loss of DBP as well as 25(OH)D in the urine as the maximal transport capacity of the megalin/cubulin system is saturated. Reports in the literature have not included direct measurement of free 25(OH)D in these conditions, but a small study of nephrotics showed lower total and free $1,25(OH)_2D$ compared to people with normal renal function (125).

Clinical Conditions Not Associated With Altered DBP Levels

Obesity

High BMIs are associated with reductions in total and free 25(OH)D but not DBP or elimination of half-life measurements of 25(OH)D (126). The underlying mechanism for these changes is unknown but may be related to the pro-inflammatory state and circulating cytokines present in obesity, although increased volume of distribution (into fat) has also been invoked.

DBP Haplotypes

Investigations using direct measurements of free 25(OH)D have detected statistically significant but not marked differences in free 25(OH)D concentrations between healthy individuals with the six common DBP haplotypes (**Figure 4**). This is in contrast to the marked differences between haplotypes reported with calculated free 25(OH)D levels (122, 127). As noted previously with directly measured free 25(OH)D, the lowest free 25(OH)D is seen with the Gc 2/2 haplotype and the highest levels with the 1s alleles. Per cent free was highest with the 1f/1f haplotype in our studies (40) (see **Figure 4**).

Nursing Home Subjects

In a vitamin D dose titration study (128) of nursing home residents, who are older, have more chronic co-morbidities, and receive more medications than younger people or community-dwelling elderly, free 25(OH)D levels rose along with increases in total 25(OH)D. The per cent free was higher than in younger adults. Relationships between free and total 25(OH)D were also steeper than those of normal subjects or younger outpatients suggesting altered affinity of 25(OH)D to DBP in this group. Slightly lower albumin concentrations may have also had a small contribution. Inflammation and/or elevated cytokines that

accompany very old age or multiple morbidities may have also contributed to altered affinity of 25(OH)D to DBP in this group (129).

Associations With Markers of Vitamin D Biologic Function

PTH is generally found to be negatively correlated with free 25(OH)D as well as total 25(OH)D. Reports variably conclude that one or the other shows a slightly more significant relationship, but neither explains more than a small amount of the variability in the relationship. Moreover, if the megalin/cubilin complex is operative in the parathyroid gland as it is in the kidney, PTH levels may not be able to distinguish between free and total 25(OH)D with respect to biologic action. However, further insight into the impact of free vs. total 25(OH)D on PTH levels may be gained from several recent studies showing that with high dose D supplementation, changes in iPTH were significantly related to changes in directly measured free 25(OH)D but not to changes in total 25(OH)D (128, 130, 131), suggesting that free 25(OH)D might be a better marker of the biologically available fraction at higher total 25(OH)D concentrations or when 25(OH)D is changing. Data on relationships between directly measured free 25(OH)D and bone density or markers of bone turnover are inconsistent.

Other Conditions

There are limited data on the effect of oral contraceptives or hormone replacement therapy with estrogen, but free 25(OH)D levels and relationships between total and free 25(OH)D do not appear to be significantly influenced by the use of these agents at currently prescribed dosages and routes of administration. Similarly, stable medical conditions such as hypertension, prediabetes, diabetes, osteoporosis, or mild renal disease do not appear to significantly alter relationships between free and total 25(OH)D.

Summary of Clinical Studies

The impact of clinical conditions on free 25(OH)D is that the absolute level, the percent free 25(OH)D and the relationship between free and total 25(OH)D concentrations, differ in pregnant women, 336 people with cirrhosis, and elderly people with multiple morbidities compared to normals or community-dwelling outpatients. These relationships are affected to a much smaller extent by BMI in all groups. It is key that while DBP haplotype variation is associated with differences in per cent free 25(OH)D, the DBP haplotype effects are far smaller in magnitude than those of pregnancy, cirrhosis, or very old nursing home residents with multiple chronic conditions. Thus, total 25(OH)D measurements may be misleading in persons with altered total-to-free relationships, although for other clinical conditions the relationship between total and free 25(OH)D may be less affected.

CONTRIBUTION TO THE FIELD

25(OH)D measurements in the blood currently provide the standard assessment of vitamin D status. Nearly all 25(OH)D circulates as the bound form, with the vitamin D binding protein (DBP) accounting for approximately 85% of the binding, with albumin accounting for most of the rest. However, it is the very small percentage that is not protein bound (0.03% in normal individuals) that is able to cross the membrane of most cells. Conditions that alter levels of DBP or its binding to 25(OH)D alter the relationship between free and total levels. If the free concentration provides a more accurate assessment of vitamin D status, measuring only total 25(OH)D levels may be misleading in situations where the relationship between total and free 25(OH)D levels is altered as in liver disease and pregnancy or in individuals with different DBP alleles. This review examines the impact of different DBP alleles and clinical conditions that do the relationship between free and total 25(OH)D levels, concluding that in a number of clinical situations measuring the free level may provide a better index of vitamin D status than total levels in such situations.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Vitamin D Binding Protein and Inflammatory Injury: A Mediator or Sentinel of Tissue Damage?

*Richard R. Kew**

Department of Pathology, Stony Brook Cancer Center, Renaissance School of Medicine, Stony Brook University, Stony Brook, NY, United States

Neutrophils are the most abundant type of white blood cell in most mammals including humans. The primary role of these cells is host defense against microbes and clearance of tissue debris in order to facilitate wound healing and tissue regeneration. The recruitment of neutrophils from blood into tissues is a key step in this process and is mediated by numerous different chemoattractants. The neutrophil migratory response is essential for host defense and survival, but excessive tissue accumulation of neutrophils is observed in many inflammatory disorders and strongly correlates with disease pathology. The vitamin D binding protein (DBP) is a circulating multifunctional plasma protein that can significantly enhance the chemotactic activity of neutrophil chemoattractants both *in vitro* and *in vivo*. Recent *in vivo* studies using DBP deficient mice showed that DBP plays a larger and more central role during inflammation since it induces selective recruitment of neutrophils, and this cofactor function is not restricted to C5a, as prior *in vitro* studies indicated, but can enhance chemotaxis to many chemoattractants. DBP also is an extracellular scavenger for actin released from damaged/dead cells and formation of DBP-actin complexes is an immediate host response to tissue injury. Recent *in vitro* evidence indicates that DBP bound to G-actin, and not free DBP, functions as an indirect but essential cofactor for neutrophil migration. DBP-actin complexes always will be formed regardless of what initiated an inflammation, since release of actin from damaged cells is a common feature in all types of injury and DBP is abundant and ubiquitous in all extracellular fluids. Indeed, these complexes have been detected in blood and tissue fluids from both humans and experimental animals following various forms of injury. The published data strongly supports the premise that DBP-actin complexes are the functional neutrophil chemotactic cofactor that enhances neutrophil chemotaxis *in vitro* and augments neutrophilic inflammation *in vivo*. This review will assess the fundamental role of DBP in neutrophilic inflammation and injury.

Keywords: inflammation, neutrophil accumulation, chemotactic factor, vitamin D binding protein (DBP), tissue injury

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Giovanni Lombardi,
Istituto Ortopedico Galeazzi (IRCCS), Italy
Stefano Pagano,
University of Perugia, Italy

***Correspondence:**
Richard R. Kew
richard.kew@stonybrookmedicine.edu

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HISTORICAL CONTEXT AND BRIEF BACKGROUND

The vitamin D binding protein (DBP, also abbreviated VDBP) was initially named the group-specific component of serum (abbreviated as Gc-globulin). Jan Hirschfeld is credited with the first publication in 1959 describing the migration pattern of an unknown serum protein using agarose gel electrophoresis and rabbit anti-human serum (i.e., immunoelectrophoresis) (1). This report described qualitative differences in the electrophoretic migration of α_2-globulin proteins in serum obtained from 10 normal healthy blood donors (1). These immunoreactive proteins would display a consistent migration pattern (either fast, intermediate, or slow) that was specific to an individual donor, and thus was named the group-specific component of serum (1). This paper, together with two subsequent papers by Hirschfeld published the following year (2, 3), initiated this six decade long investigation into the structure and function of DBP. A major milestone for DBP research occurred in 1975 when Diager et. al. reported that the serum group-specific component binds vitamin D, and consequently, the authors proposed to name the protein the vitamin D binding α-globulin (4), later shortened to the vitamin D binding protein. A few years later several reports described what initially was thought to be a larger cellular form of DBP (5–7) but subsequently was shown unequivocally to be plasma DBP binding to G-actin monomers in a 1:1 molar complex (8). Thus, by 1980, DBP was known to have two distinct binding functions: vitamin D and G-actin. Structural studies would later confirm the vitamin D sterol and G-actin binding pockets in DBP (9, 10). Beginning in the mid-1980's and continuing into the early 1990's, numerous reports identified DBP and plasma gelsolin as part of the actin scavenging system in blood. These proteins work in tandem to clear extracellular actin released from dead or damaged cells for removal from the circulation, largely in the liver (11). Furthermore, several studies demonstrated that serious injury (burns, traumatic injury, acetaminophen-induced hepatotoxicity) generate large amounts of extracellular actin that consumes DBP and reduces its plasma concentration (12–15). Low levels of plasma DBP directly correlate with poor overall survival in these studies (12–15). Although the actin binding properties of DBP have been clearly established and shown to be physiologically relevant, this role largely has been overshadowed by the vitamin D sterol binding function, particularly over the past 15 years with increasing awareness of the essential role of vitamin D in health and disease. Besides, the vitamin D binding protein is not the best choice of name to promote its actin binding function! There are many facets of this multifunctional plasma protein, several of which are discussed in other reviews of this series. The topic herein will focus primarily on the actin binding capacity of DBP, how this function plays a role in chemotaxis enhancement of neutrophils and may act as a possible mediator of inflammatory tissue injury.

DBP-ACTIN COMPLEXES AND TISSUE INJURY

Tissue injury often causes cell damage and death leading to the release of intracellular contents into extracellular fluids. Many intracellular molecules (HMGB1, mitochondrial DNA, ATP, etc.) have different roles in the extracellular environment and function as "alarmins" (also known as danger-associated molecular patterns or DAMPs) to signal the immune system to the presence of tissue injury (16). Although numerous alarmins have been described and their role in tissue injury well-characterized, it is quite surprising that very little attention has been paid to actin, the major cytoskeletal protein that is the most abundant intracellular protein in any organism. Actin essentially exists in two states inside a cell: monomeric globular actin (G-actin), or G-actin polymerized into filaments (F-actin) (17). These two forms are in a constant state of polymerization and depolymerization that is highly regulated by several intracellular actin binding proteins, and this dynamic process is very prevalent in motile cells such as leukocytes (18). During tissue injury large quantities of actin can be released into extracellular fluids where the protein escapes normal intracellular regulatory mechanisms and the protein spontaneously will form F-actin filaments. In animal models, extracellular F-actin filaments have been shown to alter the coagulation and fibrinolytic systems leading to occlusion and damage of the microcirculation (particularly in the lung) in a manner similar to how fibrin damages the microvasculature in disseminated intravascular coagulation (15, 19). Accordingly, an effective extracellular scavenging system has evolved to dispose of actin released from dead or damaged cells (15). The system is composed of two plasma proteins that have complementary functions: gelsolin, which severs F-actin filaments into G-actin monomers, and DBP that binds G-actin in a high affinity (K_d of 10^{-9} M) 1:1 molar complex for transport and eventual clearance primarily in the liver. This process consumes DBP and a decreased plasma concentration of actin-free DBP has been shown to be an effective but indirect marker of tissue injury in cases of severe trauma in both humans and animal models (15). Although there has been interest in the potential therapeutic role of gelsolin as an extracellular actin scavenger to treat several conditions, less attention has been focused on its partner DBP, despite the fact that over the past 30 years numerous studies (>400 listed on PubMed) have reported that DBP is associated with various acute and chronic diseases in several different organs [reviewed in (20, 21)]. There is no apparent common connection in these various studies, but a very broad view of all these reports would suggest that DBP has a fundamental role during tissue injury, perhaps due to its actin scavenging function.

The vitamin D binding protein is an abundant plasma protein that is part of the albumin gene family and shares considerable amino acid homology and structural similarity with these proteins (11). Several groups have published the crystal structure of DBP that revealed an α-helix triple domain arrangement (characteristic of the albumin family) that forms a broad U-shaped or saddle-shaped molecule (9, 22, 23). The N-terminal (domain I) and the C-terminal domains (III) form the front and back of the saddle and domain II the seat (10, 22, 23). This shape is designed to perfectly fit a molecule of G-actin, and this has been confirmed by crystal structure analysis of DBP bound to G-actin (10). Vitamin D sterol binding pocket in domain I is distinct from the actin binding domain, and a molecule of DBP can bind both ligands simultaneously without

apparent alteration in binding affinity or protein function (24). Although DBP mRNA expression has been reported in many different tissues, plasma DBP is synthesized by hepatocytes in the liver and circulates in blood with a range of 5–9 μM (300–500 μg/ml) (11). It has a relatively rapid turnover in plasma with a half-life of about 2 days (compared to 22 days for albumin) (25). In contrast to albumin whose plasma levels decrease during inflammation, DBP levels in blood are stable or rise slightly during the acute phase response of inflammation (26). Moreover, DBP is ubiquitous *in vivo* and significant quantities (0.1–1 μM range) have been detected in all extracellular fluid compartments (cerebrospinal, bronchoalveolar, synovial, etc.) (25, 26). Although DBP is the primary transport molecule for vitamin D sterols in the blood and extracellular fluids, its concentration in plasma is much higher than vitamin D. Normally only 1–2% of the total circulating DBP pool has vitamin D bound and this percentage never rises above 5% (27). This is in contrast with other transport proteins in blood where about 50% of the total protein pool is bound with ligand (25). This fact prompted many to speculate that DBP must have other essential functions, perhaps related to its ability to scavenge G-actin (28). Indeed, significant tissue injury can result in a large percentage of circulating DBP complexed with actin (12–15). Actin-induced depletion of plasma DBP to levels below 3.5 μM (200 μg/ml) have been shown to be an effective but indirect marker of tissue injury that correlates with poor prognosis in cases of sepsis, multiple trauma and acetaminophen-induced liver failure (12–15). Clinical outcome and decreased plasma levels of DBP have a statistical correlation similar to the other outcome metrics such as the APACHE II score (sepsis), Kings College criteria (liver failure) and the TRISS-like method (multiple trauma) (13–15).

The vitamin D binding protein and actin are abundant and ubiquitous proteins of the intra- and extracellular compartments, their expression is stable during inflammation and cell injury causes immediate complex formation. Thus, global transcriptome analysis of mRNAs increased or decreased during inflammatory injury most likely would not identify actin or DBP (29). For these reasons we believe that the potential role of DBP-actin complexes during inflammation has been overlooked. Varying levels of DBP-actin complexes are continually formed *in vivo* as a result of minor tissue trauma, menstrual cycles, infections, surgery, etc. Hence, low levels of circulating DBP-actin would be a routine physiological process and should be transient and inconsequential. On the other hand, prolonged generation and/or high concentrations of DBP-actin in extracellular fluids potentially could act as a danger signal (alarmin) of ongoing and significant tissue injury. Although previous research has focused on actin-free DBP in plasma, the role of DBP-actin complexes in tissue injury has not been determined, most likely because it has been assumed that these complexes are inactive by-products of cell damage that are rapidly cleared from the circulation. Interestingly, a previous study examining fulminant hepatic necrosis (FHN) in humans showed that 72% of plasma DBP was complexed with actin in patients who died of the disease whereas only 22% of total DBP was bound to actin in FHN survivors (12). However, while this data was very compelling, the authors of this study focused on actin-free DBP, not the level of DBP-actin complexes. Although DBP-actin complexes generally have been viewed as benign by-products of cell injury, recent studies from our lab (both *in vitro* and *in vivo*) have shown that these complexes may serve as an alarmin and possess a cytokine-like function. Thus, the accumulated evidence has shown that DBP, via its actin binding function, likely plays a role in both the mediation and resolution of tissue injury.

CELL-ASSOCIATED DBP

The vitamin D binding protein interacts with many different cell types to achieve its diverse functions (delivery of vitamin D sterols, binding and/or clearance of G-actin, chemotactic cofactor function, macrophage activating-factor), and it appears that the protein must first bind to its target cell surface in order to mediate these effects. Numerous studies have reported DBP on the surface of most cell types including B-lymphocytes (30–32), T-lymphocytes (33), testicular cells (34), placental cytotrophoblasts (35, 36), pancreatic acinar cells (37), monocytes (38), neutrophils (39), and renal proximal tubule cells (40). It is clear that cell-associated DBP is not a novel cellular form but rather circulating DBP bound to the cell surface (41). Although binding to the plasma membrane is the first step in the interaction of DBP with cells, it is unlikely that a cell surface binding site for DBP would be a specific high affinity ($K_d \leq 10^{-8}$ M) receptor. DBP is abundant and ubiquitous in all fluid compartments and circulating blood leukocytes are bathed in 5–9 μM DBP (11). Therefore, a relatively low affinity binding site with a K_d significantly above the plasma concentration of DBP would seem most logical, otherwise leukocytes in blood could act as a "sink" for DBP and diminish the circulating pool. Several studies have shown that DBP binds with low affinity to megalin, also known as LDL receptor related protein 2 (LRP2) (42–44). Megalin is a large scavenger receptor that is primarily expressed on the surface of epithelial cells in the kidney (proximal tubules), endocrine glands, and reproductive organs (45). Megalin on renal proximal tubules binds and captures DBP (both free and bound to vitamin D) in glomerular filtrate for re-uptake into the circulation (45). DBP also binds specifically, but with low avidity, to chondroitin sulfate glycosaminoglycans (CS-GSGs), and more specifically to the CS-GAG group on CD44, which is widely expressed on most leukocytes (46, 47). However, several papers have reported that DBP bound to the surface of cells cannot be removed by numerous high salt and/or detergent washes, suggesting a relatively tight binding site (30, 33, 38).

Our lab has extensively investigated the binding of DBP to human neutrophils and myeloid cell lines (U937, HL-60) since cell binding is the critical first step in the process of DBP functioning as a chemotactic cofactor. DBP does not follow the kinetics of saturable binding to a single high-affinity receptor, but instead displays a multiphasic, time-dependent interaction with neutrophils over the course of 60 min at 37°C: weak binding (5–20 min), strong binding (20–50 min), shedding into the extracellular fluid (>45 min) (46). There is very little (if any) binding of radiolabeled DBP to cells incubated in an ice bath

(1°C), but at 37°C three distinct phases of DBP binding are observed, indicating that a dynamic process is required to express this binding site. In addition, if neutrophils are first stimulated with chemotactic factors or calcium ionophores at 37°C prior to incubation with labeled DBP, the delay in tight binding phase is essentially eliminated. The initial weak binding of DBP to neutrophils probably is mediated by low avidity molecules such as CD44 or other chondroitin sulfate proteoglycans upregulated from internal stores in neutrophil cytoplasmic granules (46, 48). These weak binding molecules may serve to capture DBP from extracellular fluids and loosely tether it to the cell surface.

The biochemical characterization of the tight binding site for DBP on the plasma membrane of neutrophils, and mechanisms of the subsequent protease-mediated shedding, were very extensive (46, 48–50) and showed that: (a) DBP bound to the plasma membrane can only be dissociated using harsh denaturing conditions; (b) solubilization of cells revealed that the bound DBP partitions to the detergent insoluble fraction, which contains the actin cytoskeleton; (c) confocal immunofluorescence microscopy revealed that DBP and actin co-localize on the surface of neutrophils; (d) immunoprecipitation of DBP bound to (or shed from) cells, followed by mass spectrometry analysis showed that the major binding partners were actin, CD44 and annexin A2. Annexin A2 is known as a molecular facilitator since it binds multiple diverse molecules (including CD44 and actin) to assemble plasma membrane complexes in a phospholipid and calcium-dependent manner (51). Interestingly, two reports published more than a dozen years before our studies on neutrophil cell surface DBP binding site, described that DBP was tightly bound to actin on the surface of human B lymphocytes (30) and monocytes (38).

The third phase, DBP shedding from the cell surface, was shown to be due to the action of the enzyme elastase on the DBP binding site, since DBP is not modified (cleaved) during this process (49). Elastase is a physiologically important protease with broad substrate specificity, its expression is restricted mainly to neutrophils and abundant quantities of this protease are stored in the azurophil (primary) granules in the cytoplasm of both immature and mature neutrophils (52). Inhibition of elastase will inhibit shedding and cause DBP to accumulate on the plasma membrane, and also will inhibit the chemotactic cofactor function of DBP (49). Previous reports have described proteolytically active elastase on the cell surface of neutrophils *in vitro* (53, 54). Interestingly, both DBP binding, and shedding of the binding site, are constitutive processes and occur in the absence of an inflammatory or chemotactic stimulus, however these stimuli accelerate the process (46, 49). Moreover, as will be discussed below, DBP binding to cell surface actin on neutrophils and subsequent elastase-mediated shedding from the plasma membrane, may be a key step in understanding its function as a chemotactic cofactor.

In addition to DBP uptake from fluids, we also have previously reported that human neutrophils contain an intracellular store of DBP in specific (secondary) granules (39), and this finding was verified in a follow-up study investigating the DBP binding site in neutrophils (46). Neutrophil specific granules contain a diverse array of molecules including several binding proteins such as haptoglobin and vitamin B_{12} binding protein (55). It is not clear why these cells have DBP stored in intracellular granules formed during myelocyte stage of neutrophil development in the bone marrow (56). Perhaps cells utilize this store of DBP during chemotaxis and phagocytosis when there is dynamic rearrangement of cell structures and release of granule contents (56). The amount of DBP was calculated to be 3 ng/10^6 neutrophils, a rather small quantity but considering that an inflammatory exudate may contain billions of neutrophils, the amount of DBP that neutrophils potentially could release in an inflammatory lesion may not be insignificant.

NEUTROPHILS, INFLAMMATORY INJURY, AND THE CHEMOTACTIC COFACTOR FUNCTION OF DBP

Neutrophils are the primary "rapid response" cells of the innate immune system that are essential for host defense (57, 58). Individuals with a marked reduction in circulating neutrophils (neutropenia), either due to a rare congenital abnormality or more commonly a consequence of cancer chemotherapy, are highly susceptible to severe and often life-threatening bacterial and fungal infections. The importance of maintaining adequate numbers of circulating neutrophils is highlighted by the fact that ∼60% of the total bone marrow output of all blood cells is dedicated just to produce neutrophils (59). It is estimated that the average healthy person has a steady-state production of 100 billion neutrophils per day under homeostatic conditions, whereas cell production increases significantly and rapidly during an infection, a process known as emergency granulopoiesis (60, 61). However, it has been well-known for more than a century that excessive tissue accumulation of neutrophils is observed in many inflammatory disorders (neutrophilic inflammation). Cytotoxic products from activated neutrophils mediate significant tissue injury and are linked to the pathogenesis of numerous acute and chronic diseases (57–59). The destructive potential wrought by excessive numbers of responding neutrophils is highlighted by the fact that this cell type can liquify and obliterate tissue, i.e., a cavity resulting from an abscess (62). There has been remarkable progress over the past 5–8 years in understanding various aspects of neutrophil biology, from their birth in the bone marrow to their death at sites of inflammation and everything in between. Perhaps most interesting is that neutrophils possess considerable plasticity and are far more adaptable to their environment than previously thought. Furthermore, neutrophils can display several disparate functions that contradict their long-time moniker as "masters of tissue destruction." Many excellent reviews on various aspects of neutrophil biology have been published recently (63–66).

Migration of neutrophils from the bloodstream into various tissues is a critical stage during inflammation triggered by both infectious and non-infectious stimuli (63). Although much is known about the chemoattractants and their receptors that initiate and direct the neutrophil migratory response, little is known about factors in physiological fluids that regulate tissue recruitment of these cells. More than 35 years ago

several investigators had demonstrated that normal human serum possesses a heat stable chemotactic enhancing activity for complement activation peptide C5a (67–69). A 60 kDa protein (called co-chemotaxin) was partially purified from human serum and shown to be capable of enhancing the neutrophil chemotactic activity of C5a and its stable degradation product C5a des Arg (70). Previously, we were the first of several groups to identify that DBP was the serum co-chemotaxin for C5a (71, 72), and several other groups subsequently confirmed our initial observations that DBP can augment the chemotactic activity of C5a/C5a des Arg (73–79). These studies all reported that DBP does not possess chemotactic activity but requires chemoattractants to exert its cofactor activity. Other studies also reported that DBP is not able to augment other C5a-mediated functions in neutrophils such as oxidant generation and degranulation (release of cytoplasmic granule contents) (71, 79). But most curious was the observation that DBP could not enhance the chemotactic activity of other major neutrophil chemoattractants: formylated peptides, CXCL8 (IL-8), leukotriene B_4 or platelet activating factor (71, 72), leading to the general consensus that the chemotactic enhancing properties of DBP appeared to be restricted to C5a/C5a des Arg. All of these reports used an *in vitro* chemotaxis assay, employing either blind-well or Boyden chambers, to measure an increase in neutrophil migration when DBP was added. Although this assay is very sensitive and quantitative, it has limitations that resulted in the mistaken conclusion that DBP was specific for complement peptide C5a. C5a is a very potent neutrophil chemotactic factor and on a molar basis is 10–50 times more potent than other major chemoattractants when tested *in vitro* (71, 80). Therefore, the chemotactic enhancing activity of DBP during the short incubation (30 min) filter-based assays was particularly noticeable when C5a was used as the stimulus. However, utilizing a different *in vitro* assay (under agarose), that requires a longer incubation period (4 h), revealed that DBP could enhance neutrophil movement to other chemoattractants as well (81). Moreover, *in vivo* studies using the DBP null mice (discussed below) have shown that the chemotactic cofactor activity of DBP is not specific for C5a as previously thought but can augment the chemotactic activity of perhaps many leukocyte chemoattractants. These recent *in vitro* and *in vivo* studies described below have helped to better define how DBP functions to enhance neutrophil chemotaxis that may contribute to neutrophilic inflammation.

LESSONS FROM THE DBP NULL MOUSE

The initial reports describing the chemotactic cofactor function of DBP were received enthusiastically at that time in the inflammation research community (71, 72). However, that high level of interest faded with the subsequent failure to describe a clear mechanism of chemotaxis enhancement, and to provide confirmation that DBP enhances neutrophil chemotaxis *in vivo*. Previous attempts to define a mechanism using *in vitro* approaches were not successful. For example, our lab performed numerous chemical cross-linking and co-immunoprecipitation experiments that demonstrated clearly DBP does not physically-associate with the C5a receptor (50). In addition, we reported that and DBP does not bind to C5a, and cell binding of C5a and DBP are independent events (82). Other groups have shown that DBP does not alter the number of neutrophil C5a receptors or the receptor K_d for C5a, thereby discounting another obvious explanation for its co-chemotactic effect (79, 83). The question of physiological relevance and whether DBP enhances neutrophil recruitment to tissues *in vivo* needed to wait more than 10 years until a DBP null mouse was generated.

The vitamin D binding protein has been very well-studied in multiple different populations worldwide for over 50 years, but no natural homozygous deficiency of DBP had been reported in humans, or to the best of our knowledge, any mammal. There was no clear reason why a DBP-deficiency was never identified but it was widely speculated that a DBP deletion may be embryonic lethal. Nevertheless, Nancy Cooke's lab at the University of Pennsylvania produced the first mice that were homozygous null for DBP (84). These mice were healthy, of similar size and appearance as wild-type mice, and the DBP null males and females were fertile and produced normal sized litters (84). This phenotype was somewhat surprising at the time given the fact that a natural DBP deficiency had not been observed. Analysis of blood chemistry values revealed that DBP null mice had essentially the same levels of serum calcium, phosphorus, PTH, and alkaline phosphatase as DBP sufficient wild type mice when fed a standard vitamin D replete mouse chow diet (84). However, the serum of DBP null mice had >95% reduction in both 25(OH) and 1,25 $(OH)_2$ vitamin D (84). Moreover, when placed on a vitamin D deficient diet, the DBP null mice quickly developed secondary hyperparathyroidism and bone mineralization defects such as osteoid thickening that were not seen in DBP sufficient wild-type mice (84). The initial DBP null strain was backcrossed onto a C57BL/6J background for 10 generations and was used in further studies which showed that the lack of circulating DBP does not alter the tissue distribution, uptake, activation or biological potency of vitamin D (85). Thus, DBP does not alter bioavailable vitamin D but appears to function as a circulating reservoir of 25(OH) vitamin D.

Since no case of homozygous deficiency in humans has been reported, a long-standing unresolved question has been: can DBP null mice function as a murine model for DBP deficiency in humans? Surprisingly, during the preparation of this review, the first report of a DBP deficient human was published (86). The individual is a 58 year-old female who presented with long-standing and progressive ankylosing spondylitis and severe vitamin D deficiency that did not respond to vitamin D supplementation. Laboratory results showed a deletion of the DBP (*GC*) gene and corresponding absence of circulating DBP. Although this individual had a profound deficiency in both 25(OH) and 1,25 $(OH)_2$ vitamin D, her blood calcium levels were normal (86). The bone abnormalities and blood chemistry values of this DBP deficient woman were similar to those observed in DBP null mice fed a vitamin D-deficient diet (84, 86). *In vitro* studies using dermal fibroblasts from this DBP null patient showed that DBP does not alter cellular uptake of bioactive vitamin D and expression of the responsive gene

CYP24A1, a very similar result also was previously reported using cells from DBP null mice (85). However, no information was provided about this patient's response to infections or tissue injury. This report provides some degree of validation that DBP null mice can act as a model for DBP deficiency in humans.

DBP NULL MICE AND INFLAMMATORY INJURY MODELS

Our *in vivo* studies of inflammatory injury utilized the DBP null mouse strain developed by Nancy Cooke's lab (84, 87). This DBP null strain was re-derived the by *in vitro* fertilization using $DBP^{-/-}$ sperm and a wild-type C57BL/6J female (DBP+/+) to produce $DBP^{+/-}$ hemizygotes which were cross-bred to generate the DBP null ($^{-}/^{-}$) and wild type (+/+) mouse colonies (81). These $DBP^{-/-}$ and $DBP^{+/+}$ mice have been used in three injury models: acute lung injury, multiphasic (acute, chronic, fibrotic) lung injury, and acute muscle injury (81, 88). Each model clearly showed that DBP null mice always have less inflammation, and most noticeably, significantly fewer neutrophils at the site of injury. The markedly reduced neutrophilic inflammation observed with DBP null mice is very consistent among the injury models and reproducible between experiments, but the underlying cellular and molecular mechanisms that are responsible for generating this phenotype is only partially understood. A proposed tentative model is described in a separate section below.

The first study to examine the *in vivo* role of DBP in inflammatory injury used a model of acute complement-dependent alveolitis, induced by either immune complexes or purified mouse C5a (81). In both alveolitis models, DBP null mice had significantly reduced (∼50%) neutrophil recruitment to the lungs compared to their wild-type $DBP^{+/+}$ counterparts, and lung histology showed significantly less inflammation in the null mice (81). Another important observation is that addition of exogenous DBP to the lungs of DBP null mice completely rescued their neutrophil recruitment defect. The same study also showed that bronchoalveolar lavage (BAL) fluid from wild-type mice had extensive DBP-actin complexes (∼75% of total DBP) 4 h after induction of alveolitis. Although as predicted there were no DBP-actin complexes in DBP null mice, there was detectable actin in the BAL fluid from these animals, indicating actin release from damaged cells (81). These results indicate that DBP null mice have impaired neutrophil recruitment due to lack of DBP and not a cellular defect since the total number, receptor expression and chemotaxis of circulating DBP null neutrophils are essentially identical to cells from their wild-type $DBP^{+/+}$ counterparts.

A second study investigated if a systemic DBP deficiency could attenuate multiphasic lung injury and tissue remodeling induced by bleomycin (81). Wild type and DBP null mice received bleomycin by oropharyngeal aspiration; lung injury was evaluated after 7, 16, or 21 days. DBP null mice all survived to day 21 and did not display overt signs of morbidity whereas all wild-type mice died between day 13 and 16 and showed clear signs of respiratory distress. Bronchoalveolar lavage (BAL) fluid from wild-type mice had extensive DBP-actin complexes (60–75% of total DBP) whereas DBP null mice had no complexes but had evidence of free actin. Analysis of BAL fluid on days 7 and 16 post-treatment showed that both mouse strains had similar numbers of lung macrophages and lymphocytes, but DBP null animals had significantly fewer lung neutrophils. Histological analysis of the lungs on day 16 showed that DBP null mice had a 50% decrease in fibrosis and collagen deposition as compared wild-type animals. This study demonstrated that a systemic deficiency in DBP provides significant protection from bleomycin-induced inflammation and fibrosis in mice.

The third *in vivo* study utilized an acute muscle injury model induced by injection of 50% glycerol into the thigh muscle (88). All animals survived the procedure, but intramuscular glycerol injection showed lysis of skeletal myocytes, and inflammatory cell infiltrates in both strains of mice. The muscle inflammatory cell infiltrate in DBP null mice had remarkably few neutrophils as compared to wild-type mice. The neutrophil chemoattractant CXCL1 was significantly reduced in muscle tissue from DBP null mice. Plasma obtained 48 h after glycerol injection revealed that DBP null mice had significantly lower levels of systemic cytokines IL-6, CCL2, CXCL1, and G-CSF. Multiplex analysis of 36 cytokines indicated that DBP null mice had a less inflammatory and more pro-reparative cytokine profile than their wild-type $DBP^{+/+}$ counterparts (88).

These *in vivo* studies comparing null mice to wild-types showed that DBP may have a central role during inflammation since it induces selective recruitment of neutrophils, and the DBP cofactor function is not restricted to C5a as prior *in vitro* studies indicated, so the physiological implications are much broader. So how does DBP contribute to inflammation by enhancing neutrophil recruitment? The accumulating *in vitro* and *in vivo* evidence appears to suggest that DBP-actin complexes may act as an alarmin and trigger pro-inflammatory functions. DBP is the major extracellular scavenger for actin released from damaged/dead cells and formation of DBP-actin complexes is an immediate host response to tissue injury. All of the *in vivo* injury models discussed above had evidence of DBP-actin complexes only in wild-type mice, and DBP repletion in the acute alveolitis model reversed the neutrophil recruitment defect in the DBP null mice. The currently accepted actin scavenger hypothesis states that injury-induced depletion of plasma DBP will diminish the actin binding capacity in blood and extracellular fluids, leading to formation of actin filaments that obstruct flow and damage small blood vessels (15). Furthermore, if the current actin scavenger hypothesis is correct, it follows that DBP null mice should succumb rapidly to an intravenous bolus of actin which would obstruct the pulmonary vasculature. However, contrary to what would be predicted by the actin-free DBP hypothesis, we observed that DBP null mice do not succumb to a bolus of purified actin injected intravenously (89). In fact, DBP null mice were largely resistant to the lung inflammation and injury observed in wild-type mice, and the null mice appeared utilize plasma gelsolin to clear the actin bolus. Wild-type mice also had a large percentage of their total plasma DBP pool (78%) bound to actin 1.5 h after i.v. injection. Moreover, *in vitro* studies showed that purified DBP-actin complexes added to cultured

endothelial cells caused direct cell damage (at 4 h) or death (at 24 h), providing clear evidence that DBP-actin complexes have a direct detrimental effect on cells (89). Thus, results from DBP null mice provide strong evidence to refute the prevailing actin scavenger hypothesis and suggest that the inverse hypothesis may be valid, i.e., an increase in DBP-actin complexes, and not a reduction in actin-free DBP, correlates with inflammation and injury.

A POSSIBLE ROLE FOR VITAMIN D IN INFLAMMATORY INJURY?

Vitamin D is known to regulate numerous genes that are involved in the immune and inflammatory response. *In vitro* and *in vivo* studies have shown that active vitamin D produces a tolerogenic, anti-inflammatory, and reparative phenotype as evidenced by immune cell activation status and cytokine profiles (90, 91). DBP null mice have almost no detectable serum vitamin D but actually are vitamin D sufficient when fed a vitamin D replete chow diet (84, 87). Although plasma DBP does not alter the tissue availability of vitamin D, the effect on immune cells in the blood is not known. The lack of plasma DBP in mice may permit greater delivery of bioactive vitamin D to immune cells during their transit in the blood, potentially altering their transcriptomes to dampen inflammation and limit tissue damage. Superimposed on this possible scenario of transcriptional regulation by vitamin D is a lack of DBP-actin complexes during tissue injury. Perhaps both mechanisms together cause DBP null mice to have less neutrophilic inflammation and resultant tissue damage following injury. However, these possibilities remain to be investigated. Finally, it is interesting to note that we previously reported that bioactive vitamin D (1,25 dihydroxy-vitamin D_3) bound to DBP at physiologically relevant concentrations of 10 and 100 pM, completely abolished the DBP chemotactic cofactor function of human neutrophils *in vitro*, but had no effect on chemotaxis to optimal concentrations of four different chemoattractants (80). In contrast, 25-hydroxy-vitamin D_3 bound to DBP had no effect on the chemotactic cofactor function (80), thus providing evidence of a direct inhibitory effect of bioactive vitamin D on the DBP chemotactic cofactor function for neutrophils.

PROPOSED MODEL OF DBP-ACTIN COMPLEXES AND INFLAMMATORY INJURY

Recent evidence indicates that DBP bound to G-actin, and not free DBP, functions as an indirect but essential cofactor for neutrophil migration, thus, providing a possible mechanism to explain how DBP functions to enhance neutrophil migration *in vitro* and recruitment to sites of inflammation *in vivo* (81, 88, 89). However, it is not clear how DBP-actin complexes enhance neutrophil recruitment and inflammation. It is interesting to speculate that DBP-actin complexes may augment a chemotactic signal and cause release of other proinflammatory molecules stored within neutrophils. Perhaps the most attractive candidate in this scenario is calprotectin, a 24 kDa heterodimer composed of S100A8 and S100A9 that can be rapidly released from neutrophils (92). Abundant quantities of S100A8 and S100A9 are stored in the cytosol of neutrophils, and upon cell activation these molecules can be released into the extracellular space as active heterodimers or heterotetramers (92). S100A8/A9 has multiple proinflammatory functions and has been shown to mediate both neutrophil bone marrow development and facilitate chemotaxis of mature circulating cells, most likely by binding to toll-like receptor 4 (TLR4) on the plasma membrane (92). The proposed mechanism involving DBP-actin complexes has clear physiological relevance since DBP is abundant and ubiquitous in all fluid compartments, and release of G-actin from damaged/dead cells is a consistent feature in all types of inflammatory injury. Moreover, we have previously reported that DBP binds to actin on the neutrophil plasma membrane followed by elastase-mediated shedding of these complexes, perhaps as microvesicles, into the extracellular fluids (46, 48, 49). We propose that DBP-actin complexes can bind (or re-bind following shedding) to a neutrophil surface receptor that triggers S100A8/A9 release. In turn, S100A8/A9 binds in an autocrine or paracrine manner to neutrophil TLR4 inducing a signal that synergizes with the chemoattractant receptor signal to enhance migration. Furthermore, it is well-known that TLR4 ligation and signaling synergizes with signals from chemoattractant receptors to enhance leukocyte chemotaxis both *in vitro* and *in vivo* (93, 94).

This provisional model may explain how a deficiency of DBP results in significantly decreased neutrophilic inflammation. However, there are several "unknowns" with this model that need to be investigated. First, the putative receptor for binding DBP-actin complexes has not been identified, but other studies have shown that the receptor Clec9A binds F-actin and is involved in sensing damaged cells (95–97). It is not known if the Clec9A receptor also binds G-actin complexed with DBP or if even if this receptor is expressed on neutrophils. Second, it is not known if the putative DBP-actin receptor signals release of S100A8/A9 when it is ligated with complexes. Third, a question remains do all DBP-actin complexes function the same or are there modifications that differentiate between inflammatory and benign complexes. Finally, do other cell types besides neutrophils and endothelial cells also respond to DBP-actin complexes, particularly hepatocytes and Kupffer cells in the liver (the primary site of DBP-actin clearance).

FUTURE DIRECTIONS

Many questions about the functions of DBP remain to be answered. However, new investigative tools and experimental approaches will be needed to decipher these functions. For example, new mouse models with tissue specific inducible expression of DBP, or a mouse model constructed with selective deletions in functional regions within DBP (vitamin D, actin, cell binding regions) by utilizing CRISPER-Cas9 gene editing technology. Another essential tool currently needed is an antibody that only recognizes a neoepitope on DBP-actin complexes, and not DBP or G-actin monomers.

This antibody then could be used to develop an ELISA that specifically detects only DBP-actin complexes in biological fluids. In closing, research into the biological functions of DBP has progressed far since Hirschfeld's initial description 60 years ago, but perhaps in the near future new experimental approaches using advanced technologies (single cell transcriptomics, mass cytometry, advanced microscopy, and *in vivo* imaging, etc.) and bioinformatic analysis may reveal what DBP actually has been doing all along.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Vitamin D Binding Protein and the Biological Activity of Vitamin D

Rene F. Chun[1], Albert Shieh[2], Carter Gottlieb[1], Vahe Yacoubian[1], Jeffrey Wang[1], Martin Hewison[3] and John S. Adams[1]*

[1] Department of Orthopaedic Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA, United States, [2] Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, United States, [3] Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, United Kingdom

Vitamin D has a long-established role in bone health. In the last two decades, there has been a dramatic resurgence in research interest in vitamin D due to studies that have shown its possible benefits for non-skeletal health. Underpinning the renewed interest in vitamin D was the identification of the vital role of intracrine or localized, tissue-specific, conversion of inactive pro-hormone 25-hydroxyvitamin D [25(OH)D] to active 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]. This intracrine mechanism is the likely driving force behind vitamin D action resulting in positive effects on human health. To fully capture the effect of this localized, tissue-specific conversion to 1,25$(OH)_2$D, adequate 25(OH)D would be required. As such, low serum concentrations of 25(OH)D would compromise intracrine generation of 1,25$(OH)_2$D within target tissues. Consistent with this is the observation that all adverse human health consequences of vitamin D deficiency are associated with a low serum 25(OH)D level and not with low 1,25$(OH)_2$D concentrations. Thus, clinical investigators have sought to define what concentration of serum 25(OH)D constitutes adequate vitamin D status. However, since 25(OH)D is transported in serum bound primarily to vitamin D binding protein (DBP) and secondarily to albumin, is the total 25(OH)D (bound plus free) or the unbound free 25(OH)D the crucial determinant of the non-classical actions of vitamin D? While DBP-bound-25(OH)D is important for renal handling of 25(OH)D and endocrine synthesis of 1,25$(OH)_2$D, how does DBP impact extra-renal synthesis of 1,25$(OH)_2$D and subsequent 1,25$(OH)_2$D actions? Are their pathophysiological contexts where total 25(OH)D and free 25(OH)D would diverge in value as a marker of vitamin D status? This review aims to introduce and discuss the concept of free 25(OH)D, the molecular biology and biochemistry of vitamin D and DBP that provides the context for free 25(OH)D, and surveys *in vitro*, animal, and human studies taking free 25(OH)D into consideration.

Keywords: vitamin D, free vitamin D, bone, immunology, DBP, CYP27B1, VDR

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****Correspondence:***
Rene F. Chun
rchun@mednet.ucla.edu

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INTRODUCTION

The benefits of vitamin D for mineral homeostasis and bone health are well-established. Deficiency of vitamin D, rickets in children and osteomalacia in adults, can be treated or prevented with oral supplements of vitamin D. Despite promising pre-clinical observations and vitamin D-deficiency association studies, the impact of vitamin D on other aspects of human health such as common

cancers, cardiovascular disease, type 2 diabetes obesity, autoimmune disorders, and infectious disease remains controversial (1–5). Randomized, controlled supplementation trials are required to better define the extra-skeletal roles of vitamin D. However, these trials are complicated by two unanswered questions: (1) what is the best marker of vitamin D status and (2) what constitutes a level that is sufficient to promote the health benefits of vitamin D?

ENDOCRINE VITAMIN D METABOLISM AND ACTION

The name "vitamin D" in this review refers to a collection of secosterol molecules detectable in the serum of vertebrates (left panel, **Figure 1**). Briefly, cholecalciferol or vitamin D3 (vitamin D) results from the ultraviolet B (UVB; 290–315 nm)-mediated photolytic-conversion of 7-dehydrocholesterol (DHC) in skin (6–8). Vitamin D can also be obtained from (i) food, principally from fortified dairy and juice products, (ii) consumption of fresh caught fish (e.g., salmon) (9), and (iii) oral vitamin D supplements. Vitamin D2 (ergocalciferol) is naturally found in fungi (e.g., mushrooms) and sometimes used in food fortification and supplementation regimes. Regardless vitamin D2 proceeds through the same modifications as described for vitamin D3 below.

Once in the general circulation vitamin D is bound to its serum carrier, vitamin D binding protein (DBP) and to a lesser extent albumin (10, 11), and is subject to a first hydroxylation step by vitamin D substrate-dependent 25-hydroxylase [CYP2R1 (12) and possibly a yet unidentified hydroxylase(s) (13)] in the liver resulting in 25-hydroxyvitamin D (25(OH)D). Very little 25(OH)D (∼5%) is secreted in to the bile (14). Rather, the bulk of 25(OH)D re-enters the circulation, once again bound to either DBP or albumin for endocrine transport to target tissues. DBP- and albumin-bound 25(OH)D in urine is reclaimed by tubular epithelial cells in the kidney (15). Here internalized 25(OH)D is freed from its carrier protein(s), becoming substrate for (i) the low capacity 25-hydroxyvitamin D-1-alpha-hydroxylase (CYP27B1) and production of the active, hormonal form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) or (ii) the high calpacity 25-dihydroxyvitamin D-24 hyxdoxylase (CYP24A1) to form the largely non-biologically active metabolites 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) and 1,24,25-trihydroxyvitamin D, respectively (16). The various hydoxylated forms gain access to the general circulation bound to DBP or albumin. Rheostatic endocrine control over the reciprocal production of 1- and 24-hydroxylated vitamin D metabolites is exerted by parathyroid hormone and FGF23. Parathyroid hormone increases activity of CYP27B1-hydroxylase (17, 18), decreases product output by CYP24A1 (19, 20); thus, increases the "activation" quotient of product $1,25(OH)_2D$:substrate 25(OH)D in the serum. On the other hand, FGF-23 blunts CYP27B1 activity (21) and promotes CYP24A1 activity; thus, decreases the $1,25(OH)_2D$:25(OH)D activation quotient and increases the $24,25(OH)_2D$:25(OH)D inactivation quotient (22, 23).

As noted above, $1,25(OH)_2D$ can be chaperoned in an endocrine mode to potential target tissues that employ serum bound, extracellular $1,25(OH)_2D$ as a specific ligand for transactivation of the vitamin D receptor (VDR) in the target cell driving $1,25(OH)_2D$-VDR directed differential gene expression (24). A major caveat in the concept of direct endocrine action of $1,25(OH)_2D$ is the fact all adverse physiological consequences of vitamin D deficiency in humans are associated with a low serum 25(OH)D, not a low $1,25(OH)_2D$ level [**Table 1**; (25)]. In fact, in the basal state, before treatment to raise 25D levels, subjects with low serum 25D often have serum 1,25D levels that are relatively elevated as a consequence of compensatory secondary hyperparathyroidism. In this instance the increase in the host's circulating concentration of PTH drives an increase in renal 1,25D production. This suggests that 25D deficiency in the serum is a cause for "endocrine resistance" to circulating levels of the 1,25D hormone at the level of the gut. After vitamin D restoration treatment with return of 25D balance to normal and resolution of secondary hyperparathyroidism, there is an increase in intestinal calcium absorption even though there is a relative decrease, or no change in the circulating serum 1,25D level. This suggests that there may be local conversion of 25D to 1,25D in the gut outside of the serum compartment that is driving intestinal calcium absorption and/or that only measuring the total amount of vitamin D metabolite(s) in the serum, may be an inadequate biomarker of response to restoration of 25D levels in the blood to normal.

LOCAL VITAMIN D METABOLISM AND ACTION

In non-renal tissues, the CYP27B1 converts 25(OH)D to $1,25(OH)_2D$ for local usage in paracrine, autocrine, and intracrine regulated activities (26). Perhaps the most relevant physiological/pathophysiological example of these events (e.g., those confined to the local tissue microenvironment outside of circulating serum compartment) are the human granuloma forming diseases like sarcoidosis and tuberculosis (27). In these disease states, cells of the innate immune response, principally macrophages, express the same metabolic machinery to synthesize $1,25(OH)_2D$ intracellularly when presented with a CYP27B1 activating signal and with sufficient 25(OH)D in the extracellular space to serve as substrate for the CPY27B1. When the extracellular concentration of 25(OH)D falls below the equivalent of ∼20 $ng{\cdot}mL^{-1}$ or 50 nM, the *intracrine* production of $1,25(OH)_2D$ via the CYP27B1-hydroxylase becomes limiting; unlike the renal CYP27B1, the enzyme in the macrophage is highly substrate-drive (28). Taking the human granuloma-forming, macrophage dominant infectious disease tuberculosis (TB) as an example, in the face of deficient extracellular substrate 25(OH)D the macrophage CYP27B1 is unable to generate enough active $1,25(OH)_2D$ metabolite to effectively ligand sufficient VDR in that cell to promote expression of vitamin D-dependent antimicrobial genes (29, 30). The end result is failure of the macrophage to mount an effective autophagy-related, vesicular killing response to ingested *Mycobacterium*

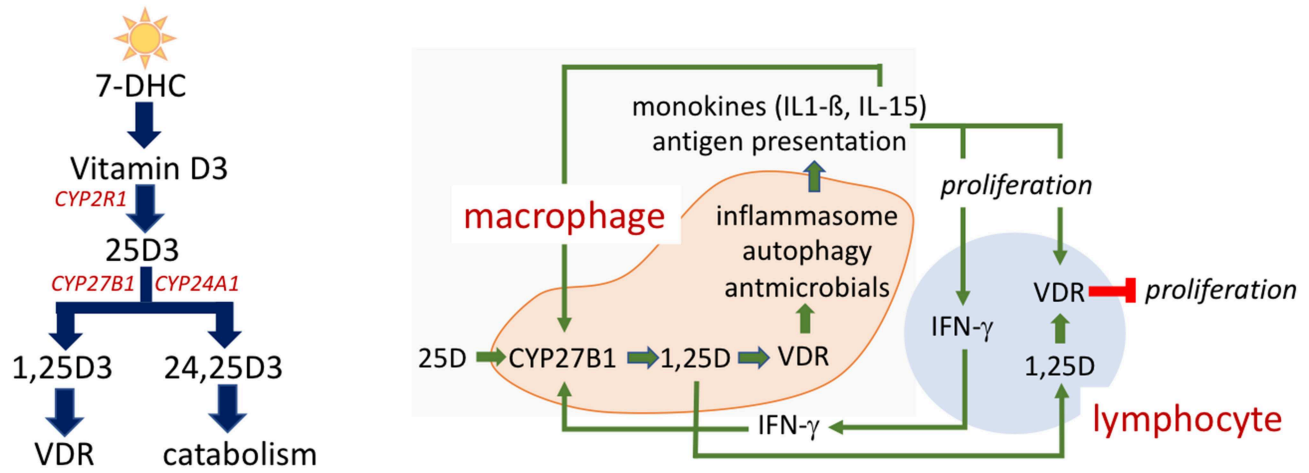

FIGURE 1 | Vitamin D metabolism and action. Vitamin D metabolites **(Left)**. 7-dehydrocholesterol (7-DHC) is photoconverted to vitamin D_3 by UV exposure of skin. CYP2R1 in the liver hydroxylates vitamin D_3 to 25-hydroxyvitamin D_3 (25(OH)D). Another hydroxylation to 25(OH)D_3 by CYP27B1 occurs in the kidney but also in a number of extra-renal tissues produces the active 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). 1,25$(OH)_2D_3$ is the cognate ligand for the vitamin D receptor (VDR), a nuclear transcription factor, that directs 1,25$(OH)_2D_3$ regulated gene transcription. CYP24A1 is responsible for the hydroxylation that yields 24,25-dihydroxyvitamin D (24,25$(OH)_2D_3$) and subsequent catabolism to non-biologically active metabolites. Intracrine mechanism in immune cells **(Right)**. Vitamin D action in immune cells is reliant upon the intracrine (local) production of active 1,25(OH)2D within the macrophage. Vitamin D status of the host as defined by serum, extracellular 25(OH)D levels impact immune response, as 25(OH)D is the substrate for CYP27B1. A complex interplay between monokine signaling, that can both be responsive to and stimulatory of 1,25$(OH)_2D$ synthesis, and regulatory signaling among innate and adaptive immune cells is shown.

TABLE 1 | Vitamin D and human health.

Adverse health outcomes significantly associated with low serum 25(OH)D level	
Low bone density	Obesity
Hip fractures	Insulin resistance
Non-vertebral fractures	Type 1 diabetes
Heart attack	Type 2 diabetes
Hypertension	Cancer
Stroke	Preterm delivery
Neurocognitive dysfunction	Pre-eclampsia
Proximal muscle weakness	Inflammation/infection
Autoimmune diseases	Multiple sclerosis

Numerous health conditions have been associated with low total serum levels of 25(OH)D and are listed in the table. Conditions pertaining to bone health are indicated in blue. Table is based on reviews by Rosen et al. (2) and Rosen and Taylor (4).

tuberculosis (M. tb) (31, 32). *In vitro* this failure can be rescued in a 25(OH)D concentration-dependent fashion by exchanging vitamin D deficient human serum with vitamin D sufficient serum (>30 ng·mL-1 or 75 nM); in other words, rescue of the macrophage innate immune is achieved by conditioning activated macrophages *ex vivo* in serum from the same host after treatment of the host with vitamin D *in vivo* (30). Demonstrating successful rescue from 25(OH)D deficiency *in vivo* in humans exposed to or in the very early phases of infection with *M. tb.* would support the value of 25(OH)D-driven innate immune competence in prevention and use as adjunctive therapy early in the course of this disease (33).

The local antimicrobial capacity of the macrophage is subject to intracrine, autocrine, and paracrine feedforward and feedback immune regulatory circuits. This regulatory network is depicted schematically in the right panel of **Figure 1**. Stimulation of the Toll-like receptor signaling pathway by pathogen-associated molecular pattern (PAMP) molecules induces expression of CYP27B1 and VDR as well as of monokines (e.g., IL-15 and IL-1β). In an *autocrine* mode, these two monokines act to amplify expression of the CYP27B1 and 1,25$(OH)_2D$-VDR-directed generation of antimicrobial peptides (34). In a *paracrine* fashion IL-1ß mobilizes and activates cells of the adaptive immune response (35–37). Activation of the Th1 subset of "helper" lymphocytes promotes: (1) production of IFN-γ, the most potent known stimulator of the macrophage CYP27B1-hydroxylase (38); and (2) induction of expression of the VDR in adaptive immune response cells (39, 40). When IFN-γ-driven production of 1,25$(OH)_2D$ in the macrophage is robust enough to allow escape of the active vitamin D metabolite into the local, pericellular inflammatory microenvironment, this 1,25$(OH)_2D$ is sufficient to drive VDR-dependent gene expression in activated lymphocytes such as inhibiting proliferation of those lymphocytes. As such, the predominant *paracrine* action of 1,25$(OH)_2D$ in this setting is to modify the adaptive immune response (41) and turn down IFN-γ and macrophage CYP27B1 gene expression, preventing a potential overzealous adaptive (auto)immune response harmful to the host. Therefore, 25(OH)D "sufficiency" in the serum of the host appears to be paramount in providing the optimal IFN-γ-mediated feedback control on 1,25$(OH)_2D$ synthesis by the macrophage and appropriate antimicrobial response to ingested microbes. For example, failure of this normal feedback control in disseminated infection with *M. tb.* may result in escape of 1,25$(OH)_2D$ from the local immune microenvironment into the serum tuberculosis, resulting in a form of endocrine-acting

"1,25$(OH)_2$D intoxication" and life-threatening hypercalcemia. This form of extra-renal 1,25$(OH)_2$D intoxication can occur in certain granuloma-forming diseases such as tuberculosis (where pathogen is known) and sarcoidosis (where pathogen is unknown).

These scenarios indicate that the serum level of bioavailable 25(OH)D to macrophages is a key determinant of normal/abnormal physiological control of innate and adaptive immunity in the host with low serum 25(OH)D levels. Cell and molecular biology experiments have established co-existent expression of the CYP27B1 and VDR in the same cell (**Table 2**). This observation makes all of these cell-types potential candidates for intracrine metabolism and action of 1,25$(OH)_2$D generated from 25(OH)D available to that cell from its extracellular microenvironment *in vivo*. Beyond tuberculosis just discussed, many human diseases are associated with "low" serum 25(OH)D levels compared to matched controls without the disease (**Table 1**). In cardiovascular disease, the leading cause of mortality in the US, mortality is inversely related to serum 25(OH)D <20 ng/ml (42, 43), though this is disputed (44). All of these disease states are associated with altered host immunity. Perhaps this is the common link to adverse outcomes in this otherwise diverse set of disorders.

DEFINING VITAMIN D STATUS

The total serum 25(OH)D level (the sum of 25(OH)D that is bound to carrier proteins and is free in the circulation) is the currently accepted marker of choice for defining vitamin D status in any given individual. Its preferential use is based on (i) analysis of the existing body of clinical research, (ii) assay advances that confer easy and accurate measurements and (iii) the fact that it is the most abundant and stable of the various vitamin D metabolites in serum; 25(OH)D has a relatively long half-life (15 days) compared to 1,25$(OH)_2$D (15 h). However, health organizations across the globe differ significantly in their definition of what level of total circulating 25(OH)D constitutes sufficiency and deficiency for a "normal" population without evidence of active bone disease (e.g., osteoporosis, hyperparathyroidism, etc.). In North America, the Institute of Medicine has recommended the value of ≥20 ng/ml 25(OH)D (50 nmol/L) for sufficiency (45), whereas the Endocrine Society recommended for ≥30 ng/ml (75 nmol/L) (46). The Vitamin D Council states that individuals should strive for levels above 40 ng/ml (100 nmol/L) (47). In contrast to this, the UK Science Advisory Council on Nutrition defined vitamin D deficiency as serum levels of 25(OH)D <10 ng/ml (25 nmol/L), but did not recommend an optimal level for human health (48). Although the 10 ng/ml level is held by some other European nations, some have recommended higher levels (49). Investigators in the vitamin D field have highlighted the limitations of the total 25(OH)D as the routine biomarker for vitamin D status in various commentary and review articles (50, 51) and discussed other potential markers and the possible need of a "vitamin D panel" (52, 53).

Human data indicates that the threshold for detection of a relative increase in the serum iPTH at the individual and population level occurs when the total serum 25(OH)D level falls below ∼30 ng/ml (54, 55). Thus, PTH offers a measurable biological consequence of "low" 25(OH)D. However, PTH levels are not exclusively controled by 25(OH)D as serum concentration of ionized calcium is sensed at the parathyroid gland by the calcium-sensing receptor (CaSR). When serum calcium levels drop, CaSR signal transduction in the parathyroid yields an increase in PTH production that then enters the general circulation resulting in PTH's endocrine effects.

Another reason for the uncertainty concerning the validity of total 25(OH)D as an exclusive serum marker for vitamin D health is due to the complex molecular biology and biochemistry of 25(OH)D-associated bioactivities. This is particularly relevant to the intracrine, paracrine or endocrine conversion of 25(OH)D to active 1,25$(OH)_2$D, because (i) the local concentrations vitamin D metabolites outside of the serum compartment cannot be easily measured *in vivo* and (ii) the subsequent molecular actions of 1,25$(OH)_2$D in conjunction with its binding by the VDR is dependent on diverse mechanisms beyond simple variations in circulating 25(OH)D. These include: (1) the transport and target tissue uptake of 25(OH)D; (2) the directed intracellular transport of 25(OH)D to the inner mitochondrial to CYP27B1 for enzymatic conversion of 25(OH)D to 1,25$(OH)_2$D; (3) export if 1,25$(OH)_2$D from the mitochondia and binding of 1,25$(OH)_2$D to VDR; and (4) competing catabolism of 1,25$(OH)_2$D by the enzyme CYP24A1 also located on the inner mitochondrail membrane. In this review, we will briefly discuss the molecular biology and biochemistry behind these processes, with particular emphasis on the role of free 25(OH)D as a key determinant of the downstream actions of 1,25$(OH)_2$D, specifically in bone and mineral health.

TABLE 2 | Cell types that express both CYP27B1 and VDR.

Cells co-expressing a functional CYP27B1 and VDR	
➡Macrophage	Enterocyte
Dendritic cell	Decidual stromal cell
Parathyroid cell	Fetal trophoblast
Osteoblast	Prostate epithelial cell
Osteoclast	Vascular endothelial cell
Keratinocyte	Pancreatic β cell
Mammary epithelial cell	Renal tubular cell

In contrast to the endocrine action based on kidney production of 1,25$(OH)_2$D that travels through the general circulation to VDR-possessing target cells, a number of cell types harbor both CYP27B1 and VDR making intracrine metabolism and action possible. The red arrow indicates the macrophage, the cell type in which the intracrine metabolism of 25(OH)D to 1,25$(OH)_2$D and 1,25$(OH)_2$D-directed gene expression has been most clearly demonstrated ex vivo.

MOLECULAR BIOLOGY AND BIOCHEMISTRY OF VITAMIN D ACTION

1,25$(OH)_2$D is the active vitamin D molecule with 25(OH)D being its immediate precursor (panel A, **Figure 1**). It is 1,25$(OH)_2$D that drives vitamin D-regulated gene expression in target cells. Under normal conditions, the level of serum

$1,25(OH)_2D$ is tightly regulated within a narrow range (30–60 pg/ml) at a level that is 1,000X less plentiful than its 25(OH)D precursor. In non-pregnant humans, $1,25(OH)_2D$ in the serum comes almost exclusively from expression of CYP27B1 in the kidney. Circulating $1,25(OH)_2D$'s endocrine actions are to regulate the serum level of calcium by optimizing intestinal calcium absorption and/or calcium resorption from the skeleton (56). Not surprisingly then, when kidney failure occurs, there is (i) an accompanying decrease in renal CYP27B1 capacity, (ii) a fall in $1,25(OH)_2D$ production and (iii) a reduction in the serum calcium level. In the opposite case, when a pathophysiological extra-renal source of CYP27B1 (e.g., the macrophage in granuloma-forming diseases; see right panel, **Figure 1** and **Table 1**) becomes dysregulated and dominant, serum $1,25(OH)_2D$-driven hypercalcemia and/or hypercalciuria [$1,25(OH)_2D$ intoxication] occurs overriding the calcium-lowering actions of the CaSR in the parathyroid gland and in the kidney (57). Only in these two abnormal calcemic states is the serum level of $1,25(OH)_2D$ of the host informative to the clinician in evaluating a patient.

At the molecular level, $1,25(OH)_2D$ binds VDR with the highest affinity among all vitamin D metabolites regardless of whether $1,25(OH)_2D$ is coming from the serum outside the target cell (endocrine mode), from the local microenvironment outside of the serum compartment (paracrine mode), or from the inside of the cell (intracrine mode) (58). In the $1,25(OH)_2D$-liganded state the VDR preferentially forms heterodimers with retinoic acid X receptor (RXR). In the $1,25(OH)_2D$-occupied state the VDR and its unliganded RXR partner heterodimer become transacting complexes binding to specific cis-acting vitamin D response elements (VDREs) in the genome. The heterodimer interacts with the transcriptional machinery resulting in $1,25(OH)_2D$-regulated (positive or negative) gene expression and corresponding bioactivities. Due to the three-dimensional "looping" nature of DNA-protein interactions, VDRE-like enhancer and repressor motifs can found at considerable distance from the transcriptional start site of a vitamin D regulated gene (59, 60).

Owing to a rapidly growing skeleton with a high demand for calcium and phosphate for skeletal mineralization and in the face of normal VDR and CYP27B1 activity, children suffering from low 25(OH)D levels and secondary decreases in optimal intestinal absorption in dietary calcium and phosphate can develop vitamin D deficient rickets (61). This is commonly observed in subpopulations of impoverished, dark-skinned children (i) who require up to 10 times more cutaneous sunlight exposure that lightly pigmented children to make the same amount of vitamin D in their skin and (ii) in whom consumption of natural vitamin D-rich foods (e.g., fish) and vitamin D supplemented foods is compromised. In nations of lower income, children usually consume a diet rich in grains; grains are a rich source of phytates known to chelate ingested calcium further decreasing intestinal calcium absorption. Vitamin D deficiency rickets can also be hastened in children subjected to religious/cultural practices that (e.g., occlusive garb) that effectively eliminate skin exposure to sunlight. In vitamin D deficient rickets the serum calcium and phosphate is low and PTH and $1,25(OH)_2D$ usually elevated for the subject's age (62). A low 25(OH)D level (usually <10 ng/ml) is the distinguishing marker for vitamin D deficient rickets, distinguishing it from Human Vitamin D Resistant Rickets (HVDRR; high $1,25(OH)_2D$) and Pseudo Vitamin D Deficient Rickets (PDDR; low $1,25(OH)_2D$) (63). Appropriate dietary supplementation with calcium and vitamin D to normalize the serum 25(OH)D level (e.g., >30 ng/ml) alleviates nutritional rickets (61, 64).

Prevention of nutritional rickets in children and osteomalacia in adults is the primary concern behind the recommendations on vitamin D intake and serum 25(OH)D level attainment. Various medical organizations have evaluated the existing body of research data to support their positions and are exhaustively reported elsewhere (45, 48). However, two key studies are emblematic of the basis for the determinations. In one line of evidence, PTH is used as a biomarker of bone health. In some studies, an inverse association between PTH (declining) and 25(OH)D (increasing) has been observed (55, 65, 66). In one study involving 1,536 post-menopausal women (55) an inflection point where the decline in serum PTH levels off was identified at a 25(OH)D level of 30 ng/ml, leading some authorities in the vitamin D field to call for this to demarcate the cutoff for vitamin D sufficiency (46). Another often quoted study involved post-mortem ($N = 675$) determination of 25(OH)D levels and comparisons to histomorphometric analysis of transiliac crest biopsies (67). From this dataset, the IOM (45) concluded that at a serum 25(OH)D level of 20 ng/ml and above, 99% of the normal population (e.g., without a known bone disease) should have no pathological accumulation of osteoid (unmineralized portion of bone indicative of rickets in children and osteomalacia in adults). Interestingly, the original authors (67) using different criteria to analyze the same data concluded that 30 ng/ml 25(OH)D as the recommended level for optimal skeletal health.

TOTAL 25(OH)D OR FREE 25(OH)D?

The measured total 25(OH)D concentration in serum is present at nearly 1,000-fold higher levels compared to $1,25(OH)_2D$ in serum and easily and accurately measured from a small amount of sample (25–50 μl) (68). As such, total 25(OH)D has become the de facto biomarker of the state of vitamin D deficiency or sufficiency of the host. However, the majority (>99%) of serum 25(OH)D is bound (**Figure 1**, left box) to carrier proteins (∼85% to vitamin D binding protein [DBP]; ∼15% to albumin). The affinity of DBP for 25(OH)D is ∼1,000-fold greater than that of albumin for 25(OH)D (11). Cells that express the cell surface receptor proteins megalin and cubulin can internalize the DBP-bound-25(OH)D complex into an endolysosome with ultimate release of 25(OH)D from DBP into the cell interior for further metabolism and/or catabolism of 25(OH)D. This mechanism of endocytosis, intracellular release of 25(OH)D from acid-hydrolyzed DBP has been most clearly demonstrated at the luminal membrane of the tubular epithelial cells in the kidney

(15). Here internalized 25(OH)D is the substrate used by the CYP27B1 to form 1,25(OH)$_2$D for endocrine distribution. In cells not expressing megalin, 25(OH)D entry into its target cell is proposed to be accomplished by diffusion of the unbound, free 25(OH)D across the lipid bilayer of the plasma membrane to the cell interior (15). Thus, the biologically relevant substrate for some cells is DBP-bound 25(OH)D (essentially equal to total 25(OH)D) and for others it is the free 25(OH)D or a related metric of bioavailable 25(OH)D (sum of free 25(OH)D and albumin-bound-25(OH)D).

Though the serum protein carriers of vitamin D metabolites are well-characterized, it is now clear that vitamin D metabolites also reside in non-serum locales such as body fat and inside cells, suggesting that specific vitamin D binding proteins other than DBP and albumin may also play a role in vitamin D biology. Notably intracellular vitamin D binding proteins (IDBPs) were identified as a result of investigations into apparent vitamin D resistance in New World Primates [NWP; reviewed in Adams et al. (69, 70)]. The high serum levels of steroid hormones in general and vitamin D in particular, relative to Old World Primates (OWP), was shown to be associated with a form of target tissue insensitivity to 1,25(OH)$_2$D (71). The first indications of a functional role for IDBPs was the observed diminished ability of NWP cells to effectively upregulate VDR-target genes, like the *CYP24A1*, despite having comparable amounts of VDR (72, 73) and VDR functionality (74). Using cell extracts from NWP and OWP in VDR-1,25(OH)$_2$D radiolabel binding assays, a protein in NWP cell extracts was observed to prevent VDR-1,25(OH)$_2$D binding when mixed with OWP extracts. This inhibition of binding was abolished after trypsin digestion or heat denaturation (75). Upon further characterization, IDBP was found to (i) bind 25(OH)D as well as other steroid hormones (76) and (ii) be part of the heat shock protein 70 family (77, 78). With cDNA constructs for IDBP obtained, transient and stable overexpression *in vitro* in tissue culture studies revealed that IDBP could increase 1,25(OH)$_2$D synthesis, possibly by chaperoned delivery of 25(OH)D to the CYP27B1 (79). The ATPase domain of IDBP was essential to this function (80, 81), with the BCL2-associated athanogene 1 (BAG1) serving as an IDBP co-chaperone (82).

Measurement of the free, unbound form of 25(OH)D in serum is challenging, because its levels are low (4–8 pg/ml range) and has historically required radioactive tracers of 25(OH)D with equilibrium or centrifugal dialysis methods that are cumbersome and impractical for use in clinical laboratory testing services (10, 11). Free and bioavailable 25(OH)D levels in the serum can be mathematically calculated using equations that incorporate the binding affinity of DBP for 25(OH)D and the concentration of DBP in the serum (11, 83). This approach of calculating the free, biologically active fraction of 25(OH)D has also been taken with testosterone (84) and thyroid hormone (85). Unfortunately, for free 25(OH)D, it was discovered that one commonly used DBP ELISA kit used to calculate for free 25(OH)D had differential sensitivities to the common phenotypic variants of DBP (discussed later in this review) that resulted in inaccurate measurement of DBP concentrations in some samples leading to inaccurate calculated free 25(OH)D levels in those serum samples.

TABLE 3 | Hormone binding proteins found in human serum.

Binding protein	Metabolites		Percent free
DBP 4.5–5.5 μM[a]	Total 25(OH)D 25–75 nM[a]	Free 25(OH)D 5–20 pM[a]	0.02
	Total 1,25(OH)$_2$D 50–198 pM[b]	Free 1,25(OH)$_2$D 325–525 fM[c]	0.5
TBG 241–722 nM[b]	Total T4 58–154 nM[b]	Free T4 11–23 pM[b]	0.02
	Total T3 1.1–2.8 nM[b]	Free T3 3.0–6.8 pM[b]	0.3
SHBG 16.5–55.9 nM[b] (male)	Male total T 9.2–31.8 nM[b]	Male free T 30–87 pM[b]	0.3
	Male total E2 28–156 pM[b]	Male free E2 <1.7 pM[d]	1
SHBG 24.6–122.0 nM[b] (female)	Female total T 0.3–1.7 nM[b]	Female free T <15 pM[b]	0.9
	Female total E2 46–609 pM[b]	Female free E2 1.6–18.5 pM[d]	3

[a] *Nielson et al. (88).*
[b] *https://www.labcorp.com/test-menu/search.*
[c] *Bikle et al. (89).*
[d] *https://www.questdiagnostics.com/testcenter/TestCenterHome.action.*
Vitamin D binding protein (DBP), thyroxine binding globulin (TBG), and sex hormone binding globulin (SHBG) are tabulated with their serum concentration levels. The respective metabolite concentrations total, free and percent free are also presented. Albumin can bind all the hormones listed and transthyretin can bind T4 albeit at lower affinity compared to their primary carrier proteins. The bioavailable concept has been applied to these hormones and comprise the sum of albumin-bound-hormone and free hormone.

Direct measurement of the various free hormones of clinical importance have been developed (86, 87). However, this aspect of clinical chemistry remains challenging due to (i) the low concentration of these metabolites in the serum (**Table 3**) and (ii) variability of serum composition (e.g., lipids and other potentially interfering molecules) between patients that can introduce error into the measured value. Recently, an ELISA based method for detection of free 25(OH)D has been developed (90) and has been utilized in a number of clinical studies that we will summarize in the clinical studies portion of this review. Most recently, a high-throughput method to measure bioavailable 25(OH)D has been developed (91). However, procedures to ensure accuracy and precision across a wide range of sample conditions both for both of these assays have not been yet developed; as a consequence, these assays are deemed for research use only.

MOLECULAR BIOLOGY AND BIOCHEMISTRY OF DBP

The vitamin D binding protein (DBP) is a multi-functional protein also known as Group Specific Component (GC) [reviewed in Chun (92) and Delanghe et al. (93)]. DBP is a member of the albumin superfamily of proteins. DBP is a moderately abundant protein (∼5 μM in humans) in serum of

TABLE 4 | Molecular biology of most common DBP polymorphisms.

SNP name	GC name	Codon variant	Amino acid variant
rs4588	GC1	ACG	Thr-436
	GC2	AAG	Lys-436
rs7041	GC1F	GAT	Asp-432
	GC1S	GAG	Glu-432

Two single nucleotide polymorphisms (SNP) account for three of the major forms of DBP (originally known as GC-globulin). Table also includes the specific codon and amino acid variation that define these variants.

vertebrates. In humans the *GC/DBP* 13 exon *gene* is located at 4q13.3. Its gene product, DBP, is highly expressed in the liver and exported into the circulatory system. DBP contains 474 amino acids of which the 16 N-terminal amino acids function as a signal peptide. DBP can be glycosylated (94) to varying degrees depending on genotype. The glycosylation pattern has been suggested to be structural basis for the macrophage activating factor activity of DBP (95, 96).

DBP's most well-characterized role is that of a carrier protein for vitamin D metabolites. Its rank order avidity for the vitamin D and it metabolites are (10, 11, 89, 97) as follows: 24,25$(OH)_2$D>25(OH)D>1,25$(OH)_2$D>vitamin D. DBP binds vitamin D3 and its metabolites with greater affinity than vitamin D2 and it metabolites (97). DBP can also bind actin (98). This biological action is suggested to be that of scavenging of exposed actin, preventing overzealous extracellular polymerization after tissue injury (99). DBP can also bind circulating fatty acids (100) and C5a des Arg, the latter of which enhances complement activation (101).

DBP migrates at ∼52–59 kDa in electrophoretic gels (102). It was variations in DBP mobility in isoelectric focusing (IEF) gels that garnered initial research attention prior to its function being determined. One banding pattern was termed GC-1F (faster), another GC-1S (slower), and still another GC2 (103). GC2 migrated less rapidly toward the anode compared to either GC1 forms. The different forms, due to single amino acid differences (**Table 4**), were (i) used to determine allelic frequency for samples worldwide (104) and (ii) found to associate with the racial background of the source human serum. In the Kamboh study, black subjects were more likely to have the GC1F forms (67–79% of alleles in USA blacks) while white subjects more frequently yielded the GC1S pattern (49–57% of alleles in USA whites). The GC2 allele was observed were more frequently seen in white subject samples (21–31% in USA whites) compared to blacks (8–13% in USA blacks). These three classic forms (GC1F, GC1S, and GC2) account for the vast majority of the variation DBP across human populations. Since these early studies, many other SNPs (105) have been found in GC of which a small percentage encodes a missense mutation that changes the amino acid code.

Affinity Differences and DBP Genotype

The biological significance of DBP's different allelic forms is unclear. Conceptually, if the different genotypic forms of DBP had different affinities for vitamin D metabolites, then the levels of those metabolites could be influenced by the genotype. However, in terms of experimentally measured differences in affinity for vitamin D metabolites, one study reported large differences (106) but three others did not (107–109). Media supplemented with serum that contained different alleles of DBP had differing impacts on assessed immunological readouts consistent with affinity differences in three *in vitro* studies (110–112). The mechanism for the observed differences were not investigated in those studies; thus, non-affinity dependent mechanisms cannot be ruled out. Using an ELISA based assay to detect free 25(OH)D, some small differences in percent free were detected between the genotypes (113). However, these differences were smaller in magnitude than the differences anticipated if the report of large affinity difference among genotype was accurate.

DBP Serum Concentration and DBP Genotype

The other mechanism by which DBP genotype could impact free 25(OH)D levels is through differentials in the genotype-dependent DBP concentration. It is on this basis that Powe et al. (114) proposed how black Americans, with lower total serum 25(OH)D levels and substantially lower DBP levels compared to white Americans, could ultimately have similar free 25(OH)D levels. However, it was eventually determined that the monoclonal antibody-based ELISA used in their study was less sensitive to the GC1F DBP, the form most frequently found in blacks (88, 115). This resulted in an underestimation of serum DBP levels in black subjects and a consequent overestimation of the calculated free and bioavailable serum 25(OH)D levels in their serum. Nonetheless, there is evidence of some genotypic effect on DBP concentrations. One early study (116) of Danish women found DBP concentration ranked according to the presence of GC1 alleles (GC1-GC1 > GC1-GC2 > GC2-GC2). Consistent with those findings was a study in a population male subjects with modest racial diversity where GC2/GC2 subjects exhibited the lowest serum DBP concentration (88).

In another recent study utilizing serum samples from both women and men with modest racial diversity, the presence of the GC2 allele in one of three allelic combinations resulted in lower DBP levels compared to the three allelic combinations without GC2 (113). Currently, it is thought that DBP's role in determining free 25(OH)D levels is largely through its concentration [i.e., for a fixed total 25(OH)D, more DBP yields less free 25(OH)D] and perhaps to a small degree by genotype. As such, in most cases total 25(OH)D and free 25(OH)D are highly correlated. However, there are some conditions where DBP levels diverge from typical levels. For instance, patients with liver disease have lower levels of DBP while pregnant women have higher DBP levels (89, 117) resulting in higher free 25(OH)D levels in those with lower DBP concentrations. In the more extreme case of patients before [lower DBP, lower 25(OH)D] and after [higher DBP, higher 25(OH)D] liver transplants, the percentage of free 25(OH)D relative to total 25(OH)D is higher before transplantation. Recently, a case report described a subject with

a homozygous deletion of DBP (118). Compared to unaffected and heterozygous siblings, the affected patient had no DBP and very low serum levels of 25(OH)D and $1,25(OH)_2D$. Despite the extremely low serum levels of vitamin D metabolites, the subject had normal calcium balance with only relatively small alterations in bone health and mineral metabolism. This case report is the strongest evidence to date that the total serum 25(OH)D and $1,25(OH)_2D$ level can be disconnected from "normal" vitamin D status. One explanation is that albumin, or some other chaperone in the serum, assumes the role of delivering 25(OH)D and maintaining viable levels of free 25(OH)D and $1,25(OH)_2D$, as was measured in this subject, to target tissues for further metabolism and action.

ANIMAL STUDIES OF FREE 25(OH)D AND BONE

Dbp heterozygous and homozygous knockouts in mice do not have any obvious bone phenotype relative to wild type (119). However, with the loss of a single copy of *Dbp* the levels of circulating total serum 25(OH)D and $1,25(OH)_2D$ are diminished and when both copies were absent, the levels of serum 25(OH)D and $1,25(OH)_2D$ were extremely low, phenocopying the $DBP^{-/-}$ subject just described above. Interestingly, neither $DBP^{+/-}$ or $DBP^{-/-}$ mice exhibited any skeletal abnormalities or problems with calcium and phosphate balance (119). These findings support the hypothesis that free hormone levels alone are adequate for sustaining skeletal health. Consistent with this was another study with *Dbp* homozygous knockouts where the levels of $1,25(OH)_2D$ was measured in the intestinal tissues (120). Even though the double knockout animals had very low total serum $1,25(OH)_2D$ levels compared to wild type, the levels of $1,25(OH)_2D$ measured in the intestinal tissues were very similar. These findings suggest that trace amounts of free metabolites and/or enhanced local conversion of 25(OH)D to $1,25(OH)_2D$ are sufficient as long as sufficient amounts substrate 25(OH)D are available to the host. The fact that mice with no DBP are still viable suggests that albumin, though having a much lower affinity for vitamin D metabolites, could serve as the carrier protein in place of DBP. When *Dbp* null mice were raised on vitamin D3-free diets, they developed secondary hyperparathyroidism and bone mineralization defects much more rapidly than paired wild-type mice (119). This result indicated that DBP's function is to maintain a stable reservoir of circulating extracellular vitamin D metabolites. Owing to its higher affinity for vitamin D metabolites, DBP is more effective in this role than albumin, despite both DBP and albumin being filtered into the urine and reclaimed by megalin.

Another test of the biological impact of free 25(OH)D in mice (121) was based on the difference in affinity of DBP for D2 or ergocalciferol (vitamin D found in fungi) vs. D3 or cholecalciferol (vitamin D found in animals) forms of vitamin D metabolites. Mice raised on diets containing exclusively D2 or D3 would result in the mice having only 25(OH)D2 and 25(OH)D3 circulating in their serum. Since DBP affinity for D2 forms is lower relative to D3 forms, the free 25(OH)D2 levels were expected to be higher in animals raised on D2 diets. In this study, mice were placed on D2 or D3 diets (1,000 IU/kg) beginning at week 3 and tested at week 8 and week 16 for 25(OH)D3 and 25(OH)D2 serum levels and bone phenotype by histomorphometry. These mice had similar total 25(OH)D levels at week 8 (26.6 ± 1.9 ng/ml 25(OH)D2 vs. 28.3 ± 2.0 ng/ml 25(OH)D3) and at week 16 (33.3 ± 4.4 vs. 31.7 ± 2.1 ng/ml). However, as anticipated, they differed in their free 25(OH)D levels with free 25(OH)D2 greater than free 25(OH)D3 at week 8 (16.8 ± 0.65 vs. 8.4 ± 0.63 pg/ml, $P < 0.001$) and at week 16 (17.4 ± 0.43 vs. 8.4 ± 0.44, $P < 0.001$). Histomorphometric analysis of their bones detected that at week 8, the D2 fed mice had significantly higher osteoclast surface/bone surface, eroded surface/bone surface, and mineral apposition rate (high bone turnover) compared with mice raised on the D3 diets. Additionally, osteoblast surface/bone surface, an index of bone formation, was higher in week 8 D2 in females only. The reason underpinning this sexual dimorphism in bone formation rates remains unknown. The bone phenotype at week 16 revealed significantly higher bone volume/total volume and trabecular number in the D2 mice relative to the D3 mice. Taken together, despite similar total serum 25(OH)D levels, bone phenotype differences were observed in association with different free 25(OH)D levels (higher in D2 mice) suggesting the relevance of free 25(OH)D to bone health.

To the best of our knowledge, there have been no studies on the effects of free 25(OH)D and the bone health of animals besides the studies in mice summarized above. However, the existence of nocturnal bats that roost in dark locations (122) could be informative. These bats have very low total serum 25(OH)D (<5 ng/ml) as their normal state suggesting that "deficient" (by human standards) total 25(OH)D levels presumably still yields adequate free 25(OH)D levels to sustain normal bone and mineral homeostasis for this species. Interestingly, these bats are fully capable of attaining higher 25(OH)D levels when housed in conditions that expose them to sunlight. Perhaps these animals have lower DBP concentrations or metabolite affinities for DBP to compensate for their naturally low total serum 25(OH)D levels. A test of these animal's serum with the free 25(OH)D assay could be informative. Other possible adaptations that permit compensation to the very low circulating levels of 25(OH)D include: (1) a highly efficient CYP27B1; (2) hyper-sensitive VDR to ligand; (3) heightened transactivation potential of VDR-interacting co-activators; and/or (4) diminished functional activity of the CYP24A1 catabolic machinery. There is one study comparing DBP affinity for 25-hydroxyvitamin D among several animals; these investigators found the DBP from rat and cattle exhibited higher affinity to 25(OH)D compared to horse and rhesus monkey with humans having the lowest affinity of species tested (123). What the levels of free 25(OH)D are and its importance to these species have not yet been examined.

HUMAN STUDIES OF FREE 25(OH)D

Revitalized interest on the impact of vitamin D on human health beyond bone was spurred by studies that investigated

vitamin D's regulatory role in the adaptive and innate immune system (26, 29, 124). Three *in vitro* studies have demonstrated that decreasing free 25(OH)D by increasing DBP in the culture media diminished immune functions of adherent monocytes (110), dendritic cells (111), and T-lymphocytes (112). Because of these findings *in vitro*, the parameter of free 25(OH)D began to receive greater interest in clinical research. In non-bone health association studies, the results have been mixed. In some studies free serum 25(OH)D levels were inversely associated with coronary artery disease (125), pediatric inflammatory bowel disease (126) and ulcerative colitis (127), insulin sensitivity (128), reduction in lipid markers in statin patients (129), and acromegaly (130). However, in some other studies free 25(OH)D was inferior in asthma (131) and no better than total 25(OH)D for colorectal cancer in African-Americans (132).

Concerning bone and mineral health, a 2011 report (133) was the first to utilize free 25(OH)D and the related concept bioavailable 25(OH)D (sum of free 25(OH)D and albumin-bound 25(OH)D) for analytical purposes. They found that these two metrics of serum vitamin D status were more closely and directly associated with BMD in the individual than total serum 25(OH)D. This group then followed up with another report (134) that showed that compared to measures of total 25(OH)D bioavailable 25(OH)D had a significant direct association with serum calcium (corrected for the serum albumin level) and a significant inverse association with PTH. These investigators suggested that this could address the long-standing paradox of how black Americans with lower total serum 25(OH)D levels have higher BMD and similar PTH levels compared to white Americans. In additional work, they measured DBP serum concentrations in black Americans and found them to be lower than those in white Americans. They concluded that the resultant higher bioavailable 25(OH)D may account for the nominal differences in BMD and serum PTH (114) despite what appeared to be sub-optimal total serum 25(OH)D levels; unfortunately, a high-throughput assay to directly measure free 25(OH)D was not available at that time. Thus, in these reports, measured DBP, albumin and total 25(OH)D values were input into mathematical equations (11, 84) to calculate values for free 25(OH)D and bioavailable 25(OH)D. Disappointingly, one of the popular ELISA kits for DBP quantitation at the time relied upon a monoclonal antibody that turned out to possess reduced sensitivity to the DBP polymorphism (GC1F) most frequently found in black Americans (88, 115). Because of this characteristic of the ELISA, the DBP concentration was under-reported for these subjects leading to an over-estimation of the calculated free and bioavailable 25(OH)D. The manufacturer of this monoclonal ELISA has re-designed their ELISA and released a new version in January 2017 addressing this problem (135). Even with these complications, these early studies sparked interest in examining bone and mineral health by markers of vitamin D status other than the traditional total serum 25(OH)D.

Investigators have continued to use calculated free and bioavailable 25(OH)D in serum in association studies. In light of the difficulties with the monoclonal antibody-based ELISA for DBP, immunological methods based on polyclonal antibodies (less influenced by DBP polymorphisms) and techniques independent of antibodies entirely (mass spectrometry) can be employed to measure DBP for calculation of bioavailable 25(OH)D in serum (88, 115). Additionally, an ELISA method has been developed that measures free 25(OH)D directly (90) such that some investigators use this assay exclusively in their studies though many also include data from calculated free and/or bioavailable 25(OH)D.

HUMAN CLINICAL STUDIES OF FREE 25(OH)D AND BONE

This section surveys the recent literature examining whether free (directly measured or calculated using the polyclonal DBP assay) vs. total 25(OH)D is more consistently associated with various measures of bone health, including intestinal calcium absorption, parathyroid hormone secretion, and bone mineral density.

Intestinal Calcium Absorption

To our knowledge, there has only been one study to date examining the relation between free and total 25(OH)D with intestinal calcium absorption. Aloia et al. randomized 71 adults to receive either placebo, 800, 2,000, or 4,000 IU/days of vitamin D3 over 8 weeks. At both baseline and follow-up, neither free nor total 25(OH)D nor $1,25(OH)_2D$ in the serum was associated with intestinal calcium absorption efficiency (136). This supports the concept that VDR-directed increases in intestinal calcium absorption are controlled locally, outside of the serum compartment.

Parathyroid Hormone

Multiple investigators have assessed whether free vs. total 25(OH)D is more strongly correlated with the serum PTH level, with results being inconsistent. For example, in an analysis of 155 subjects that included 24 cirrhotics and 20 pregnant women, Schwartz et al. reported that both free and total serum 25(OH)D were similarly, inversely correlated with the serum PTH (117). Similar findings have been reported in: (1) healthy pre- (137) and postmenopausal women (136); (2) individuals with obesity (138); (3) patients cirrhosis of the liver (139); (4) children (2–18 y/o) in Spain (140); (5) blacks and whites in a supplementation study (placebo, 2,000, 4,000 IU/days for 16 weeks) (141); (6) a RCT of prediabetics (142); and (7) pregnant white women in Germany (143). While the above studies reported similar correlations between serum free and total 25(OH)D with serum PTH, others have favored free 25(OH)D. For example, Schwartz et al. conducted a 16-weeks trial in which 81 older women and men received vitamin D3 at doses of 800, 2,000, or 5,000 IU/days or 50,000 IU/weeks. At the end of the study, free, but not total serum 25(OH)D was inversely associated with the serum PTH; however, free 25(OH)D explained only a small amount of the variability in PTH [$R^2 = 0.08$; (144)]. In two, smaller trials – one in which 38 participants received 500,000 IU of vitamin D2 or D3 over 10 weeks (145) and another in which 35 participants received 2,400 IU/day of vitamin D3 or 20 mcg/day of 25-hydroxyvitamin D3 (146)—Shieh et al. reported

that longitudinal increase in free 25(OH)D was significantly associated with concurrent decrease in serum PTH during the first 8–10 weeks of supplementation (when 25(OH)D levels change most rapidly), whereas increase in total 25(OH)D was not. In adults with primary hyperparathyroidism, Wang et al., similarly found that the free serum 25(OH)D was inversely correlated with circulating PTH levels, but total 25(OH)D was not (147). Further complicating the picture are studies favoring total 25(OH)D over free 25(OH)D. In a cohort of Hungarian adults assessed at the end of winter total, but not free 25(OH)D, was inversely correlated with PTH (148). In a study of UK whites and south Asians, total, but not free 25(OH)D, was inversely correlated with PTH (149). In a study of pregnant adolescents (13–18 y/o), the inverse association of PTH with free 25(OH)D was weaker than that observed total 25(OH)D (150).

Bone Mineral Density

While a change in the serum PTH level is the outcome that has been most frequently tested in relation to free vs. total 25(OH)D, some cross-sectional studies have examined bone mineral density (BMD) as well. As was the case with changes in the serum PTH, results with BMD have been inconsistent. For example, Jemielita et al. reported that in 304 adults, neither total nor free serum 25(OH)D at a single point in time was associated with BMD assessed by DXA, or peripheral quantitative CT (151). In contrast, in a cross-sectional analysis comparing the correlations between free vs. total serum 25(OH)D with BMD and composite indices of femoral neck strength, Alwan reported that higher free 25(OH)D levels were correlated with greater BMD (lumbar spine, femoral neck, total hip), and femoral neck strength ($r = 0.24$–0.34, $p < 0.05$), but total 25(OH)D was not (152). On the flip side, Michaelsson et al. reported that in women from Sweden (mean age 68 years) higher total, but not free, serum 25(OH)D was associated with greater BMD (153).

CHALLENGES AND PROSPECTS

Undoubtedly, further clinical studies should be conducted using bone/mineral outcomes as well as non-skeletal health readouts to assess the utility of free 25(OH)D. As described in the above section pertaining to bone and mineral health, human studies comparing whether free vs. total serum25(OH)D is more frequently associated with intestinal calcium absorption, parathyroid hormone secretion, or bone mineral density have yielded inconsistent results ranging from no difference, to those favoring either free, or total 25(OH)D. We propose that there are two major challenges that contribute to these inconsistencies. First, is the lack of a specific "readout" of vitamin D bioactivity. While circulating PTH levels are influenced by vitamin D status (154–156), it is also regulated by the calcium sensing receptor. Additionally, attempts to associate BMD with vitamin D status are complicated by the fact that BMD in adults is principally determined by attained peak bone mass that is partly dependent on vitamin D status (157). Thus, it is difficult to discern the relative importance of free vs. 25(OH)D in human cross-sectional studies using these parameters. As such, even when studies report statistically significant correlations, the r values tend to fall in the range (−0.3–0.1 and 0.1–0.3) that are deemed "weak relationship" by statisticians. Second, many human studies do not employ subjects who are 25(OH)D deficient. These subjects would have the most to gain physiologically from treatment to return 25(OH)D in the serum to normal. Ideally, supplementation studies must include subjects that are clearly at insufficient levels and then have it demonstrably shown that their levels are raised into the sufficient range. It is through longer-term longitudinal analyses of intra-individual changes in serum total and free 25(OH)D after an aggressive vitamin D supplementation regimen that would have a greater likelihood to detect any associations.

Lastly, there is currently only one method of direct measurement of free 25(OH)D (90) with reasonable throughput. Though very promising, this assay is based on antibody interaction with 25(OH)D and needs further validation on the wide variety of sample quality (i.e., time from collection to testing, temperature of storage, variability of potentially interfering serum components among patients, etc.) encountered in clinical laboratory practice (158). In the future, perhaps a mass spectrometry-based method could be developed as is occurring in the measurement of other free hormones such as estradiol (159), thyroid hormone (160), and testosterone (161).

AUTHOR CONTRIBUTIONS

JA developed the overall organization of and approved this publication. JA, RC, AS, CG, and MH wrote sections of this review. VY and JW participated in copy editing.

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REVIEW
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Vitamin D Binding Protein: A Historic Overview

Roger Bouillon[1*†], *Frans Schuit*[2†], *Leen Antonio*[1,3†] *and Fraydoon Rastinejad*[4†]

[1] Laboratory of Clinical and Experimental Endocrinology, Department of Chronic Diseases, Metabolism and Ageing, KU Leuven, Leuven, Belgium, [2] Gene Expression Unit, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium, [3] Department of Endocrinology, University Hospitals Leuven, Leuven, Belgium, [4] Nuffield Department of Medicine, Target Discovery Institute, University of Oxford, Oxford, United Kingdom

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***Correspondence:**
Roger Bouillon
Roger.bouillon@kuleuven.be

†ORCID:
Roger Bouillon
orcid.org/0000-0002-6446-3763
Frans Schuit
orcid.org/0000-0003-3225-6695
Leen Antonio
orcid.org/0000-0002-1079-2860
Fraydoon Rastinejad
orcid.org/0000-0002-0784-9352

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Vitamin D and all its metabolites are bound to a specific vitamin D binding protein, DBP. This protein was originally first discovered by its worldwide polymorphism and called Group-specific Component (GC). We now know that DBP and GC are the same protein and appeared early in the evolution of vertebrates. DBP is genetically the oldest member of the albuminoid family (including albumin, α-fetoprotein and afamin, all involved in transport of fatty acids or hormones). DBP has a single binding site for all vitamin D metabolites and has a high affinity for 25OHD and 1,25(OH)2D, thereby creating a large pool of circulating 25OHD, which prevents rapid vitamin D deficiency. DBP of higher vertebrates (not amphibians or reptiles) binds with very high affinity actin, thereby preventing the formation of polymeric actin fibrils in the circulation after tissue damage. Megalin is a cargo receptor and is together with cubilin needed to reabsorb DBP or the DBP-25OHD complex, thereby preventing the urinary loss of these proteins and 25OHD. The total concentrations of 25OHD and 1,25(OH)2D in DBP null mice or humans are extremely low but calcium and bone homeostasis remain normal. This is the strongest argument for claiming that the "free hormone hypothesis" also applies to the vitamin D hormone, 1,25(OH)2D. DBP also transports fatty acids, and can play a role in the immune system. DBP is genetically very polymorphic with three frequent alleles (DBP/GC 1f, 1s, and 2) but in total more than 120 different variants but its health consequences, if any, are not understood. A standardization of DBP assays is essential to further explore the role of DBP in physiology and diseases.

Keywords: vitamin D, vitamin D binding protein (DBP), megalin, actin, 25-hydoxyvitamin D, 1,25-dihydoxyvitamin D

INTRODUCTION

The vitamin D binding protein (DBP) is a multifunctional protein that is well-conserved in the evolution of vertebrates. We review the history of the discovery of this gene and protein and summarize its main functions as known today. The major milestones in the discovery of this gene and protein and in the elucidation of its function are summarized in **Table 1**.

TABLE 1 | Major milestones in the discovery of the role of the vitamin D binding protein.

1	Antirachitic activity in serum migrates with α-globulin mobility	(1)
2	Radioactive vitamin D or 25OHD migrate with different electrophoretic mobility in serum from mammals, birds, amphibian or fish (α or β-globulin or albumin mobility)	(2)
3	Discovery of a major serum protein with genetically different polymorphism called, Group-specific Component (GC)	(3)
4	Identity of GC with the vitamin D binding protein of human serum	(4)
5	Purification and characterization of human DBP and other species, including high affinity-high capacity properties van DBP from higher vertebrate species	(5–7)
6	Immunoassays of DBP in humans and other species	(8)
7	DBP is a member of the albuminoid family	(9)
8	Description of the gene (and protein) structure of GC/DBP	(10)
9	DBP binds actin and plays a crucial role in depolymerization of extracellular actin filaments	(11)
10	Sex hormones regulate the concentration of DBP in a species-specific way. The concentration of total $1,25(OH)_2D$ fluctuates in line with the total concentration of DBP so that free $1,25(OH)_2D$ is feedback regulated	(12, 13)
11	Free 25OHD concentrations are extremely low (<0.1 % of total concentration) and not feed-back regulated	(14)
12	DBP binds to megalin at the luminal site of renal tubuli and thereby avoid urinary loss of 25OHD	(15)
13	DBP null mice are viable and healthy but have extremely low total concentrations of 25OHD and $1,25(OH)_2D$	(16)
14	DBP binds fatty acids, and unsaturated fatty acids impair the binding of $1,25(OH)_2D$ and 25OHD to DBP	(17, 18)
15	DBP binds to membranes proteoglycans of leucocytes and thereby enhances complement C5a-stimulated chemotactic activity in activated neutrophils	(19)
16	Crystal structure of human DBP	(20)
17	DBP can be measured by mass-mass spectrometry	(21, 22)
18	First human subject with homozygous deletion of the GC gene	(23)

DISCOVERY OF GC PROTEIN AS AN α-GLOBULIN AND ITS IDENTITY WITH THE SERUM VITAMIN D BINDING PROTEIN

In 1961, when serum proteins were still mainly characterized by their electrophoretic mobility as α, β, or γ globulins, further identification of the major proteins led to the discovery a highly polymorphic protein with genetically defined small differences in electrophoretic mobility, and therefore named "Group-specific component" or GC (24). Initially, only GC1 and GC2 were identified, but later on GC1 was found to be a mixture of GC1f (fast) and GC1s (slow), because GC1f has a slightly faster electrophoretic mobility than GC1s (**Figure 1**). Using polyclonal antibodies to detect GC and due to improved sensitivity for detecting small differences in the isoelectric point by isoelectric focusing of sera from subjects from around the world, more than 120 different variants were detected (**Figure 1**) (25–27). This technique allowed using this protein to study genetic links between populations and to use it in forensic medicine or paternity disputes (28).

Many authors studied independently the electric mobility of "vitamin D" activity in serum either by measuring the anti-rachitic activity, or later on, by using radiolabeled vitamin D or 25-hydroxyvitamin D (25OHD). Based on such data, lipoproteins were found to be the main transport mechanism in cartilaginous fish and amphibians (29). In most other vertebrate species, an α-globulin was the major transport protein in mammals and a β-globulin mobility was found in birds, whereas in some species, radioactive vitamin D (metabolites) migrated with albumin mobility (29, 30). The first major link between GC and DBP was made by Daiger et al. Indeed, from the perfect parallelism between the electrophoretic mobility of GC (measured by immunologic techniques) and the mobility of [^{14}C]vitamin D, they concluded that GC is the major transport protein for vitamin D (4). Peterson et al. had reported the purification of small amounts of human DBP and mentioned that GC was only a minor contaminant of "his" protein (31). However, the fact that the GC protein was identical to DBP was soon confirmed by three different laboratories reporting the purification of DBP from human serum, monitored by prior addition of [^{3}H]25OHD (5–7). We saturated the binding capacity of human serum by adding, in addition to radiolabeled 25OHD, sufficient stable 25OHD to avoid elimination of apoprotein during the purification process, thereby achieving a higher recovery rate (5). All these groups were able to show that the protein they isolated based on its binding of [^{3}H]25OHD was immunologically and electrophoretically identical to GC protein. The three groups initially applied different names, such as transcalciferin (5), but soon agreed upon DBP as abbreviation for the serum vitamin D binding protein (8). DBP was subsequently also purified from rat (32), chick (33) and many other species.

In summary, GC was discovered in the early 1960s as a polymorphic serum protein and at the same time a serum protein (DBP) was identified transporting vitamin D and its metabolites. In 1985 and 1986 GC/DBP were found to be the same protein.

GENE CODING FOR GC/DBP

From early onwards, the three major forms of GC were shown to be the result of a pair of co-dominant autosomal alleles, resulting in homozygous GC1f-1f, GC1s-1s, GC2-2 or a mixture of these genes in most subjects. The gene for DBP/GC is localized on human chromosome 4q11-q13 as shown by *in situ* hybridization techniques, whereas the gene is localized on chromosome 5 or 13 in the mouse and rat, respectively (34). The gene is positioned close to the genes for albumin, α-fetoprotein and afamin (also known as a-albumin), with a centromere-DBP-albumin-α-fetoprotein-afamin-telomere orientation. Their protein products are mainly synthesized and secreted by hepatocytes. The DBP gene is also expressed in kidney, testis, endocrine pancreatic cells, and fat cells (35). Genetic analysis of the evolution of these sets of genes indicates that DBP might well be the oldest member of the family (**Figure 2**). Human and rat DBP have 13 introns and a 42 kb gene structure. The human gene codes for a 1690 nucleotide mRNA and a 458 amino acid long single chain protein, preceded by a 16 amino-acid signal propeptide.

In summary, the structure of the GC/DBP protein was identified, located on human chromosome 4 close to the other members of the albumin gene family.

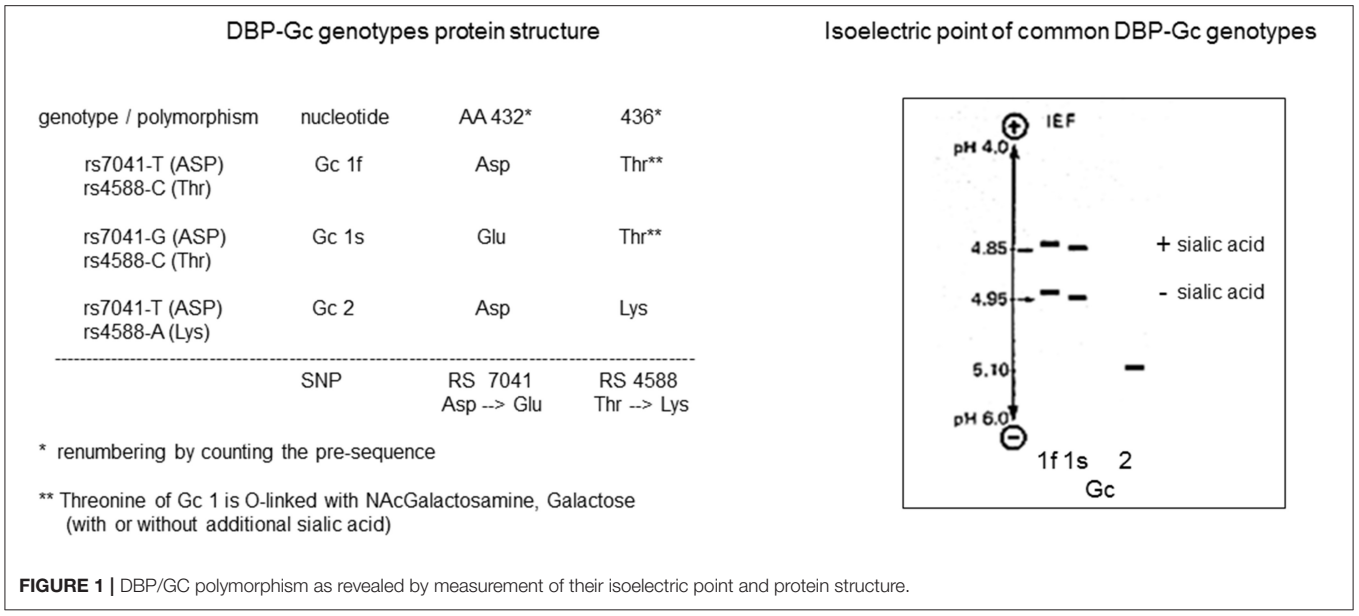

FIGURE 1 | DBP/GC polymorphism as revealed by measurement of their isoelectric point and protein structure.

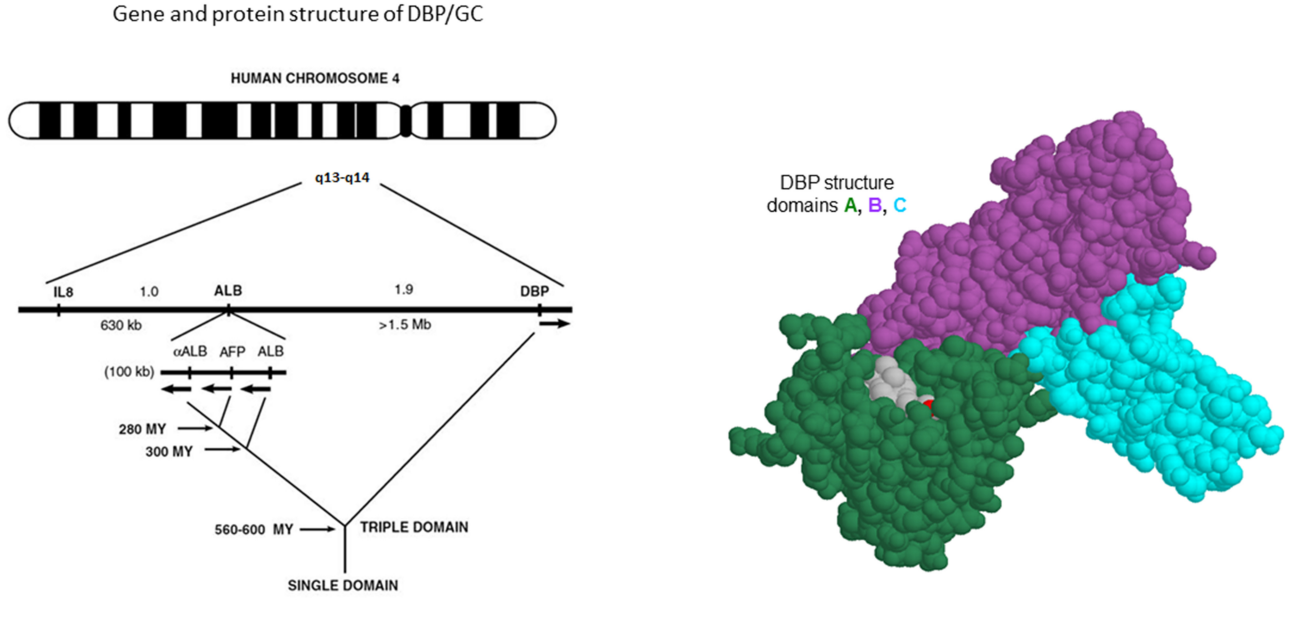

FIGURE 2 | Gene and chromosome structure of GC/DBP and adjacent albuminoid family genes.

EVOLUTION OF VERTEBRATE GC/DBP

One of us (FS) studied the vertebrate GC gene and evolution of the predicted DBP protein primary structure, whereas another co-author (FR) evaluated the 3D structure of GC/DBP and its interaction with the vitamin D metabolites. The National Center for Biotechnology Information, U.S. National Library of Medicine (NCBI Gene) listed on June 7th 2019 GC gene sequences of 210 vertebrates (nine bony fishes, one amphibian, 14 reptiles, 59 birds and 127 mammals). In all clades, we ascertained the correctness of the searched gene not only by sequence homology but also by syntheny with its two neighboring genes, SLC4A4 and NPFFR2. A complete domain 1 primary structure (AA1-110) of DBP was found in 191 of these sequences. Here we summarize the essential conclusions, as a detailed analysis will be presented in a separate paper. First, in all major clades of vertebrates the predicted GC/DBP gene can be found, whereas [as expected from the literature (36)] the paralogous albumin gene is absent all fish, amphibia, lizards, snakes, and turtles. Second, by far the best conserved part of the primary structure of DBP (virtually 100% conserved in all species with a complete sequence) was a series of 28 cysteines that are

ordered over the three protein domains as a typical repetition of ".....C....CC....C.....". Two of these repetitions are present in the domain 1 primary structure. The evolutionary importance of cysteine pairs to form disulfide bridges in the primary structure was already suggested earlier, based on fewer gene and protein (six species for GC/DBP) structures (9, 37). Third, the highest protein homology was present in domain A, reflecting its crucial role to transport vitamin D and its metabolites, which are mainly hydrophobic. For instance, $25OHD_3$ has only two polar hydroxyl groups (on C3 and C25). These two critical recognition features involve in mammals residues Tyr-32 and Ser-76 to recognize the 25-OH and 3-OH moieties, respectively, which are located at two extreme ends of its ligand. Tyr-32 is not only well-conserved among mammalian species but also in bony fishes; however, the reptilian branch of vertebrates evolved to a different well-conserved asparagine. For residue 76, the conservation is the OH-group from either a serine or the slightly larger residue threonine. Moreover, amino-acid residues 8, 12, 24, 35, 68, and 107 that contribute to van der Waals interactions with vitamin D or its metabolites are also highly conserved among mammals and often substituted conservatively by other hydrophobic amino acids (Ile-12 or Val-12; Leu-107, or Met-107) that would form similar van der Walls interactions with the ligand. This conservative nature of the amino acids involved in the binding cleft of DBP explains the high affinity of DBP for 25OHD in all clades of vertebrates.

In summary, GC/DBP is found in nearly all vertebrate species with well-conserved structure over the 500 million span of vertebrate evolution. This is especially the case for the ligand (25OHD) binding cleft.

DBP: ORIGIN, TURNOVER, AND SERUM MEASUREMENTS AND CONCENTRATION

Origin and Turnover

Vitamin D binding protein, as well as its close family members, is mainly produced in the liver, although the gene and protein are also expressed in very low concentrations in other tissues as mentioned above. The pool of DBP in rabbits is about 54 mg/kg with a half-life of 41 h and distributed between the intravascular pool (1/3) and the extravascular pool (2/3). The distribution is largely limited to the extracellular volume, in line with albumin (38). The half-life of DBP in human plasma is about 1.7 days and thus markedly shorter than the half-life of 25OHD, which is estimated at about 15 days, based on studies with deuterium labeled 25OHD in healthy subjects from the UK and Gambia (39). The estimated daily production of DBP is about 700–900 mg/d for an adult person (10 mg/kg/d). In comparison, the total body albumin is about 280 g for a normal adult and thus more than 300 times greater than that of DBP. About 40% of albumin is intravascular, with the remaining 60% distributed in the interstitial space of various organs (primarily muscle, adipose tissue, connective tissue, and skin) with an average interstitial concentration of about 60–70% of that of plasma. Rabbit and human data suggest a similar compartmentalization of DBP (38, 40). The absolute synthesis rate of albumin is about 150 mg/kg/day or 10.5 g/day for a 70 kg human. About 8.5% of plasma albumin and 4% of the total body albumin are synthesized each day, corresponding to a total body albumin turnover time of about 25 days or a half-life of 17.3 days. This is about 10 times longer than that of DBP but shorter than other circulating proteins (hemoglobin has a life span of 120 days) and similar to that of γ-globulins (41). The mRNA of DBP in liver is rather low (similar to the situation of albumin mRNA), suggesting a slow turnover of this mRNA comparing with the protein turnover. The clearance site of DBP is not fully understood, but DBP is partially filtered in the glomerulus and reabsorbed in the tubuli mediated by a carrier receptor mechanism (megalin, see below), followed by intracellular degradation. In rabbits, DBP holoprotein and apoprotein are cleared at about the same rate (42). The half-life of DBP is thus markedly shorter than the half-life of 25OHD and this implies that 25OHD is recirculated after degradation of DBP. Removal of sialic acid does not changes its plasma clearance (40).

Protein Structure and Polymorphisms of DBP

The mature DBP structure of humans is 458 amino acids (AA) long, whereas rat, mouse, and rabbit DBP all are 460 AA long. DBP has a highly conserved number of cysteines and disulfide bridges. It has three domains, much like albumin, and these domains are probably the results of gene duplication of a singly common ancestor structure (34). Unexpectedly, the 3D structure of DBP differs substantially from that of albumin (20). The A domain has a cleft structure allowing to bind 25OHD with high affinity. The structure of the holoprotein (DBP plus 25OHD or analogs) confirms the predicted AA sequences (AA 35–49) responsible for binding of 25OHD and all other vitamin D metabolites. Indeed, there is only a single binding site for all D metabolites. DBP has the highest affinity for 25OHD-lactones, followed by 25OHD = $24,25(OH)_2D$ > $1,25(OH)_2D$ (5, 6, 43). The binding site of DBP for 25OHD is show in **Figure 3**. The structure of DBP with a vitamin D with a pentanor side chain modification, known from *in vitro* binding studies to have a high DBP affinity, allows to explain why such analog has a higher affinity (for detailed discussion see (20, 34). The binding site of vitamin D for DBP is totally different from that of the binding site of the vitamin D receptor (VDR) (44). The main characteristics of DBP are summarized in **Table 2**. Human DBP has an isoelectic point (IEP) of about 4.89, but this varies according to DBP/GC genotype. The stability of DBP at high temperature is markedly enhanced by binding to 25OHD. The holoprotein (DBP.25OHD complex) has a different IEP compared with the apoprotein, and this indicates that the protein undergoes a structural modification when bound to vitamin D metabolites (5, 45). DBP is highly polymorphic as it was originally discovered by this characteristic and therefore received its initial name of group-specific component. The three most common alleles and protein structures are shown in **Figure 1**. GC1 (1f or 1s) has a high degree (about 10–25%) of O-glycosylation in threonine position 436 with a linear trisaccharide (NeurNAc-Gal-GalNAC) whereas residue 434 is much less glycosylated (1–5%) by a disaccharide (without the final sialic acid). DBP/GC is

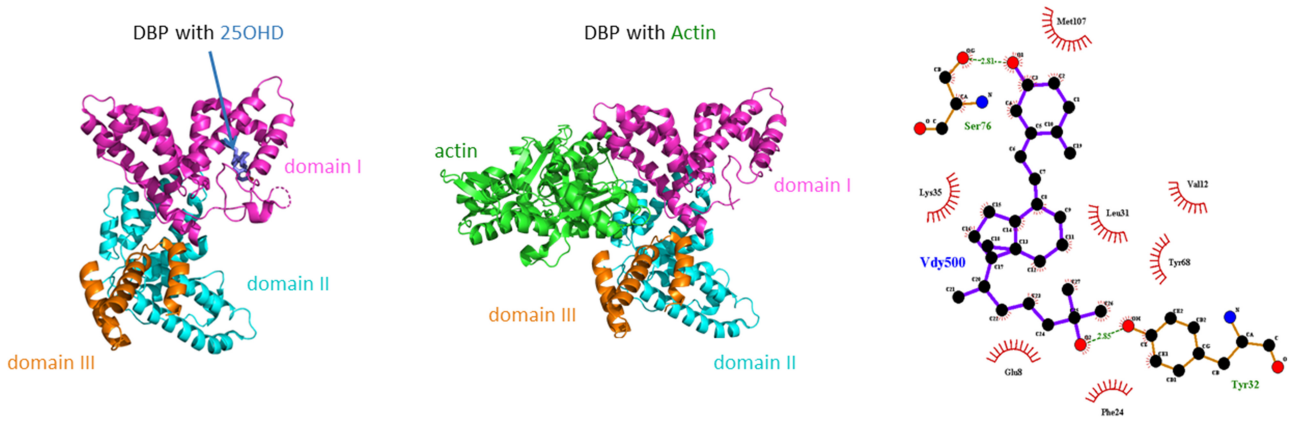

FIGURE 3 | Crystal structure of human DBP in combination with 25OHD or actin. In addition the main amino acids involved in the binding of 25OHD to the cleft in the A domain of human DBP as shown.

similarly (poorly) glycosylated on AA 434 but not on AA 436 (being lysine rather than threonine) in DBP/GC1. The terminal sialic acid of DBP/GC 1 can be present or absent and therefore both DBP/GC1f and DBP/GC1s are present in serum in double bands with a very small difference in isoelectric point (**Figure 1**). Neuraminidase treatment can remove sialic acid and thereby eliminate this double band on isoelectric focusing (45, 46). The genetic or molecular (pre-or posttranslational) origin of the large number (>124) of variants of DBP in humans is largely unknown (34, 47) and the implication for the functions of DBP (see below) is unknown. The most common genetic variants (GC1s/1f/2) are due to polymorphisms in the third domain, whereas the few other variants are due to polymorphisms in the second domain [reviewed in (34)]. The best-known variant (GC1A1) is one found in Aboriginals and some South African blacks (48). Genetic polymorphism of DBP has also been documented in other species such as rats (32, 49), monkeys (50), swine, rabbits (24), chicks, and horses.

Measurement of DBP

DBP is usually measured by one of the many methods based on specific anti-DBP antibodies. This frequently requires species-specific antibodies when measured in different species. In the author's laboratory, polyclonal antibodies were generated for DBP from humans, rats, chicks, rabbits, mice, guinea pigs, and dogs. The binding capacity of serum for 25OHD can also be estimated by using radiolabeled 25OHD using a Scatchard plot to calculate its affinity and binding capacity. In most cases, the measured binding capacity was lower than the immunoassay values (51) but later technological improvements allowed to generate very similar concentrations between binding and immunoassays.

We used radial immunodiffusion as this avoids the need for major dilution of serum samples, but others used a wide variety of other assay methods, such as rocket electrophoresis, turbidimetry, or nephelometry, ELISA or even radioimmunoassay. More recently, DBP has also been measured by tandem mass spectroscopy after prior peptide digestion. This method requires specific synthesis of a mixture of labeled peptides common to all GC/DBP isoforms and in addition other peptides which are genotype-specific (21, 22, 52, 53). Based on a close collaboration between a team of the Leuven University, NIST (Karen Phinney and Lisa Kilpatrick) and Dr. Hoofnagle's group in Seattle, a reference standard for DBP (based on protein purified from homozygous GC1f, GC1s, and GC2 volunteers) and a reference method for an MS/MS based assay of DBP has been developed (53). An Elisa technique by R&D (54, 55) used monoclonal antibodies and was widely used to measure serum DBP concentration. The results obtained with this method surprised many experts as this assay showed race- and DBP/GC-specific results, whereby blacks or African-Americans (mostly having DBP/GC1f genotype) had markedly (−50%) lower serum DBP than whites (54). The authors, therefore, concluded that free 25OHD concentrations in African Americans were similar to those in Whites and that this could explain the paradox of "low" vitamin D status but solid bones and low fracture rate in African-Americans. These data and at least 50 other manuscripts using this assay all seemed to conclude that "we" all used wrong methods (total 25OHD) instead of free 25OHD to estimate the real vitamin D status (56). However, previous studies using polyclonal antibodies did not find racial differences in serum DBP (57). Extensive studies thereafter, using polyclonal and mass spectrometry based assays (22, 58, 59) all convincingly demonstrated that the R&D monoclonal DBP assay discriminates against DBP/GC 1f and that all results in genetically heterogeneous populations should be "retracted" or re-interpreted. The senior author of the Powe et al. paper later on agreed that the DBP results, based on the monoclonal R&D assay, from these studies were wrong (60).

The concentration of DBP in normal human serum is in the μmolar range (~6 μmol/l or about 300 ml/l) but different laboratories have reported mean concentrations varying between 200 and 600 mg/l. Most of these differences are probably due to lack of standardization of DBP assays and reference material. Hopefully, this will soon be corrected by using the NIST reference preparation (53). Most assays use polyclonal

TABLE 2 | Major characteristics of the human vitamin D binding protein.

Gene	- Located on chromosome 4q11–q13, close to albumin, α-fetoprotein, and afamin genes and in syntheny with its two neighboring genes, SLC4A4 and the neuropeptide receptor 2, NPFFR2 - Autosomal co-dominant gene transmission - Highly polymorphic gene/protein with more than 120 variants in human populations around the world. Polymorphisms also found in several other species including rodents
Protein	- 458 amino acids−58 kD–preceded by 16 amino acid signal propeptide - Three domains: a highly evolutionary conserved A domain with a cleft able to bind all vitamin D metabolites. The B and C domains can bind actin with high affinity - Single binding site at the surface of the A domain, creating a cleft for all vitamin D metabolites - Isoelectric point 4.6–5.0 depending on gene polymorphism or posttranslational modifications - Pool size: 2.8 g - T½: 1.7 days - Daily production: 10 mg/kg
Serum concentrations	- μmolar concentration (∼200–600 mg/l) in normal adults - Mainly apoprotein configuration and <5% as holoprotein
Biological functions	- Binding/transport of all vitamin D metabolites with high affinity (25OHD lactone > 25OHD = 24,25$(OH)_2$D = 25,26$(OH)_2$D > 1,25$(OH)_2$D > vitamin D, and vitamin D_3 metabolites > vitamin D_2 metabolites) - Binding of actin monomers and enhanced clearance of fibrillar actin - Binding of fatty acids and especially unsaturated fatty acids - Binding to megalin-cubilin receptor complex - Binding to membrane of leucocytes and activation of complement C5 system
Clinical implications	- DBP concentration and affinity define the free concentration of all vitamin D metabolites - Low concentration in fetus and neonates - Increased serum concentration when exposed to estrogens - Decreased serum concentration in case of liver diseases, nephrotic syndrome, malnutrition, severe acute trauma, or disease - Complete genetic absence leads to very low serum concentrations of all vitamin D metabolites without a clinical phenotype

antibodies and therefore the effect of protein polymorphism in the final measurement is minimal. As mentioned above, one assay, however, used monoclonal antibodies and reported mean DBP concentrations that were very different according to the genetic (or racial) differences in DBP (54). Other monoclonal antibodies, however, do not discriminate GC/DBP isoforms (61, 62). When measured with a variety of polyclonal antibodies, DBP concentrations in humans with GC/DBP2 genotype are slightly (10–20%) lower than in GC/DBP 1 carriers. This was already know in 1966 and confirmed in many later studies [reviewed in (63)].

DBP circulates in serum in much higher concentrations than the combined concentration of all vitamin D metabolites, as serum DBP concentration is in the mid μmolar concentration whereas the serum concentration of the major metabolite, 25OHD, is usually below 100 nmol/l. This implies that <5, and usually <2% of DBP is a holoprotein (DBP plus vitamin D metabolite) and nearly all DBP circulates as apoprotein. This is quite different from the other binding proteins for ligands of nuclear receptors. The DBP gene transcription is regulated by a balance between hepatic nuclear factors (HNF) 1α and 1β, as is the case for other liver abundant proteins. However, unlike the regulation of albumin, HFN1α is a positive regulator and HFN1β is a dominant negative regulator of DBP expression (64). DNA methylation of the promotor region can also modify the DBP gene expression (65). DBP is already expressed in the yolk sac (as documented in animals) and later on in the fetal liver and thus appears in the fetal circulation. Indeed, DBP can be found in serum of human fetuses in the 3th trimester and its concentration in (human) cord serum is only about half that of maternal serum as reported by several authors (13, 34).

Regulation by Hormones and Other Factors

The concentration of DBP in human serum is in the μmolar (micromolar) range as measured by immunoassay or, more recently, by MS/MS (34). In humans, exposure to estrogens increases serum DBP but androgens have no effects. Decreased serum DBP concentrations are found in patients with liver cirrhosis, malnutrition, peritoneal dialysis, and nephrotic syndrome (see also below DBP-megalin interaction). The concentration in the human fetus and cord serum is lower than in adult serum. Exposure to estrogens increases the serum concentration of DBP (e.g., due to intake of contraceptive estro-progestogens or during pregnancy). Therefore, the serum DBP concentration in pregnant women at the time of delivery is about twice the concentration found in cord serum (13, 14). Exposure to or loss of androgens does not change serum DBP concentrations in humans. Vitamin D deficiency or excess, or vitamin D resistance, idiopathic hypercalcemia of infancy, or osteoporosis and many other diseases (such as hyperparathyroidism or hyperthyroidism, sarcoidosis, cancer, Addison's disease, or growth hormone deficiency) have no effect on serum DBP concentration. DBP is slightly increased in acromegaly (66) and in some inflammatory diseases (such as in rheumatoid arthritis) (67). DBP concentrations are slightly decreased in serum of type 1 diabetes patients (68) and even more severely in BB or streptozotocin-induced diabetic rats (69), but better diabetes control can restore these concentrations (70). Decreased concentrations of DBP are also found in patients with a variety of kidney diseases or in case of renal loss of DBP. Patients with nephrotic syndrome therefore not only lose massive amounts of albumin but also large amounts of DBP (having a lower molecular weight than albumin). This urinary loss of DBP also entrails urinary loss of 25OHD and thus results in vitamin D deficiency (71). Low DBP concentrations are also found in animals or patients with genetic or acquired loss of megalin or cubilin, wo proteins functioning as cargo receptor to reabsorb serum proteins, filtered in the glomerulus and recovered in the renal tubuli (15) (see below). The genotype of DBP also has a small effect on the serum DBP concentrations as subjects with homozygous GC2-2 genotype have about 5–10% lower serum

DBP concentrations compared to GC1 carriers (63). Whether this is due to a lower hepatic synthesis or more rapid clearance (lower glycosylation) is unknown.

DBP is a positive acute phase reactant after infections or minor trauma (72, 73). Severe trauma (e.g., hip fracture) or severe illness (such as patients requiring intensive care treatment) decreases serum DBP by more than 10%. Whether this is due to an increase in distribution volume, decreased synthesis or increased clearance as DBP or DBP-actin complex is not fully understood. Indeed, major tissue injury causes the release of intracellular actin into the blood stream, creating actin-DBP complexes that are rapidly cleared from the circulation (74), so that the positive effect of increased synthesis is largely compensated by even more rapid destruction and thus resulting an decreasing DBP concentrations. During ICU stay, serum DBP slowly increases and reaches normal levels again after about 10 days (Ingels et al., submitted). The serum concentration of DBP has a modest influence on the half-life of 25OHD as demonstrated by using deuterium-labeled 25OHD in healthy men living in the UK or The Gambia (39). This is not surprising as lower DBP concentrations, while serum 25OHD remaining similar, generate higher free 25OHD concentrations and a slightly higher catabolic rate. This study also showed that the half-life of $25OHD_2$ was slightly shorter than that of $25OHD_3$, in line with lower affinity of $25OHD_2$ for DBP.

Evolutionary Aspects of DBP

Early in the evolution of vertebrates, after whole genome duplication, all elements of the vitamin D endocrine function became gradually operational (75). The vitamin D receptor, (VDR), coming from an ancestor nuclear receptor involved in detoxification, acquired a critical role in maintaining a normal calcium homeostasis and bone metabolism as to cope with an environment with lower access to calcium and a need for solid but light weighted bone in a terrestrial rather than an aquatic milieu. The CYP P450 gene family was broadened to include several genes with specific role in the activation and degradation of vitamin D (CYP2R1, CYP27B1, and CYP24A1). Finally, a specific transport protein for the lipid soluble vitamin D and its metabolites in serum is found in most fish and all terrestrial vertebrates studied so far. Not discussed here is the broad range of genes that are under the direct or indirect control of the vitamin D hormone (76) and which may explain why a poor vitamin D status not only results in a skeletal/calcium but also extra-skeletal phenotypes.

The VDR and the crucial CYPs for the metabolic activation of vitamin D are already present in cartilaginous fish. However, based on gel electrophoresis of radiolabeled 25OHD, Hay and Watson (29) concluded that the vitamin D metabolites in fish with a cartilaginous skeleton are transported by lipoproteins and not by a specific DBP as in higher vertebrates. Allewaert et al., however, found two vitamin D binding proteins in fish (carp) but only one resembles DBP (high 25OHD affinity and actin binding) (77). In amphibians ($n = 12$ species), Hay concluded that vitamin D was transported by lipoproteins. Allewaert, however, found in sera of amphibia (two rana species, Bufo marinus and salamandra) and reptiles, a 25OHD-binding protein with high affinity for 25OHD but with a low concentration (about 0.03 μmol/L or about 100-fold lower than in higher vertebrates) (78). DBP from reptiles is able to bind actin (as in higher vertebrates, see below), as revealed by sucrose gradient ultracentrifugation, but DBP from all amphibian binding proteins did not show actin binding. In reptiles (iguana, varanus, python, and geomyola), a DBP like protein able to bind 25OHD and actin was found as in chicks and mammals (78, 79). In turtle, trachemys scripta (a member of reptiles), DBP binds thyroxine as well as vitamin D (80). The situation in birds is complex as most species use a β-globulin, and a minority use a protein with an α-globulin or albumin mobility. DBP is present in bird serum and chick (gallus domesticus) but chick DBP has a slightly higher molecular weight than human DBP. Although avian DBP has a β-globulin mobility, it has a high degree of homology with human or rat DBP at protein and gene level (33).

Older studies, before the use of gene structure to define protein structures, revealed that mammals (65 species of 14 mammalian orders, including marsupials) use a transport protein with α-globulin ($n = 65$) or albumin mobility ($n = 7$ such as pacific dolphin or orcas, and three species of new world monkeys) (29). The DBP concentration in mammals (other than humans) are also in the μmolar range (as in birds). DBP levels are very low in rat fetuses and at birth. Thereafter, its concentration increases more than five-fold in adult rats. After rat "puberty," androgens increase serum DBP whereas the DBP concentration does not change when rats are exposed to endogenous or exogenous estrogens (12). Vitamin D deficiency does not influence the concentration of DBP in rodents (nor in humans). Serum DBP concentrations in rats and mice increase when these rodents are exposed to androgens, contrary to what happens in humans and birds. Indeed, mouse DBP as measured by a mouse specific immunoassay, is higher in adult males compared to adult females. The DBP concentration did not markedly vary among different mouse strains [6–10 μmol/l in males and 5–9 μmol/l in females (81)]. Cortisol binding protein in rats, however, increases when animals are exposed to estrogens (as in humans). In guinea pigs, DBP concentrations decrease during pregnancy by nearly 50% (contrary to humans). Fetal and neonatal DBP concentrations of DBP are very low, similar to what has been observed in rats, despite the much greater maturity of guinea pigs at time of birth. Therefore, when comparing different species, we can conclude that DBP is already expressed early during fetal life but the DBP concentration at birth is much lower than later in life. Sex hormones change the concentration of DBP in all species and usually estrogens increase DBP concentrations except in rodents where androgens increase serum DBP.

DBP is found in all primates and migrates as an α-protein (as in humans), except in two New World strains. Their DBP is immunologically identical to that of other primates but has a different mobility. The genetic and molecular basis of this difference is not fully explained so far, as the molecular weight and isoelectric point are identical in these New World monkeys and humans (50).

The main lesson from all these studies is that vitamin D or its metabolites bind to lipoproteins in all vertebrates but from bony fish onwards, a protein of the albuminoid family, DBP, becomes

the major transport protein with high affinity for 25OHD. The electrophoretic mobility of this protein however can vary from α- to β-globulin or albumin mobility. DBP concentrations are low in amphibia and reptiles (0.03 μmolar concentration) but are high in birds and mammals (∼5 μmolar concentration).

In summary, GC/DBP is mainly produced in the liver and circulates in high concentrations in serum of birds and mammals. It is usually measured by immune assays but now also by mass spectrometry. Its concentration in serum is stable and regulated by sex steroids. Decreased concentrations are found in liver or kidney diseases. Total (genetic) absence of DBP was so far only found in a single adult human without generating a bone or other clinical phenotype.

FUNCTIONS OF DBP (TABLE 2)

Vitamin D Transport

DBP Is the Major Transport Protein for All Vitamin D Metabolites

DBP, as its name suggests, is the major binding/transport protein for all vitamin D metabolites. There is only a single binding site in its A domain. Vitamin D is produced in the skin by photochemical transformation of 7-dehydrocholesterol (7DHC) into pre-vitamin D, followed by slow equilibrium with vitamin D itself. Vitamin D then binds to DBP and thereby drives the equilibrium in favor of vitamin D itself. This DBP-bound vitamin D is then rapidly taken up by the liver, and hydroxylated by CYP2R1 and possibly some other 25-hydroxylases, whereby the 25-hydroxylated metabolite is secreted into the circulation. It is then again, and now with higher affinity, bound to DBP. Vitamin D from dietary origin is absorbed in the intestine with about 70% efficacy in normal subjects. Although this process was for a long time considered to be a passive transport of a lipophilic molecule to access the lipid layer of the intestinal cells (82), more recent data suggest that vitamin D absorption and secretion from and into the lumen of the gut is medicated by carrier proteins that are also involved in cholesterol transport in the intestine (83). To what extent vitamin D esters are first digested and then absorbed or lost in the feces is still a point of discussion [see *Solanum malacoxylon* being a $1,25(OH)_2$D-glucuronide causing severe generalized calcinosis in grazing cattle in Argentina (84, 85)]. After passage thought the intestinal cell, vitamin D is transported by chylomicrons and thus uses the lymph pathway before it arrives in the general blood circulation (82). In that circulation, chylomicrons (including vitamin D) are mainly taken up by fat cells or by the liver, but there is also a gradual shift of vitamin D from chylomicrons to DBP. The metabolism and transport of vitamin D resembles the metabolic fate of iodine (also a nutritional product with only intermittent supply and essential for the generation of an essential hormone; **Figure 4**). In both situations, the prohormone, 25OHD, and thyroxin (T_4) are bound with high affinity to a specific serum transport protein, DBP and thyroxin-binding globulin (TBG), respectively. Therefore, there is a large pool of circulating precursor with a long half-life (2 and 1 week, respectively), so that transient loss of supply of the nutrient (vitamin D or iodine) does not immediately cause lack of hormonal effect of the real ligand for their nuclear receptor [$1,25(OH)_2$D and VDR, T_3 and thyroid hormone receptor, respectively (**Figure 4**)]. The affinity of DBP for 25OHD is much higher than for the VDR, thereby keeping this precursor preferentially in the serum pool, and the same is true for T_4. By contrast, the active hormones are ligands for their nuclear receptors to which they bind with high affinity, whereas their affinity for the serum binding protein is much lower. In consequence, the distribution volume of 25OHD is very close to that of albumin and DBP (∼extracellular volume), whereas the distribution volume of $1,25(OH)_2$D and T_3 is close to that of the intracellular volume.

Whether the role of DBP is essential for the overall metabolism and action of the vitamin D endocrine system can best be evaluated by data generated in animals or humans with total lack of DBP. Absence of other transport proteins for ligands of nuclear receptor are well-known and absence of TBG, cortisol, or sex hormone binding proteins does not create hormonal dysfunctions. This is explained by the very low total serum concentrations of the ligands for these binding proteins, so that the free (= not bound to their serum binding protein) hormone or hormone precursor is virtually normal. Therefore, the target tissues "see" normal free hormone concentrations (and in fact the concentration of these hormones are virtually normal when measured in tissues (86). Such data are the strongest arguments in favor of the free hormone hypothesis, posing it that the unbound ligands and not their total concentrations are physiologically important. For a long time, out of hundreds of thousands of human sera, not a single case of complete DBP deficiency was found, suggesting even that this protein might be essential for normal life. DBP null mice, however, did not have a phenotype and their concentrations of "total" 25OHD and $1,25(OH)_2$D were extremely low (close to the detection limit) (16). Nevertheless, such mice did not have rickets or another phenotype when kept on a vitamin D replete diet. In line with the role of DBP as responsible for generating a pool of circulating precursor, DBP null mice are much more prone to vitamin D deficiency when kept in conditions of vitamin D deficiency (no UVB light exposure and no vitamin D in their diet). In addition, these mice were more resistant to the toxic effect of exposure to high doses of vitamin D, probably by more rapid catabolism of vitamin D and its metabolites. In 2019, the first case of true total DBP deficiency was described in a 58-year old Canadian woman, with very low serum concentrations of 25OHD and $1,25(OH)_2$D and absence of serum DBP (all measured by gold standard technology of liquid chromatography-tandem mass spectrometry) (23). Nevertheless, no history of rickets or signs of osteomalacia were present and the patient had normal serum calcium and PTH concentrations. She was found to have a homozygous deletion of the complete GC gene region and part of the adjacent NPFFR2 gene. This latter gene codes for a GPCR involved in nociception and hypothalamic-pituitary axis (87). This rare event may be explained by parental consanguinity with Lebanese background. Both genes are known to be closely linked together during the whole evolution of vertebrates (see above in chapter on genetic evolution of GC/DBP). The sibling with only one mutated allele had about half of the normal DBP concentration (in

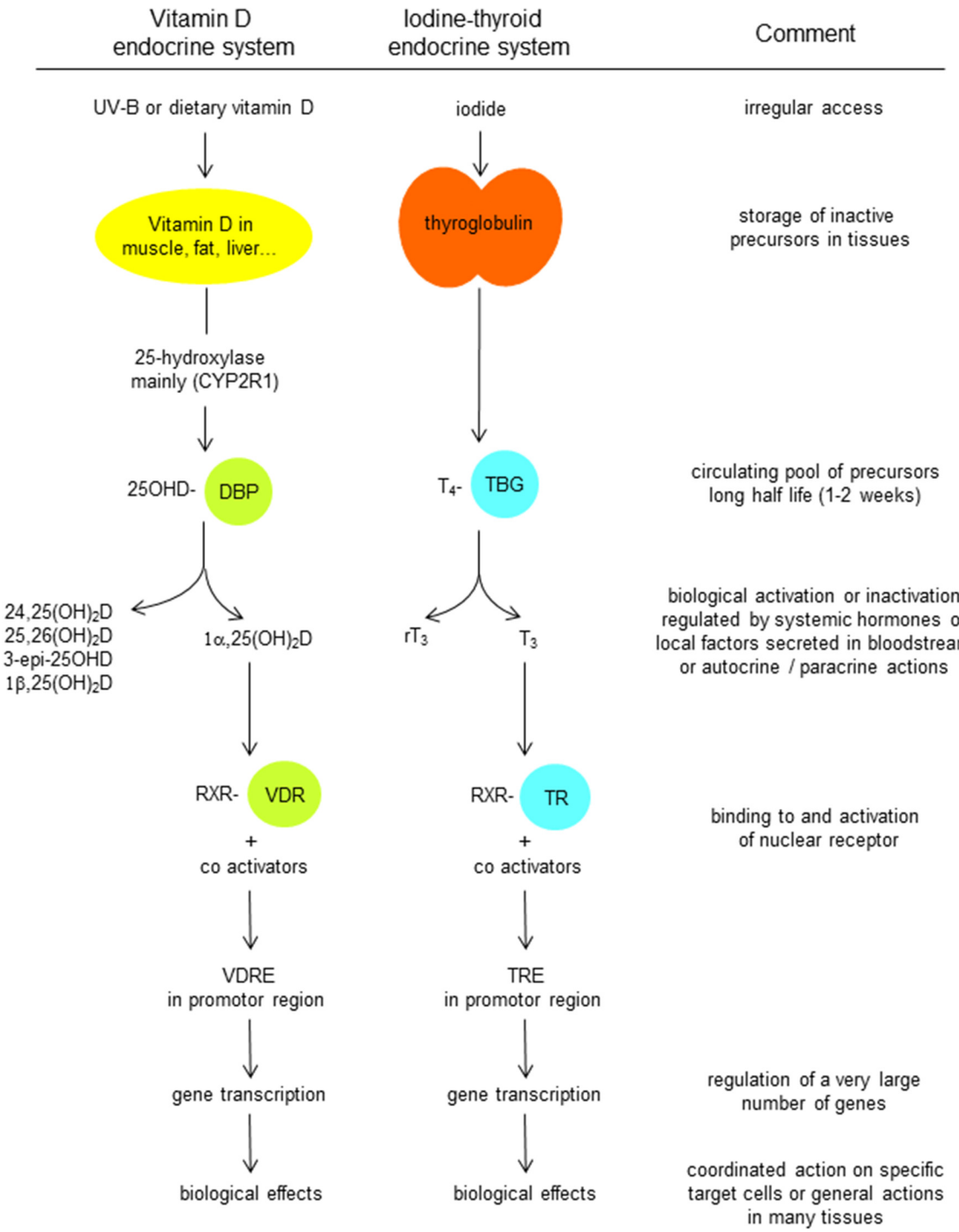

FIGURE 4 | Comparison of the transport and metabolism of vitamin D endocrine system with that the iodine-thyroid system.

line with the co-dominant inherence of both GC alleles) and serum 25OHD and $1,25(OH)_2D$ concentration were well-below the normal concentrations in the healthy other sibling. The patient also had a debilitating ankylosing spondylitis, a known autoimmune disease. It is rather unlikely that this is related to the absence of DBP, as this phenotype is not found in DBP null mice. It may be related to the loss of the adjacent gene or due to repeated exposure to very high doses of vitamin D as to "correct her apparent vitamin D deficiency" although these interventions failed to increase serum vitamin D metabolites

(23). Similarly, absence of albumin in humans is not causing a major phenotype.

Affinity of DBP for Vitamin D Metabolites

There is clearly only a single binding site for all vitamin D metabolites in DBP (**Figure 3**), in contrast to albumin which has several low affinity binding sites. 25OHD binds with high affinity but the absolute value differs slightly between species, and is dependent on the buffer medium, pH and temperature (88). Indeed, the affinity of DBP is higher (about 10 times) in barbital buffer at pH 8.6 compared to their affinity measured in different buffers at pH 7.4 (88). The affinity of DBP is highest for 25OHD-lactones, followed by about equal affinity for 25OHD and 24R,25$(OH)_2$D or 25S,26$(OH)_2$D. Its affinity for 1,25$(OH)_2$D is about 10–100 lower compared to that of 25OHD. The lowest affinity is for vitamin D itself. The structure of the cleft on human DBP largely fits with these different affinities (20). Natural variants of the side chain such as in vitamin D_2 and its metabolites may affect the binding for DBP. For humans and most mammals the difference in affinity is small, with about 20 % lower affinity for 25OHD_2 compared to 25OHD_3 (89, 90). In birds, DBP has a much lower affinity for 25OHD_2 compared to 25OHD_3 and this is supposed to be one of the main reasons for the low biological activity of vitamin D_2 for the prevention of rickets in birds (91, 92). The relative difference in affinity between 25OHD_2 and D_3 was greater when measured in diluted serum (10-fold difference) (92) than when measured for purified chick DBP (three-fold difference) (33). Vitamin D_2 is also largely ineffective in comparison with vitamin D_3 to promote the intestinal absorption of calcium-47-isotope in Cebus Albrifons monkeys but for so far unknown reasons (93). In contrast to DBP, VDR does not discriminate between 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$. Most enzymes involved in vitamin D metabolism also do not discriminate between D_2 and D_3 or its metabolites except for some non-CYP2R1 25-hydroxylases (94).

The affinity between DBP and its major metabolites has usually been studies by using [^{3}H]labeled vitamin D metabolites and either diluted serum or purified DBP. We also estimated the affinity (Ka) in rat serum as the ratio of "on rate" over "off rate" by measuring the association and dissociation rate constant (95). The dissociation rate constant was highly temperature dependent, being about 1 day at 4°C and between 2 and 12 min at 37 and 24°C, respectively. The half-association time was about 1 min at 4°C and even more rapid at higher temperature. The best estimation of the Ka based on these rate constants was very similar to the Ka measured by direct binding assay, charcoal precipitation of free ligands and Scatchard plot analysis. As the affinity is dependent on temperature, pH, buffer conditions, and methodology, variable data have been generated but there is general agreement that human DBP has a very high affinity for 25OHD, with a Ka of around 1.5×10^8 M^{-1} (34, 88) at 37°C. Whether the different isoforms of DBP have different affinities for 25OHD is a matter of debate as one group (96) concluded that DBP/GC2 has only half of the affinity of DBP/GC2s and this isoform had again only half the affinity of DBP/GC1f. Three other laboratories, however, independently, could not find significant differences in affinity for 25OHD when measured by Scatchard plot using highly labeled [^{3}H]25OHD rather that vitamin D_3 itself used by Arnaud and Constans (34, 60). When we compared the affinity of 20 human samples, only a very minor difference in affinity was found with a Ka of 1.63 ($\pm$0.29) $\times 10^{-10}$ M for GC1s homozygotes compared to 2.11 ($\pm$0.25) $\times 10^{-10}$ M for GC1f, and intermediate values for DBP/GC2 homozygotes (79). The rank order of these relatively small differences was, moreover, different when the measurements were done in a phosphate buffer compared to a Hartman buffer (both at pH 7.4) (33). A rare variant of DBP/GC (GC Aborigine or GC1A1) as found in Aboriginals and some blacks from South Africa (48) have similar Ka values (88). The affinity of DBP from different species as measured in the author's laboratory is shown in **Table 3**. The highest affinity for 25OHD is found in serum from rats [confirmed by (49)] and 5 species of amphibians (79). Lowering the pH rapidly decreased the affinity (to very low levels at pH 5), but the affinity increased at pH 8–10 with the highest values at about pH 8.6 (about 10-fold higher in mammalian DBP but not in DBP from chicks or toads). The affinity of DBP for 1,25$(OH)_2$D is about 1.5×10^7 M^{-1}, with little difference between DBP/GC genotypes and only a minor increase in affinity at higher pH (88). The affinity of purified DBP from human, rat, and chick serum was very similar to that measured in (highly) diluted serum. The variation of the assay results are about 25% of the prefix of the Ka value. When comparing methods to separate bound and free, charcoal or filter assays generated similar results (67, 88, 97). The affinity of DBP for 1,25$(OH)_2$D is about 30–50-fold lower than for 25OHD in all species studied so far. The Ka at pH 7.4 and 4°C is about 1-5-2 $\times 10^7$ M^{-1} in most species but about 10 times higher values were measured in diluted rat (49, 88) and amphibian sera (**Table 3**). In chicks (*Galllus domesticus*), the Ka of DBP for 25OHD at 4°C and pH 7.4 is 1×10^9 M^{-1} (33). Bouillon et al. could not find a specific vitamin D3-binding protein as described before by Edelstein et al. (33, 98). Indeed, labeled vitamin D behaved similarly to labeled 25OHD on simple and crossed immunoelectrophoresis of chick DBP. In DBP-free serum, both metabolites were bound to albumin.

DBP and Concentration of Unbound Vitamin D Metabolites: Free or Bioavailable D Metabolites

As the DBP has a high affinity for most vitamin D metabolites, and also has a much higher molar concentration compared to its ligands, the free concentration of all its metabolites is very low, in fact much lower than that of other ligands of nuclear receptors. The free concentration of 25OHD and 1,25$(OH)_2$D were first calculated[1] based on the measurements of total concentrations of the metabolites, total concentration of DBP, the best estimation of the affinity and the law of mass action (13, 14). Free hormones such as cortisol, thyroxin, and

[1]The formula used to calculate free hormone concentration usually refers to Vermeulen et al. (99). In reality, however, the real inventor of the formula is Walter Heyns from the Leuven laboratory of endocrinology (see his formula for calculating free cortisol (100) who taught Vermeulen's assistant (L. Verdonck), during a working visit in Leuven, how to use his formula to calculate free testosterone concentrations. The Vermeulen group later independently validated this calculation by comparing the results with direct measurements of free testosterone (Walter Heyns and Alex Vermeulen, personal communication 2018).

TABLE 3 | Affinity of DBP from different species for the major vitamin D metabolites*.

Species	25OHD	$1,25(OH)_2D$	Comments
Human	5×10^8 M^{-1}	1.5×10^7 M^{-1}	Variation between 3 and 10 $\times 10^8$, depending on buffer and DBP/genotype
Monkeys	5×10^8 M^{-1}	1.5×10^7 M^{-1}**	Measured in old and new world monkeys. In *Cebus albicans*, DBP migrates with albumin mobility but has a similar Ka
Rat	6×10^8 M^{-1}	1.4×10^8	Measured in three strains
Rabbit	1.5×10^9 M^{-1}	NM	
Chick	4×10^8 M^{-1}	1.7×10^7 M^{-1}	
Reptiles	NM	NM	
Amphibia	2–14 $\times 10^{10}$ M^{-1}	$2.6 \times 10^8 M^{-1}$	Measured in 5 species

Measured in diluted serum at pH 7.4 and 4°C, unless specified otherwise; all values are expressed as Ka at M^{-1} values.

***The Ka for $1,25(OH)_2D$ varied between 1.2 and 2.6 $\times 10^7$, depending on buffer and DBP/GC genotype.*

NM, not measured.

sex hormones are usually a better marker of their biological activity than their total concentrations (101). These free hormone concentrations are also better feedback-controlled than the total hormone concentrations. The best example is that of extreme deficiency of transport proteins of one of these ligands, resulting in a dramatic decrease in total but keeping free hormones within the normal range. Is this also applicable to the major vitamin D metabolites? Two different terminologies are frequently used for free hormones: some define free hormone as non-protein (either to the specific binding protein and albumin) bound hormone, whereas bioavailable hormone is the combination of free and loosely albumin-bound hormone.

The first major difference with other transport proteins is the combination of high affinity and very high concentration of DBP so that the free concentration of 25OHD and $1,25(OH)_2D$ are very low in absolute and relative concentrations. The free 25OHD and $1,25(OH)_2D$ concentrations are about 10 and 1 pmol/l (14). This also means that the free 25OHD concentration is <0.1% of total 25OHD and that free $1,25(OH)_2D$ is about 1% of its total concentration. Due to difference in affinity, the molar ratio of total 25OHD over total $1,25(OH)_2D$ is about 500 but free 25OHD is only 10 times higher than free $1,25(OH)_2D$. In **Figure 5**, a few formulae used to calculate the concentration of free vitamin D metabolites are shown. All formulae use the law of mass action as published by Vermeulen et al. (99) (see also footnote 1) for androgens and by Coolens et al. (100) for cortisol. The simplest way to express free hormone (vitamin metabolite) concentration is a calculation of the free hormone/metabolite as a molar ratio of molar concentration of hormone/metabolite over molar concentration of its binding protein (DBP). As DBP has such a high capacity, the concentration of the apoprotein is indeed virtually also close to that of the total DBP concentration. Such molar ratio of $1,25(OH)_2D$:DBP is nearly identical ($r >$ 0.95) to that of calculated free $1,25(OH)_2D$ (14).

Free 25OHD is not feedback-regulated in humans nor in animals. Increased intake of vitamin D or higher sun exposure increase total as well as free 25OHD concentrations, and vitamin D deficiency does not change DBP concentrations whereby total and free 25OHD concentrations decrease to about the same degree. In most studies, increase in DBP by estrogen intake does not increase serum 25OHD (14, 34). Whether free 25OHD is a better predictor than total 25OHD for health outcomes is controversial and will be discussed elsewhere in this Journal and has recently been reviewed several times (34, 60). Free 25OHD can be calculated based on DBP concentrations and the affinity of DBP for 25OHD but the DBP assay is not standardized yet and the exact value of the Ka value at 37°C is also not perfectly known (see above). Therefore, the predictive value of calculated free 25OHD is yet unknown. Free 25OHD can also be directly measured by ultrafiltration or dialysis (43, 102) or by ELISA (103). Dialysis and ultrafiltration are technically difficult and face problems due to interference of adsorption of very low vitamin D metabolites to glass or plastic tubes, and even a minute impurity of labeled metabolites represent concentrations potentially higher than the free metabolites. In normal subjects, the directly measured free 25OHD concentration has a strong correlation with the calculated free concentrations (taking into account a variation coefficient of about 10% of all measurements). In the MrOs study on elderly men, calculated free 25OHD (using polyclonal DBP assays) correlated strongly with directly measured free 25OHD (r = 0.80–0.83) (22, 59). Moreover, the calculated or measured free 25OHD concentration strongly depended on total 25OHD (r = 0.96 and 0.83, respectively). Of course, as for other free hormones, the direct measurements are especially important in case of abnormal concentrations or affinity of the binding proteins. This aspect has not yet been fully evaluated for free 25OHD. Free $1,25(OH)_2D$ concentration probably "behaves" more like free thyroxin, cortisol or sex steroid hormones. Indeed, the total $1,25(OH)_2D$ concentration correlates with DBP concentrations in most studies whereas this is not the case for total 25OHD. Increased DBP by estrogen exposure or early pregnancy increases DBP and total $1,25(OH)_2D$ very similarly, so that the free concentrations remain unchanged (14). Similarly, in sexually mature hens, DBP and $1,25(OH)_2D$ increase several fold but $1,25(OH)_2D$ increases more than the DBP so that the free $1,25(OH)_2D$ concentration is high, in line with the high calcium demands for egg shell calcification. When, however, egg shell calcification is blocked by a thread in the uterus, soft eggs are produced, calcium requirements drop, and total and free $1,25(OH)_2D$ fall back to the level of immature chicks (104). Infusion of large amounts of human DBP in blood of normal rats, increases combined rat and human DBP concentrations and also increases total $1,25(OH)_2D$ concentrations (67). Total absence of DBP in mice results in very low (nearly undetectable) total $1,25(OH)_2D$ concentrations but the tissue concentration of free $1,25(OH)_2D$ is normal (86) and serum calcium and PTH status remain normal. In the only human case of biallelic mutation of the DBP/GC gene, resulting in undetectable serum DBP concentrations, total $1,25(OH)_2D$ was nearly undetectable but without consequences for calcium or PTH homeostasis (23). Also animal studies came to the same conclusion as rabbits immunized against $1,25(OH)_2D$-conjugates as to induce antibodies for immunoassays have a more than

General principle

According to the law of mass action of interaction between 25OHD and DBP the free 25OHD levels should reflect the total 25OHD concentration ⇌ Free 25OHD + apo-DBP

Calculated free concentration depends on:

- DBP concentration (assay dependent)
- DBP affinity

Bouillon's equation for free $1,25(OH)_2D_3$ but also applicable to free 25OHD by using different value of affinity

$$K = \frac{[1,25\,.\,DBP]}{[1,25]_{free} * [DBP]_{free}} \quad (1)$$

$$[1,25]_{free} = \frac{-(K * DBP^t - K * 1.25^t + 1) + \sqrt{(K * DBP^t - K * 1,25^t + 1)^2 - 4K * 1,25^t}}{2K} \quad (2)$$

since $\begin{cases} DBP^t \sim DBP\ Free \\ 1,25^t \sim 1,25\ bound \end{cases}$

$$[1,25]_{free} = \underbrace{\frac{[1,25]_{total}}{[DBP]_{total}}}_{\substack{\text{Free } 1,25(OH)_2D_3 \\ \text{ratio or index}}} * \frac{1}{K} \quad (3)$$

Free $1,25(OH)_2D_3$ concentration

Powe's equation

$ax^2+bx+c=0$

Where:

x = $[25OHD_{free}]$ = the concentration of free 25OHD

a = KDBP· Kalb· [Alb] + KDBP

b = KDBP· $[DBP_{total}]$ – KDBP·$[25OHD_{total}]$ + Kalb· [Alb] +1

c = – $[25OHD_{total}]$

For subjects homozygous for Gc1F variant, KDBP = 1.12 x 10 E9 M-1

for Gc1S variant, KDBP = 0.60 x 10 E9 M-1

for Gc2 variant, KDBP = 0.36 x 10 E9 M-1

This polynomial may be solved for $[25OHD_{free}]$ using the quadratic equation

$$[25OHD_{free}] = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

$[25OHD_{bio}] = [25OHD_{free}] + [25OHD_{bound\ alb}]$

$= (Kalb· [Alb] + 1) \cdot [25OHDfree]$

where:

$[25OHD_{bio}]$ = concentration of bioavailable 25OHD

$[25OHDboun_{d\ alb}]$ = concentration of albumin-bound 25OHD

Kalb = affinity constant between 25OHD and albumin = 6 x 10E5 M-1

Bikle's Equation

Calculation of the free metabolite concentration is possible 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.

FIGURE 5 | Formulae to calculate the free concentration of 25OHD or $1,25(OH)_2D$ (14, 43, 54).

100-fold increase in total serum $1,25(OH)_2D$ concentrations without repercussions on calcium homeostasis (51). There are at present no publications reporting direct measurements of free $1,25(OH)_2D$. Whether free $1,25(OH)_2D$ is a better predictor than total $1,25(OH)_2D$ concentrations for health outcomes is unclear. In the MrOs study, free $1,25(OH)_2D$ concentrations were a better predictor of inflammatory markers than total 25OHD, total $1,25(OH)_2D$ or free 25OHD (105). As the concentration of DBP in maternal serum (at time of delivery) is about twice the concentration of DBP in cord serum, the maternal:neonatal ratio of DBP and vitamin D metabolites may be informative for the relative importance of free vs. total vitamin D metabolite concentrations. From previous genetic studies, it is well-documented that there is no transplacental transfer of DBP/GC (24). The total and free 25OHD concentrations show a high correlation between the maternal and fetal compartments, with *r* values usually above 0.6. The total 25OHD concentration is much higher in the mother than in the neonate but the free 25OHD is higher in cord serum. This probably reflects a good transplacental transport and a contribution of fetal 25-hydroxylase activity to the fetal vitamin D status. The correlation of total and free $1,25(OH)_2D$ between both compartments is somewhat lower than for 25OHD and the free $1,25(OH)_2D$ concentration in the fetus is slightly higher than in maternal serum. As fetal (and placental) $1,25(OH)_2D$ production may be partly responsible for the higher neonatal ionized concentration (and placental calcium transport), as shown in sheep by Care (106), such higher fetal $1,25(OH)_2D$ may reflect its biological role.

The most important implication of the free hormone hypothesis is that only the free or bioavailable hormone/ligand/vitamin D metabolite has access to the cytoplasm or nucleus of the cell. Indeed, cells have access to the total concentration of ligands bound to serum proteins when the cell uses specific receptors to internalize these complexes (**Figure 6**). As example, cholesterol carried by lipoproteins can be internalized by hepatocytes or other cells using specific LDL or HDL receptors and, therefore, these cells have access to the total concentration of cholesterol. "Free" or unbound cholesterol" is not discussed or used for clinical purposes. Other examples are shown in **Figure 6**. By contrast, most hormones enter cells by diffusion and only the unbound hormone enters most cells. This has clinical implications, as free hormone measurements are the preferred technique to estimate thyroid function. The real situation is probably slightly more complex as excess free ligands in the "nutrient type" of cellular import may influence cell function (e.g., iron overload). If cells express receptors for some binding proteins such as receptors for the sex steroid-binding protein or the presence of megalin in some cells, then such cell membrane receptors allow the import of the binding proteins (e.g., DBP) with all its ligands (see below). For some hormones, specific membrane carriers (such as membrane transporters for thyroid hormones) further complicate the model. For most situations, however, the free hormone model is physiologically and clinically relevant. We think that the DBP∗$1,25(OH)_2D$ complex belongs to the hormonal type of serum transporters as only free $1,25(OH)_2D$ influences cell function. Its free concentration in the picomolar range comes close to the affinity of VDR for its major ligand. For 25OHD, the question is different and highly relevant. Indeed, the free 25OHD concentration is around 10 pmol/l and this seems low in comparison with the affinity of the major CYPs responsible for its further metabolic activation. This raised the question whether (free) 25OHD has real access to CYP27B1 or CYP24A1 in many cells and thereby has functional implications. The role of megalin in the kidney tubuli will be discussed below (chapter "DBP binds megalin"). Most other cells do not express or have only minimal expression of megalin. Probably the best method to explore the functional role of 25OHD in the local production of $1,25(OH)_2D$ can be found by deletion or overexpression of CYPs (CYP27B1 or CYP24A1) in specific tissues and expose such mice/animals to variable serum concentrations of 25OHD and or DBP. Tissue specific deletion of CYP24A1 in mammary cells had a clear effect on the risk of breast cancer (107). Overexpression of CYP27B1 in bone cells seemed to be beneficial for bone (108, 109) and deletion of this gene in growth plate chondrocytes generated bone effects as long as the growth plate remained fully active (110). Therefore, from these few examples, it seems that 25OHD has sufficient access to some target cells and generate local actions without full understanding how such low free or bioavailable concentrations can activate these genes and cells.

When comparing four different species (humans, rats, guinea pigs, and sheep) and calculating free $1,25(OH)_2D$ based on total serum $1,25(OH)_2D$ and DBP concentrations in the maternal and neonatal compartments, free $1,25(OH)_2D$ is higher in the fetus/neonate than in their mothers. The concentrations of total and free $1,25(OH)_2D$ concentrations show a good correlation in all these species (111) so that it is likely that the fetal production of $1,25(OH)_2D$ contributes to this gradient and maybe also to the greater concentration of calcium in the fetus compared to the maternal calcium concentrations. Whether bioavailable (= sum of free and ligands loosely bound to albumin) is more important than free ligands is not fully understood and has been discussed but not "solved" in other reviews.

Whether the free hormone hypothesis also applies to the vitamin D endocrine system can be demonstrated by *in vivo* data as summarized above but also by dedicated *in vitro* studies. Addition of purified DBP or serum to culture media followed by measuring the biological response of cells or tissue to either 25OHD or $1,25(OH)_2D$ has repeatedly shown that the presence of DBP impairs the biological action, whether using the expression of a natural gene/protein or using a genetically engineered gene construct. As examples:

1. The proliferation of human lymphocytes, cultured in a DBP free medium, can be inhibited by 50% by $1,25(OH)_2D$ at 10^{10-12} concentrations, but required 100-fold higher molar concentrations if purified DBP is added at the physiologic concentration of 5 μmol/l (112). DBP counteracted the action of several vitamin D analogs with the same rank order as predicted on the basis of their affinity for DBP.
2. Resorption (measured by release of radioactive calcium) of bone tissue explants (forelimb bones from 18d old fetal rats, *in vivo* exposed to radioactive calcium) cultured in the absence

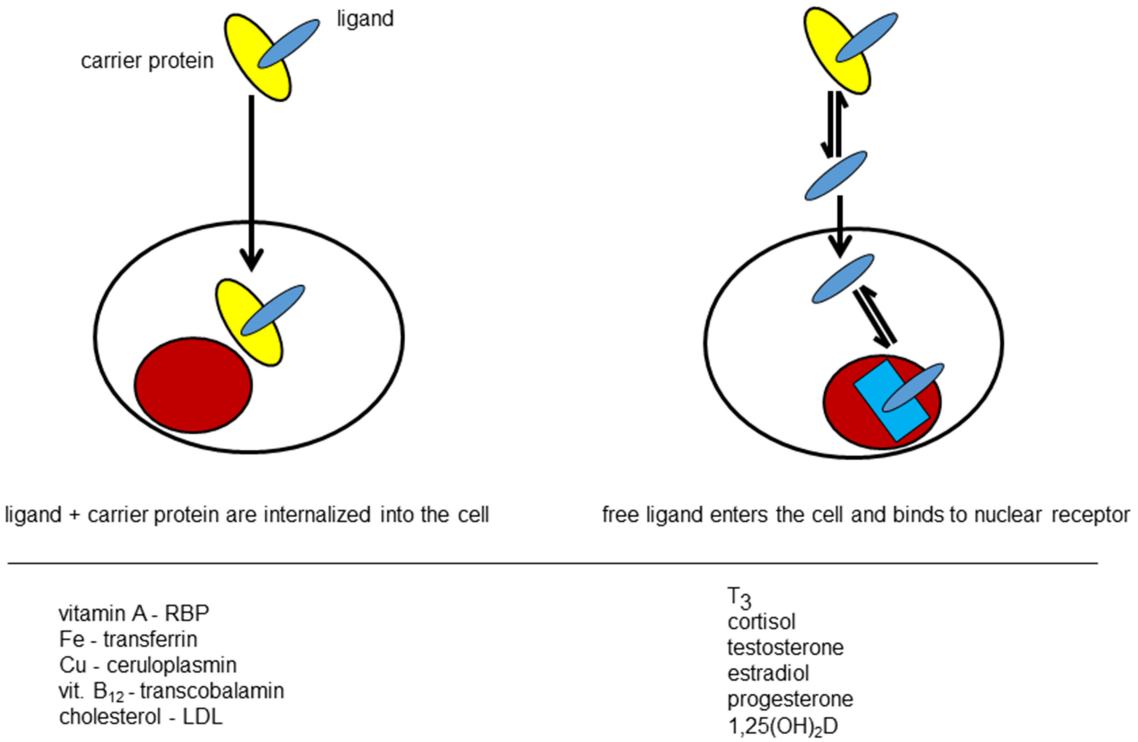

FIGURE 6 | Model of nutrient and hormonal type of access of extracellular ligands to cells.

of DBP is stimulated by low concentrations of $1,25(OH)_2D$. Adding purified rat or human DBP (5 μmol/l) impaired bone resorption but rat DBP was much more potent, in line with its 6–10-fold higher affinity for $1,25(OH)_2D$ (113).

3. Cathelicidin, a well-known target of the vitamin D hormone, is stimulated by $1,25(OH)_2D$ or 25OHD in human monocytes, cultured in serum from DBP null mice. Adding serum from normal mice however impaired this effect. The authors did not observe a megalin-mediated action (see below) (114).
4. Human foreskin keratinocytes express mRNA for CYP24A1 when exposed to $1,25(OH)_2D$. This action was markedly inhibited by culturing these cells in the presence of diluted serum compared to an albumin only based medium. Similarly, the production of radioactive $24,25(OH)_2D$ from $[^3H]$25OHD was inhibited by DBP (115).
5. Dermal fibroblasts from a patient with biallelic mutations of the DBP/GC gene or from normal volunteers were exposed to exogenous 25OHD, either in the presence of 10% normal serum containing DBP or 10% DBP deficient serum. The uptake of 25OHD in all cells was markedly inhibited by the presence of human DBP (23).

All these experiments clearly demonstrate that the presence of DBP decreased the access and actions of vitamin D metabolites in a wide variety of cells.

DBP Binds to Megalin-Cubilin Receptor

Patients with nephrotic syndrome loose massive amounts of serum proteins in their urine, exceeding the liver capacity to replace the daily loss. DBP has a smaller molecular weight than albumin and is lost together with 25OHD and other vitamin D metabolites. This renal loss of 25OHD frequently results in a poor vitamin D status as first reported in 1977 (71) and subsequently confirmed in many other studies. Megalin (global or kidney-specific) knockout mice are unable to reabsorb several proteins in the renal tubuli as it is a cargo receptor for several serum proteins, allowing to prevent their urinary loss (15). Megalin is a membrane receptor and requires cubilin as co-receptor (116), and both are expressed in several tissues but mainly in the luminal brush border membrane of the renal tubuli. Megalin is a giant protein of 600 kDalton and belong to a family of low-density lipoprotein receptors. Therefore, it is also known as LRP2. This protein was initially identified as autoantigen in an experimental model of membranous nephropathy, called Heymann nephritis (117). Megalin is a well-conserved gene/protein, already present in nematodes. In mammals, the expression of LRP2/megalin is restricted to specialized absorptive epithelia in the brain, the eye, the lung, the kidney, and the reproductive tissue. Therefore, the major phenotype of a global megalin knockout is characterized by major developmental brain abnormalities,

usually even lethal. Megalin binds and internalizes a large number of low molecular weight proteins, responsible for transporting a variety of lipids, vitamins, and hormones. This includes DBP, the retinol-binding protein, and transcobalamin. Thereby, megalin-mediated clearance of these binding proteins from the tubular lumen serves to retrieve essential vitamins and hormones bound to these carriers and to prevent uncontrolled loss of essential metabolites by glomerular filtration. More than 20 other proteins, including thyroglobulin (118) are also known to be ligands of these membrane receptors (119). In the absence of one of these co-receptors (megalin or cubilin), many low molecular weight proteins are lost in the urine, known as tubular proteinuria. Loss of these receptors can be due to a variety of acute, chronic or genetic diseases. In such diseases, DBP is also lost in the urine together with 25OHD and this loss of vitamin D metabolites may exceed the daily supply and synthesis of vitamin D and therefore cause vitamin D deficiency and rickets (15). This also implies that the renal tubuli, where CYP27B1 is transforming 25OHD into $1,25(OH)_2D$, has access to 25OHD by either the serosal site (and thus mainly access to non-DBP bound 25OHD), or via the luminal site (where DBP in complex with 25OHD is internalized by the megalin-cubilin receptor complex). Thereafter, reabsorbed proteins are broken down in lysosomes, leaving the luminal 25OHD available for metabolic activation. Megalin is then recycled to the luminal membrane. This phenomenon does not imply that the megalin pathway is essential [as suggested by some expert reviewers (120)] for the renal synthesis of $1,25(OH)_2D$, as megalin-deficient mice do not develop rickets if the urinary loss of vitamin D metabolites is compensated by sufficient intake. Neither do DBP-deficient mice develop rickets if they have access to sufficient vitamin D. Because the megalin receptor is a cargo receptor for a large number of proteins, loss of one of these membrane receptors can cause a variety of deficiencies, apart from vitamin D deficiency. Whether megalin- or cubilin-mediated uptake of DBP is also operational in other cells, such as placenta or parathyroid gland is insufficiently documented so far. However, the gene expression of megalin outside the kidney, brain, and eyes, is usually very low. The lung may be an exception and is now known to be an important target for vitamin D action (121). As discussed above, such transport mechanism, if operational outside the kidney, would expose cells expressing these cargo receptors to DBP bound 25OHD rather than free 25OHD.

DBP Binds Actin

About 50 years ago, three major binding proteins were known, the serum binding protein, now identified as DBP/GC, the intracellular/nuclear VDR (now known to be a member of the nuclear transcription factors), and an intracellular 6S binding protein (MW about 90,000 Daltons), with preferential binding for 25OHD and which was considered as a cellular "receptor" for 25OHD (122, 123). This protein was found in most tissues but also in whey and milk of humans and mammals. In 1977, however, we demonstrated that the 6S 25OHD-binding protein was in fact a complex of DBP with an ubiquitous cytosolic heat-labile compound that was by itself unable to bind vitamin D metabolites (11). This phenomenon was confirmed by using antibodies against DBP (11, 124). A few years later, while trying to identify this intracellular compound by adding different proteolytic enzymes and DNAase, Van Baelen et al. (125), found that the addition of DNAase created a triple complex DBP-unknown intracellular protein-DNAase. Based on this observation, the Leuven group identified actin as the cytosolic protein that binds with high affinity to create the so-called intracellular 25OHD-binding protein/receptor. Actin-DBP binding does not involve the presence or absence of vitamin D ligands, or vice versa. This was confirmed by resolving the 3-dimentional structure of DBP-actin by three different groups (126–128).

Many actin-binding proteins regulate or control intracellular actin polymerization or disassembly, including profilin (129). The extracellular binding proteins are, however, mainly DBP and gelsolin (also known as brevin). Profilin in also present in platelets and can act as an actin sequestrant but with a 1,000-fold lower affinity and therefore of probably minor extracellular importance (130). Gelsolin-actin forms a 2-1 complex as gelsolin is able to sever actin polymers and then one gelsolin binds and caps each filament fragment. Together with profilin and gelsolin, DBP creates a well-coordinated strategy with complementary mode of action as to rapidly remove actin (polymers). These observations have several implications. First, DBP is not found inside cells when plasma contamination is carefully avoided during tissue preparation. Thus, it is rather unlikely that extracellular DBP interferes with the key functions of intracellular actin. Second, actin-DBP complexes only happens when intracellular actin is released in the bloodstream. Indeed, Van Baelen et al. (125) clearly showed that DBP was able to depolymerize polymeric actin. Actin can easily switch from monomeric into polymeric actin in the cytosolic milieu, thereby helping in building the intracellular organizations of cells. In the plasma milieu, actin monomers are rapidly transformed into polymeric structures, which could result in clogging the microcirculation much like fibrinogen/fibrin. This process could be accelerated by the aggregation of platelets as actin-ADP binds to platelet surfaces and acts as agonist for platelet aggregation (131). DBP, however, is able to build a one-to-one complex with actin and together with other actin severing/binding proteins (e.g., brevin) in serum, allows to prevent the formulation of actin polymers in the circulation (74, 125). These proteins have different roles in actin depolymerization by either severing or capping the actin chains and creating actin-DBP complexes that are more rapidly cleared from the circulation that DBP alone (74). Actin and DBP are very tightly bound (Ka of $\sim$2–5 $\times$ 10^8 M^{-1}) (125, 132) so that these complexes are de facto not dissociated once formed in the circulation. However, actin-DBP is rapidly cleared from the circulation with a half-life of $<$1 h in rabbits (74). The damage caused by actin polymerization has been directly demonstrated in DBP null mice (133). Actin infusion in such mice resulted in more severe acute lung inflammation (vascular leakage, hemorrhage, and thickening of the vascular wall) compared with normal mice. This was confirmed *in vitro* as lung endothelial cells, exposed to DBP-actin complexes generated enhanced cell death (133). Third, the 3-D structure shows all three domains of DBP being in direct contact with

actin and interacting through a variety of contact mechanisms. The substantial surface areas from both DBP and actin that are involved in intimate protein-protein contacts explains the high affinity interaction between these two proteins. Moreover, the DBP residues involved in actin binding are less well-conserved between all species with known DBP structures than the vitamin D-binding domain. The contact surface is greater than the contact surface of actin with two other actin-binding proteins (gelsolin and profilin) together (126–128). These data confirm the actin-scavenger role of DBP in higher vertebrates. However, DBP from some fish and amphibians are unable to bind to actin and create an actin-DBP complex. The molecular reasons for that absence of actin interaction are so far unknown. Fourth, DBP-actin interaction can be used to isolate DBP from several species, as actin can be used for affinity chromatography when no species-specific antibodies against DBP are available. In clinical or research situations, the presence of actin-DBP complexes in serum or the (low) DBP concentration in serum can be used a marker for the disease severity and has prognostic significance in patients with severe illness (134). The greatest decrease in serum DBP has been found in patients with acute/fulminant hepatic necrosis (whereby serum DBP may fall to about 10% of normal values) rhabdomyolysis, sepsis, or major traumas (135–137).

DBP Binds Fatty Acids

Triglycerides are bound in serum to several lipoproteins whereas free fatty acids are mainly loosely bound to albumin. However, all members of the albuminoid family are able to bind fatty acids. DBP binds to fatty acids present in membrane phospholipids (see DBP and inflammation) of leucocytes but also bind fatty acids, and especially poly-unsaturated fatty acids (16:1, 18:1, 18:2, and 20:4) are associated with DBP/GC as measured by gas chromatography (17). When [^{3}H]arachidonic acid was added to serum about 75% was found to be bound to DBP. The molar ratio of fatty acids/DBP is about 0.4 compared to 1.8 for albumin. Mono- and polyunsaturated fatty acids impair the binding of 25OHD and 1,25$(OH)_2$D to DBP. Indeed, high concentrations (36 μmolar) of linoleic or arachidonic acid decreased the apparent affinity two to five-fold. Their effect was much greater for inhibition of binding of 1,25$(OH)_2$D than for 25OHD (18). Saturated fatty acids and many other lipid soluble serum substances do not interfere with the binding of DBP to vitamin D metabolites. Physiologic concentrations of unsaturated fatty acids may thus impair the binding of 1,25$(OH)_2$D to DBP (18). Whether this transport of fatty acids plays a real role in energy or vitamin D transport is not known.

DBP and Inflammation

DBP has no direct effects on inflammation. Its ligand, 1,25$(OH)_2$D has many immune and inflammatory effects [reviewed in (34, 35)] and DBP inhibits the cellular entry of 1,25$(OH)_2$D. DBP, however, is able to bind to membranes and chondroitin sulphated proteoglycans of leucocytes. In association with annexin A2, this binding enhances complement C5a-stimulated chemotactic activity. Quiescent neutrophils do not bind DBP and binding is dependent on prior neutrophil activation. As this activity is linked to activated leucocytes, it may play a role in local inflammation in response to cell damage by whatever mechanism (19, 138, 139). A more extensive discussion on this action of DBP has been published (34, 140) and no further data have recently been published on that topic.

DBP and Macrophage Activating Factor (MAF)

Several *in vitro* studies suggested that the deglycosylation of DBP can activate DBP to become a macrophage activating factor (MAF). A membrane bound β-galactosidase present on the surface of immune B cells and a sialidase present on T cells would remove two of the three sugar residues of DBP/GC1 protein (141). This DBP would then be able to facilitate the differentiation of monocytes to become osteoclasts and even correct the osteopetrosis phenotype of mice (142). In other circumstances DBP-MAF would activate macrophages in their battle against cancer cells or infections (143, 144). A few clinical trials tested the potential effects of DBP-MAF against HIV infections or cancer. Most of the data on DBP-MAF comes from Yamamoto's laboratory but he had to retract several major papers because of irregularities in the documentation of his data after institutional review [retracted manuscripts included (145, 146)]. Moreover, the availability of nagalase to act as endoglycosylase (and thus to deglycosylate DBP) has been questioned (147). Therefore, there is serious doubt on the existence and role of DBP-MAF despite a large number of publications [reviewed in (34, 148)].

Polymorphisms of DBP and Disease

The polymorphisms of DBP have been associated with susceptibility or resistance to a large number of chronic conditions, such as osteoporosis (149–151), type 1 and type 2 diabetes (152), thyroid autoimmunity (153), inflammatory bowel disease (154), and chronic obstructive lung disease (155). The exact role of DBP in the pathophysiology of these diseases is however not completely understood [see (156, 157) for an extensive discussion].

In summary, GC/DBP has two major functions:

1) *The transport of all vitamin D metabolites (at a single binding cleft of the A domain of the protein). Due to the combination of high affinity for vitamin D metabolites and a high protein concentration, free concentrations of all vitamin D metabolites are extremely low.*
2) *Tight binding of actin, creating a DPB-Actin complex that avoids actin polymerization in serum after tissue damage.*
3) *Other possible functions of GC/DBP need further validation.*

SUMMARY AND PERSPECTIVES

Vitamin D and all its metabolites are bound to a specific vitamin D binding protein, DBP. This discovery was made independently by studying the electrophoretic mobility of antirachitic activity or radiolabeled vitamin D (metabolites) and by studying the polymorphism of a major serum protein called Group-specific Component (GC). We now know that DBP and GC are the same protein and appeared early in the evolution of vertebrates.

TABLE 4 | Major remaining research problems and questions.

1	International standard for (gene-specific) DBP and reference method for the measurement of DBP
2	Validation of measurement of free 25OHD and its potential clinical value in comparison with total 25OHD
3	Validation of measurement or calculation of free $1,25(OH)_2D$ and its potential physiologic and clinical implications
4	Understanding of the environmental drive (and health implications) for the world-wide gene polymorphism of DBP/GC
5	Full understanding of the implications of actin-DBP binding in health and diseases
6	Understanding of the role of DBP in inflammation
7	Role of megalin and DBP-25OHD uptake, if any, in non-renal cells

DBP is genetically the oldest member of the albuminoid family (including albumin, α-fetoprotein, and afamin, all involved in transport of fatty acids or hormones). Some fish use lipoproteins as a sole transport protein but many other fish and (nearly) all amphibia, reptiles, birds, and mammals use DBP. This protein has a single binding site for all vitamin D metabolites and has a high affinity for 25OHD and $1,25(OH)_2D$. This allows creating a large pool of circulating 25OHD, which prevents rapid vitamin D deficiency when the supply of new vitamin D is compromised. DBP also regulates the access of all vitamin D metabolites to cells and tissues. In birds and mammals, DBP combines a high affinity with high concentration, whereas its concentration in early vertebrates is much lower. DBP of higher vertebrates (not amphibians or reptiles) binds with very high affinity actin, creating a 1:1 DBP:actin complex, and thereby preventing the formation of polymeric actin fibrils in the circulation after tissue damage. Megalin is a cargo receptor and is expressed in many epithelial cells, including the luminal border of the renal tubuli. Megalin and cubilin are needed to reabsorb DBP or the DBP-25OHD complex (and many other proteins and their ligands), filtered in the renal glomerulus, thereby preventing the urinary loss of these proteins and 25OHD. Absence of one of these receptors results in rickets, unless the daily supply of vitamin D is able to replace its excessive urinary loss. The total concentrations of 25OHD and $1,25(OH)_2D$ in DBP null mice or humans are extremely low (at the limit of detection) but their free concentrations in serum and tissues are probably normal. This does not impair the action of the vitamin D endocrine system on gene expression or tissue action, and therefore is the strongest argument for claiming that the "free hormone hypothesis" also applies to the vitamin D hormone, $1,25(OH)_2D$. The relative importance of free 25OHD compared to total 25OHD is not yet settled. The ongoing standardization of DBP assays and the validation of a direct ELISA for free 25OHD should allow generating the data needed to answer this question in the near future. DBP also transports fatty acids, and unsaturated fatty acids compete with vitamin D metabolites, thereby decreasing the apparent affinity of DBP for 25OHD and especially $1,25(OH)_2D$. DBP can bind to proteoglycans present in the membrane of immune cells and thereby enhance complement C5a-stimulated chemotactic activity of activated neutrophils. This role in inflammation is not fully understood. DBP null mice and a single woman with total absence of DBP are healthy and do not have a clinical phenotype related to vitamin D transport or actin, megalin, or complement binding. DBP is genetically very polymorphic with three frequent alleles (DBP/GC 1f, 1s, and 2) but in total more than 120 different variants. There is a clear North-South gradient of this polymorphism but its health consequences, if any, are not understood. There is a need for standardization of assays for serum DBP as to allow the studies needed to further explore the role of DBP in physiology and diseases. Despite the enormous progress in the deciphering of the structure of DBP and its function, there remains an impressive list of major research questions (**Table 4**).

AUTHOR CONTRIBUTIONS

RB: design and overall writing of the manuscript. FS: genetic origin of DBP and writing of several chapters of manuscript. LA: polymophism of DBP and overall correction of manuscript and references. FR: structure-function analysis of DBP and overall correction of manuscript.

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