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Behind the scenes of vitamin D binding protein: More than vitamin D binding

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Although being discovered in 1959, the number of published papers in recent years reveals that vitamin D binding protein (DBP), a member of the albuminoid superfamily, is a hot research topic. Besides the three major phenotypes (DBP1F, DBP1S and DBP2), more than 120 unique variants have been described of this polymorphic protein. The presence of DBP has been demonstrated in different body fluids (serum, urine, breast milk, ascitic fluid, cerebrospinal fluid, saliva and seminal fluid) and organs (brain, heart, lungs, kidneys, placenta, spleen, testes and uterus). Although the major function is binding, solubilization and transport of vitamin D and its metabolites, the name of this glycoprotein hides numerous other important biological functions. In this review, we will focus on the analytical aspects of the determination of DBP and discuss in detail the multifunctional capacity [actin scavenging, binding of fatty acids, chemotaxis, binding of endotoxins, influence on T cell response and influence of vitamin D binding protein-macrophage activating factor (DBP-MAF) on bone metabolism and cancer] of this abundant plasma protein.

Abbreviations: AFM, α-albumin/afamin; AFP, α-fetoprotein; ALB, albumin; AUC, area under the curve; CKD, chronic kidney disease; CSPGs, chondroitin sulfate proteoglycans; DBP, vitamin D binding protein; DBP-MAF, vitamin D binding protein-macrophage activating factor; ELISA, enzyme-linked immunosorbent assay; F-actin, filamentous actin; G-actin, globular actin; GWAS, genome-wide association study; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemotactic protein-1; NGAL, neutrophil gelatinase-associated lipocalin; RAAS, renin-angiotensin-aldosterone system; RIA, radioimmunoassay; RID, radial immunodiffusion; ROC, receiver operating characteristic; SNPs, single nucleotide polymorphisms; TSP-1, thrombospondin-1.

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Introduction

Vitamin D binding protein (DBP) is a sparsely glycosylated (0.5–1%) alpha$_2$-globulin with a molecular weight of 52–59 kDa [1–3]. Being a member of the multigene cluster that includes albumin (ALB), α-fetoprotein (AFP), and α-albumin/afamin (AFM), the human *DBP* gene is localized at 4q11-q13 on the long arm of chromosome 4 [1,2]. All four genes show a predominantly hepatic expression with overlapping developmental profiles [2]. They share a homologous three-domain structure, defined by the invariant positions of cysteine and the nearly identical disulfide bridge patterns [4], and have a conserved position of introns within the coding region [5]. The *DBP* gene is the most divergent member of the albuminoid superfamily, which probably arose by the triplication of the ancestral gene with a 192 amino acid sequence [6]. This gene is separated by at least 1500 kb from the other 3 genes and is composed of 458 amino acids. It lies in a head-to-head configuration with and has an inverted transcriptional orientation as ALB/AFP/AFM [2]. The general characteristics of DBP are summarized in Table 1.

The 3 major circulating DBP alleles (DBP1F, DBP1S, DBP2) are defined by the genetic polymorphisms rs7041 and rs4588 [7]. DBP1 and DBP2 differ from each other by four amino acids (152 Gly → Glu, 311 Glu → Arg, 416 Asp → Glu and 420 Arg → Thr) and by the attached carbohydrates [8]. DBP1F and DBP1S have an identical primary structure, except at position 416, where aspartic acid is substituted by glutamic acid. The partial glycosylation of DBP1S comprises a linear O-linked trisaccharide of the type GalNAc-Gal-Sia attached to the threonine residue at position 420 [3,9]. Besides the three common alleles, a large number (>120) of unique racial variants [10] and single nucleotide polymorphisms (SNPs) of DBP have been described [11]. The geographical variation in the DBP allele frequencies is associated with skin pigmentation and relative sun light exposure. Populations with a pale skin are characterized by a relatively lower frequency of the DBP1F allele and a higher frequency (50–60%) of the DBP1S allele. The DBP1F allele frequency is high among populations of African ancestry, whereas Caucasians have a markedly higher DBP2 allele frequency [12].

DBP is composed of three structurally similar domains with a C-terminal truncation of the third repeat. The first domain has the characteristic α-helical arrangement, which allows for binding of vitamin D$_3$ ligands. The vitamin D binding site is composed of hydrophobic residues of helices 1–6 (amino acids 35–49). This binding site at the N-terminus of DBP is a cleft located at the surface of DBP, whereas the vitamin D binding site of the vitamin D receptor is a closed pocket in the inner structure of the receptor. The second domain is similar, but a coil folding has replaced helix 7 and in the third domain only helices 1–4 are present [13]. The acting binding site is located between amino acids 373–403, spanning parts of domains 2 and 3 [14], whereas also a part of domain 1 interacts with actin [15]. Finally, C5a/C5a des Arg binding (amino acids 126–175) and plasma membrane binding domains (amino acids 150–172 and amino acids 379–402) have been identified [16].

Table 1
General characteristics of vitamin D binding protein (DBP).

	Characteristics	Reference
Family	Albuminoid superfamily, consisting of serum albumin, alpha-fetoprotein, afamin (alpha-albumin, vitamin E binding protein), and vitamin D binding protein	[1,2]
Gene localization	Long arm of chromosome 4 (4q11-q13)	[1,2]
Polymorphism	DBP1F, DBP1S, DBP2 and >120 unique racial variants	[7,10]
Molecular mass	52-58kD	[1–3]
Presence in body fluids and organs	Body fluids: serum, urine, breast milk, ascitic fluid, cerebrospinal fluid, saliva, seminal fluid and on the surfaces of lymphocytes, neutrophils and monocytes Organs: brain, heart, lungs, kidneys, placenta, spleen, testes and uterus	[19,20]
Analytical aspects	Radioimmunoassay (RIA), rocket immuno-electrophoresis, single radial immunodiffusion (RID), turbidimetry, nephelometry, enzyme-linked immunosorbent assay (ELISA), proteomics, glycoproteomics Lack of an international standard	[21–26]
Serum concentration	300–600 mg/L acute injury or sepsis: a decrease in the serum DBP concentration of 50–120 mg/L	[27,28]
Half-life	Actin-free DBP: 12–24 h, actin-bound DBP: ±30 min	[75]

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Diverse physiologically important properties have been attributed to DBP (Fig. 1). First of all, circulating vitamin D metabolites are mainly transported bound to DBP and albumin is the major secondary carrier, especially in patients with a low serum DBP concentration [17]. However, as only 1–2% of its sterol binding sites are utilized, multiple additional metabolic roles beyond vitamin D transport have been described for DBP: actin scavenging, modulation of inflammatory processes and innate immunity, binding of fatty acids and influencing bone metabolism. As we described already the interesting relationship between *DBP* polymorphisms and susceptibility to diseases [18], the purpose of this review was to give an overview of the current knowledge and evidence of the fundamental biological functions of DBP, illustrated by some examples in human pathologies.

Analytical aspects and clinical significance of vitamin D binding protein

The presence of DBP has been demonstrated in serum, urine, breast milk, ascitic fluid, cerebrospinal fluid, saliva, seminal fluid and on the surfaces of lymphocytes, neutrophils and monocytes. Differential

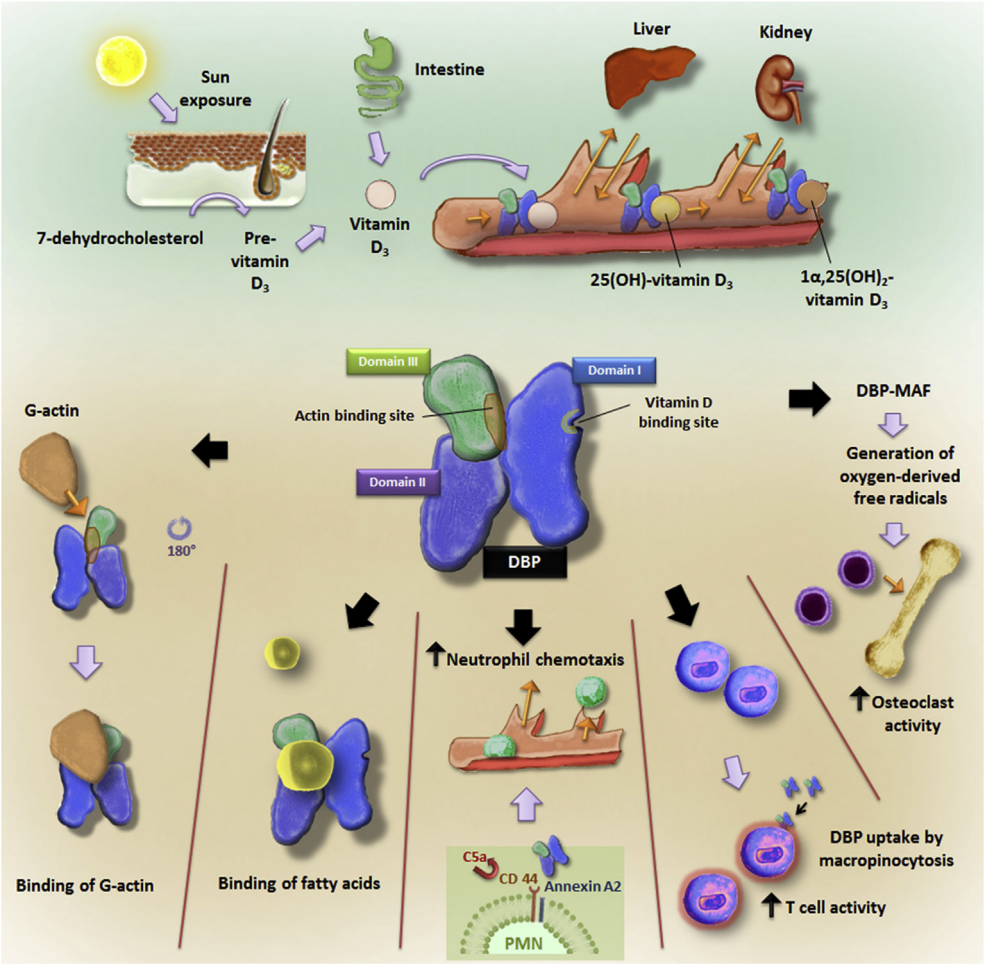

Fig. 1. Overview of the different physiological functions of vitamin D binding protein (DBP): binding of vitamin D metabolites, actin scavenging, binding of fatty acids, chemotaxis, influence on T cell response and influence of vitamin D binding protein-macrophage activating factor (DBP-MAF) on bone metabolism.

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mRNA expression has been reported in brain, heart, lungs, kidneys, placenta, spleen, testes and uterus [19,20]. The estrogen dependent synthesis of DBP is fulfilled by the hepatocytes. In comparison with blood, where the highest concentration of DBP is found, lower expression levels have been detected in other body fluids [20]. The relatively high serum concentration of DBP is well suited for immunochemical techniques. Earlier methods included radioimmunoassay (RIA), rocket immunoelectrophoresis and single radial immunodiffusion (RID). In recent years, turbidimetry, nephelometry and enzyme-linked immunosorbent assay (ELISA) have been used [21–23]. Immunonephelometry offers the advantage to combine a short assay time, an ease of use, a high sensitivity and specificity [21]. So far, an international standard for DBP is lacking. Recently, novel techniques such as proteomics and glycoproteomics have illustrated the potential value of DBP and vitamin D binding protein-macrophage activating factor (DBP-MAF) in a wide spectrum of pathologies (e.g. acute myocardial infarction [24], visceral leishmaniasis [25], and Alzheimer's disease [26]).

Serum vitamin D binding protein

DBP is predominantly expressed in serum at a concentration of 300–600 mg/L in healthy subjects [27]. In acute injury or sepsis, a decrease in the serum DBP concentration of 50–120 mg/L is observed [28]. A total deficiency of DBP has never been described in humans [29]. Changes in the level of sun exposure due to lifestyle and migration to nonequatorial latitudes or in skin characteristics might have resulted in stronger selective pressure on DBP [30]. DBP levels are stable in blacks and non-blacks, and do not change with correction of vitamin D deficiency [31]. The amount of DBP in blood is characterized by a diurnal rhythm with a decline in the morning, followed by a rapid increase during the day [32]. No age-related differences in serum DBP concentration have been reported. The anticoagulant used during sample collection has no significant influence on the test results [23]. However several other factors have an influence on the serum concentration of DBP. A 5-fold difference in the mean serum DBP concentration is present among the 3 common DBP phenotypes (with the lowest level in DBP 2-2 subjects, a higher level in DBP 2-1 subjects and the highest level in DBP 1-1 subjects), which may be explained by variations in production or metabolic rate of DBP in the different isoforms. A faster metabolic rate of DBP in DBP 2-2 subjects is observed in comparison with the sialylated DBP in the DBP 1-1 group [33]. A genome-wide association study (GWAS) of 1380 men, through linear regression of SNPs in the Illumina HumanHap500/550/610 array on fasting serum DBP, identified 2 independent SNPs located in the *DBP* gene, that were highly associated with the serum DBP concentration: rs7041 and an intronic SNP rs705117 [11]. For both SNPs, mean serum DBP concentration decreased with increasing copies of the minor allele. DBP was also associated with rs12144344 in the gene *ST6GALNAC3*, which is an accepted proxy of rs4588. Besides the influence of the DBP polymorphisms, the circulating DBP concentration is characterized by several racial/ethnic or genetic differences. Lower serum DBP levels are detected in subjects with a West African genetic ancestry. Serum DBP concentrations are also associated with the catabolic ratio of serum vitamin D [percent 24,25$(OH)_2$-vitamin D_3 (positive association)], oral contraceptive use (positive association) [16], body mass index (positive [34–36] and negative associations [16]) and lipid parameters [triglycerides, total cholesterol, LDL-cholesterol (positive association)] [37]. Finally, DBP is downregulated by a factor 3 in long-term smokers ($\geq$10 pack years) in comparison with nonsmokers [38].

Urinary vitamin D binding protein

Urinary DBP excretion, determined by ELISA, can be regarded as a tubulointerstitial inflammation and fibrosis marker. An increased urinary DBP concentration has been documented in microalbuminuric subjects and in non-diabetic chronic kidney disease (CKD) patients with overt proteinuria compared to normoalbuminuric subjects. In a small group of 52 non-diabetic CKD patients, urinary DBP excretion responded to intensification of renoprotective therapy with a dual renin-angiotensin-aldosterone system (RAAS) blockade and with a low sodium intake. However in the group of normoalbuminurics, the urinary excretion of DBP was still >100-fold lower than in the intensive treated group of CKD patients with persisting tubulointerstitial damage. Urinary DBP was associated with tubular and inflammatory damage markers such as kidney injury molecule-1

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(KIM-1), beta-2-microglobuline, cystatin C, monocyte chemotactic protein-1 (MCP-1) and neutrophil gelatinase-associated lipocalin (NGAL), independently of albuminuria [39]. The early detection and prevention power of urinary DBP for the diagnosis of diabetic nephropathy has also been evaluated. Urinary DBP levels (corrected for creatinine) were significantly elevated in diabetics showing microalbuminuria and macroalbuminuria, compared with those showing normoalbuminuria and normal controls ($P < 0.001$). Receiver operating characteristic (ROC) analysis of urinary DBP levels for the diagnosis of diabetic nephropathy rendered a cut-off value of 552 ng/mg, corresponding to a 93% sensitivity, an 85% specificity and an area under the curve (AUC) of 0.966 [40].

Increased urinary DBP levels have also been found in endometriosis, using proteomic techniques and mass spectrometry. This finding may be related to the typical systemic subclinical inflammatory process of this disease [41]. Estrogen and interleukin-1 are involved in the pathogenesis of endometriosis and influence the hepatic synthesis of DBP [42,43]. However, the potential value of urinary DBP as a diagnostic marker for endometriosis is limited [41].

Vitamin D binding protein in cerebrospinal fluid

DBP has been detected in the cerebrospinal fluid of healthy subjects with a limited passage of DBP through an intact blood-brain-barrier and a restricted passage of vitamin D-DBP complexes [44]. In patients with multiple sclerosis, two-dimensional fluorescence difference in-gel electrophoresis followed by mass spectrometry has shown that the determination of DBP in cerebrospinal fluid could serve as a specific diagnostic biomarker of progressive disease [45]. In addition, proteomics studies have reported decreased DBP levels in cerebrospinal fluid in patients with relapsing-remitting multiple sclerosis in comparison with subjects with other neurological diseases [46].

Vitamin D binding protein in ascitic fluid

The presence of DBP in ascitic fluid may be explained by the hydrostatic pressure gradient between plasma and the peritoneal cavity [47]. The determination of DBP in peritoneal fluid could be useful to monitor vitamin D deficiency in peritoneal dialysis patients. Due to a change in the permeability of the peritoneal membrane to middle-sized proteins or leakage of DBP from peritoneal inflammation, DBP losses in the peritoneal fluid have been demonstrated by two-dimensional gel electrophoresis [48]. In the peritoneal dialysate effluents of peritoneal dialysis patients with high transporter characteristics of the peritoneal membrane, a significant higher amount of DBP has been detected in comparison with subjects with other transporter characteristics [49]. Due to the loss of DBP in the ascitic fluid, a decreased serum DBP concentration could lead to alterations in the serum 25(OH)-vitamin D_3 concentration [48]. However, this statement has been criticized by another group, who showed that the peritoneal loss of DBP was not accompanied by a serum DBP deficiency due to an adapted hepatic synthesis [49].

Vitamin D binding protein: what's in a name?

As the main function of the initially unnamed serum protein, referred as group-specific component of serum (Gc-globulin), was binding, solubilization and transport of vitamin D and its metabolites, the name was changed into DBP. In comparison with vitamin D metabolites, the serum concentration of DBP is 20-fold higher, which results in a 5% occupation of the binding sites on DBP by vitamin D sterols [50]. This large molar excess of DBP has probably several potential roles: (1) protection against vitamin D toxicity and (2) acting as a buffer/reservoir for 25(OH)-vitamin D_3 [50,51]. The majority of circulating 25(OH)-vitamin D_3 (88%, $Ka = 5 \times 10^{-8}$ M) and 1,25$(OH)_2$-vitamin D_3 (85%, $Ka = 4 \times 10^{-7}$ M) is more tightly bound to DBP [51] than to albumin [25(OH)-vitamin D_3 ($Ka = 6 \times 10^5$ M^{-1}) and 1,25$(OH)_2$-vitamin D_3 ($Ka = 5.4 \times 10^4$ M^{-1}) [52]. The remaining fraction of vitamin D is bound to albumin (10–15%), and <1% of circulating vitamin D exists in an unbound form. The free hormone hypothesis states that protein-bound hormones are inactive, while unbound hormones are free to exert biological activity [53]. However in recent years, the free hormone hypothesis has been criticized as the serum

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concentration of free 1,25(OH)$_2$-vitamin D$_3$ ($\pm 10^{-13}$ M) is much lower that the concentration bound to the vitamin D receptor (Kd = 10^{-10} M) [54].

The preservation of serum 25OH-vitamin D$_3$ levels and the activation of 25(OH)-vitamin D$_3$ to 1,25(OH)$_2$-vitamin D$_3$ is regulated by the megalin mediated endocytosis of DBP-bound 25(OH)-vitamin D$_3$ in the proximal tubular cells of the kidney after filtration in the glomerulus [55]. This process is facilitated by cubilin [55] and disabled-2 (DAB2) [56]. After denaturation or proteolysis of DBP in endocytic vesicles, 25(OH)-vitamin D$_3$ acts as a substrate for CYP27B1 in the kidneys for the synthesis of 1,25(OH)$_2$-vitamin D$_3$ [57]. In contrast to the rapid turnover rate of free DBP, a limited access to target cells is reported for DBP-bound metabolites. Those complexes are less susceptible to hepatic metabolism and subsequent biliary excretion, which results in a prolonged half-life time [11]. The internalization of DBP by extrarenal tissue may be accomplished by megalin-mediated endocytosis as well as by a megalin-independent mechanisms, as observed in B-lymphocytes [58].

Among the three common DBP phenotypes, DBP1F has a greater affinity for and a more efficient transport of vitamin D metabolites [12]. Racial differences in the prevalence of common genetic polymorphisms (rs7041 and rs4588 in the *DBP* gene) provide a likely explanation for the lower 25(OH)-vitamin D$_3$ and DBP concentrations, and similar concentration of estimated bioavailable 25(OH)-vitamin D$_3$ [defined as free 25(OH)-vitamin D$_3$ plus that which is bound to albumin] observed in black Americans in comparison with whites [58]. A positive correlation is found between the serum DBP concentration and the 1,25(OH)$_2$-vitamin D$_3$ level [31,59].

Besides rs7041 and rs4588, explaining only 9.9% of the 25(OH)-vitamin D$_3$ levels, genome-wide meta-analysis has identified four SNPs, affecting 25(OH)-vitamin D$_3$ concentrations: rs2282679 (*DBP*), rs10741657 (near *CYP2R1*), rs12785878 (near *DHCR7*) and rs6013897 (at *CYP24A1*) [60,61]. As reported above, *DBP* represents the transport system of vitamin D metabolites, whereas *CRP2R1*, *DHCR7* and *CYP24A1* are involved in the vitamin D metabolic pathway [60,62]. Another genome-wide association study of 4501 subjects of European ancestry identified the association between rs2282679, in linkage disequilibrium with rs7041 and rs1155563, and the 25(OH)-vitamin D$_3$ concentration. Suggestive signals for association were also found in the following genes: *NADSYN1* (rs3829251), *DHCR7* (rs1790349) and *CYP2R1* (rs2060793) [62]. In 33,996 individuals of European descent, participants with a genotype score [combining three confirmed variants influencing 25(OH)-vitamin D$_3$ levels: rs2282679, rs12785878 and rs10741657] in the highest quartile were at increased risk of having 25(OH)-vitamin D$_3$ concentrations < 75 nmol/L or < 50 nmol/L, compared with those in the lowest quartile [60]. In Southern Chinese women, rs2282679 was associated with serum 25(OH)-vitamin D$_3$ levels and vitamin D insufficiency, whereas rs12785878 was nominally associated with vitamin D insufficiency only. The AUCs of rs2282679 and the genotype risk score were 0.561 and 0.576, respectively [62]. In Japanese rheumatoid arthritis patients, rs2282679 in the *DBP* gene was associated with lower serum 25(OH)-vitamin D$_3$ concentrations and this SNP could be a risk factor for hip fracture [63]. In a population of Chinese postmenopausal women, the variants of rs2298849 in the *DBP* gene were significantly associated with the serum 25(OH)-vitamin D$_3$ levels ($P < 0.001$) with a protective role for allele G. Among the haplotypes of rs222020–rs2298849, a positive association was found between CG and the serum 25(OH)-vitamin D$_3$ concentrations [64]. Haplotype analysis revealed that in European and Asian populations, the major DBP haplotypes carry alleles had opposite effects at rs4588 and rs2282679. In Asian populations, rs2298850 and rs11723621 were in strong linkage disequilibrium with rs4588-A, which was associated with increased and decreased levels of DBP and 25(OH)-vitamin D$_3$, respectively. Nonetheless, these variants were also in linkage disequilibrium with the C allele of rs2282679, identified in GWASs as the strongest association signal for lower 25(OH)-vitamin D$_3$ and serum DBP concentrations in Europeans [57].

As the plasma concentration of total 25(OH)-vitamin D$_3$ is considered as an indicator of the actual vitamin D status, DBP and its polymorphism should be taken into account in the interpretation of the 25(OH)-vitamin D$_3$ levels, as it could have consequences for the interpretation of blood results and the treatment in different pathologies [50]. Vitamin D deficiency is a recognized comorbidity in patients with diabetes, particularly associated with the presence of diabetic nephropathy [65]. Decreased serum DBP concentrations have been reported in diabetes type 1, although the exact consequence of this finding remains unknown until now [66]. Vitamin D deficiency or insufficiency is slightly more prevalent in diabetic subjects with albuminuria, coincident with the increase in urinary DBP excretion.

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As mentioned previously, exaggerated urinary loss of DBP could contribute mechanistically to vitamin D deficiency in this disease [65]. Using multivariate regression modeling, significant correlates of urinary DBP excretion included microalbuminuria, glycated hemoglobin, average capillary glucose and serum 1,25$(OH)_2$-vitamin D_3 concentrations [65]. However, urinary DBP loss was not associated with serum DBP or 25(OH)-vitamin D_3 and 1,25$(OH)_2$-vitamin D_3 levels, suggesting that urinary loss of DBP did not affect the vitamin D status [67]. A recent meta-analysis showed a moderate association of the DBP polymorphism with increased susceptibility to diabetes type 2 in Asians, but not in Caucasians. The following potential underlying reasons for this association have been proposed: (1) impact on the metabolite of vitamin D, affecting the amount and activity of vitamin D in the β cell, (2) impact on fatty acids, which may induce β cell abnormalities, (3) immunomodulation of DBP(-MAF) with influence on several cytokines and (4) variations in a closely linked gene on chromosome 4q12 [68]. The altered expression of DBP has also been observed in other autoimmune diseases with underlying vitamin D (un)related working mechanisms such as rheumatoid arthritis and [69] and granulomatosis with polyangiitis [26]. However in another Mendelian randomization study, DBP had no demonstrable causal effect on calcemic (osteoporosis and hyperparathyroidism) and cardiometabolic diseases (hypertension, type 2 diabetes, coronary artery disease and stroke) [70].

Vitamin D binding protein: more than just vitamin D binding?

The role of vitamin D binding protein in the extracellular actin scavenger system

Being the most abundant and highly conserved protein inside all eukaryotic cells, large quantities of actin are released into the circulation during extensive tissue damage and cell death. Besides monomeric globular actin (G-actin), extacellular polymerized filamentous actin (F-actin) is formed in association with coagulation factor Va, triggering disseminated intravascular coagulation and multiple organ dysfunction syndrome [71]. To counteract these procoagulant effects, the intravascular actin scavenging system, consisting of gelsolin and DBP, cleaves actin and inhibits repolymerization [72]. Gelsolin severs and depolymerizes actin filaments, whereas DBP is able to inhibit novel filament formation due to its high affinity (Kd = 10 nM) with G-actin and to sequester actin [73]. Functional studies that distinguish free from actin-bound gelsolin, based on the ability of the former to sever actin filaments, reveal that the binding of actin monomers to gelsolin is highly cooperative and can be prevented by prior incubation of actin with DBP, even though the apparent affinity of gelsolin for actin is 50-fold greater than that of DBP. The interaction of gelsolin with actin in cells and plasma may be regulated in part by actin monomer binding proteins. DBP-actin complexes do not bind to gelsolin. DBP removes one of the actin monomers in a 2:1 actin-gelsolin complex. DBP-actin complexes exist in blood plasma *in vivo* in the presence of free gelsolin [74] and the major DBP phenotypes have an equal actin binding affinity [12]. In comparison with the plasma half-life of actin-free DBP (12–24 h), actin-bound DBP is characterized by a plasma half-life of ±30 min [75]. Liver (parenchymal as well as non-parenchymal cells), lungs and spleen are responsible for the uptake of G-actin-DBP complexes by plasma membrane receptors [76]. Multiple studies have illustrated the important role of the actin scavenging system during severe sepsis [28,77], liver diseases [78,79], respiratory failure [80], pre-eclampsia [81,82], cardiac surgery [83] and after burn injuries [84].

The fatty acid-binding site environment of vitamin D binding protein

The shared physiologically function of all members of the albuminoid superfamily is the fatty acid binding capacity [85]. In comparison with albumin, a relatively weaker binding with fatty acids (Kd = 10^5–10^6 M^{-1}) has been demonstrated for DBP, which explains the fact that DBP plays only a contributory role in the transport of fatty acids. DBP has a single high-affinity fatty acid-binding site in comparison with the several low- and high-affinity binding sites of albumin [86]. The fatty acid-binding pockets of DBP and albumin have a different chemical/electronic environment. The fatty acid-binding site of DBP can only accommodate a polar and zwitterionic head group of palmitic acid, whereas a hydrophobic and hydrophilic head group at the carboxy-terminus are tolerated [85]. The molar ratio of fatty acids bound to albumin (1.8) is also much higher in comparison with DBP (0.4). Less

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than 5% of DBP-bound fatty acids are poly-unsaturated, in contrast to the majority of mono-unsaturated or saturated fatty acids. Arachidonic or linoleic acid, both poly-unsaturated fatty acids, compete with vitamin D metabolites [25(OH)-vitamin D_3 and 1.25$(OH)_2$-vitamin D_3] for DBP binding. This phenomenon is not observed with saturated fatty acids (e.g. palmitic acid) [87]. These results point towards a different binding and transport of various fatty acids between DBP and albumin [85].

The transport of endotoxins by vitamin D binding protein

Several endotoxin binding proteins have already been identified with the high-density lipoprotein fraction as one of the main carriers [88]. In the 1980s and 1990s, the endotoxin binding and inhibiting capacity of human DBP were also demonstrated [89–92]. In the *in vitro* limulus-amebocyte-lysate test, the inhibitory effect of DBP was dependent on its plasma concentration [92].

The role of vitamin D binding protein in chemotaxis

The recruitment of neutrophils to the inflammatory environment depends on a balance between (yet unrecognized) inhibitory and enhancing factors. Several *in vitro* [93–99] and *in vivo* studies [100] have demonstrated that DBP is involved in the complement-mediated tissue recruitment of neutrophils. Quiescent neutrophils do not bind DBP or enhance chemotaxis to C5a. During neutrophil activation, DBP binding sites are upregulated from a latent reservoir in azurophil granules [101]. A dual role of DBP in this immunological process has been proposed: (1) functioning as a direct positive regulator of neutrophil chemotaxis and (2) functioning as a neutralizer of endogenous inhibitors of chemotaxis [100].

As DBP by itself lacks chemotactic activity [101], the interaction of DBP with a binding site complex on the cell surface is essential for the chemotaxis enhancement of C5a. The formation of this binding site complex is a dynamic, multi-step and transient process, requiring cell activation and perhaps several distinct macromolecules [102,103]. DBP binds with low affinity to several cell surface ligands such as chondroitin sulfate proteoglycans (CSPGs) [102]. CD44 (a major cell surface CSPG on leukocytes) and the associated annexin A2 (a cell membrane Ca^{2+}/phospholipid binding protein) are part of the putative cell surface DBP binding site complex and mediate the chemotactic cofactor effect [104]. The binding capacity of DBP is not influenced by the C5a receptor (C5aR1/CD88)-ligand interactions [103,105]. DBP does not alter the neutrophil C5a receptor number or the Kd for C5a [97,106]. Neutrophil elastase may play a critical role in the C5a co-chemotactic mechanism, controlling the amount of DBP bound to cells, by shedding its binding site [103]. The chemotactic cofactor function of DBP is not specific for C5a and its stable degradation product C5a des Arg, as DBP can enhance the activity of other chemoattractants, including CXCL1, during inflammation [100]. In contrast to DBP, DBP-actin complexes are not chemotactic for and do not activate human neutrophils [107]. The binding of 1,25$(OH)_2$-vitamin D_3 to DBP abolishes the chemotactic cofactor function for human neutrophils [108] and oleic acid is one of the tonic inhibitors of chemotaxis in human plasma [109]. Due to its fatty acid binding capacity, DBP could scavenge the inhibitory oleic acid [100].

Besides its cochemotactic function, DBP augments the C5a-induced calcium influx in a direct way, without neutralizing an inhibitor. The C5a-induced chemotaxis and the C5a-mediated calcium influx by the DBP binding/signaling complex are facilitated by platelet-derived thrombospondin-1 (TSP-1), which binds to its cell surface receptors CD36 and CD47. Although there is no evidence that TSP-1 directly interacts with either annexin A2 or CD44, indirect associations between TSP-1 and CD44 have been proposed. One or more of the multiple ligands of both players could bridge TSP-1 and CD44, facilitating the DBP binding site complex [99].

The influence of vitamin D binding protein on T cell response

The *in vivo* influence of vitamin D on a given T cell response is complex and is probably dependent on a mixture of factors in addition to the 25(OH)-vitamin D_3 concentration: the local concentration and degradation rate of DBP, the different DBP phenotypes and the expression levels of the vitamin D

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receptor, CYP27B1 and the 1,25(OH)$_2$-vitamin D$_3$-24-hydroxylase CYP24A1 of the cells locally involved in the immune response [110].

Activated T cells take up DBP by macropinocytosis, which is a megalin-independent endocytosis. Although T cells express megalin, cubilin expression in naïve T cells is very low and is not upregulated following T cell activation [110]. In comparison with the megalin-mediated endocytosis in mammary cells and kidney cells [53,111], macropinocytosis of 25(OH)-vitamin D$_3$-DBP complexes by T cells is not followed by a conversion to 1.25(OH)$_2$-vitamin D$_3$. As the physiological concentration of 1,25(OH)$_2$-vitamin D$_3$ is not sufficiently high to affect T cell responses, a significant local production of 1,25(OH)$_2$-vitamin D$_3$ (>1000 pM) is required. The availability of 25(OH)-vitamin D$_3$ to T cells is influenced by the local concentrations and/or modifications of DBP. Inflammation-induced oxidative stress could locally lead to DBP carbonylation, impeding DBP-mediated inhibition of 25(OH)-vitamin D$_3$-induced T cell responses. The efficiency of 25(OH)-vitamin D$_3$-induced T cell responses is also influenced by the variable affinity for 25(OH)-vitamin D$_3$ of the different DBP phenotypes. *In vivo*, activated T cells interact with macrophages, which could lead to an efficient conversion of 25(OH)-vitamin D$_3$ to 1,25(OH)$_2$-vitamin D$_3$, despite the presence of DBP [110].

Vitamin D binding protein-macrophage activating factor and bone metabolism: the story is not yet completely unraveled

DBP can be converted by sialidase and beta-galactosidase treatment to DBP-MAF, as demonstrated in the original experiments with DBP-treated preparations. The concentration of DBP-MAF or O-(mono)-N-acetylgalactosaminated DBP can be determined by lectin-immunoassays [3,112] and depends partly on the DBP phenotypes [113]. Although demonstrated in earlier experiments, Ravnsborg et al. could not demonstrate an effect of DBP-MAF on cytokine release from macrophages/monocytes in the whole blood, probably due to the use of a different experimental model [3].

Current evidence of the role of DBP-MAF on bone health is limited. The effects of DBP on bone metabolism have been evaluated in only a few studies by one research group, focusing on osteopetrosis, which is a heterogeneous family of metabolic bone disorders with an increased skeletal mass due to a reduced osteoclastic bone resorption and different deficiencies in the cellular and humoral immune systems. In an animal model of two nonallelic mutations in the rat with generalized sclerotic bone, independent defects in the conversion from DBP to DBP-MAF were responsible for the enhanced bone resorbing capacity of the osteoclasts. In comparison with the *op* rats, the skeletal defects in the *ia* animals were corrected by infusions of 1.25(OH)$_2$-vitamin D$_3$ and interleukin-2. The exogenous administration of DBP-MAF had an even bigger beneficial effect on the skeletal structure in both *op* and *ia* rats in comparison with the active form of vitamin D, which might be explained by the upregulated oxidative metabolism (superoxide production) in the mutant cells (only demonstrated in the *ia* animals) [114]. Glycosylation plays an essential role for the osteoclast activating property of DBP-MAF [115], which results in the generation of oxygen-derived free radicals, stimulating bone resorbing cells [114]. Administration of a synthetic peptide fragment (consisting of 14 amino acids) derived from the human amino acid sequence at the site of glycosylation in the third domain of native DBP, to newborn rats (0.4 ng/g body weight) resulted in osteoinduction of the marrow cavity and osteogenesis of surrounding cortical and metaphyseal bone [116]. Those results could not be confirmed in an *in vivo* critical bone defect model, investigating the bone healing capacity of DBP [117]. However this study had several limitations. Although DBP-MAF exerts a stimulating effect on osteoclasts, the direct effect on the proliferation, differentiation or anabolic function of osteoblasts has not yet been demonstrated.

Vitamin D binding protein-macrophage activating factor therapy in cancer: the end of a fairy tale?

DBP-MAF is a naturally occurring protein capable of activating macrophages. Several studies and clinical trials have published extraordinary biological activities in the treatment of patients with breast-, colorectal- and prostate cancer as well as HIV [3]. However recently, the reports regarding immunotherapy with DBP-MAF have been retracted due to irregularities in the documentation for institutional review board approval [118]. As no difference has been documented in the concentration of glycosylated DBP forms between cancer patients and healthy subjects, and as DBP2 homozygotes are

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unable to glycosylate DBP on the threonine 420 residue, DBP-MAF will not have a strong impact on the occurrence of cancer in these patients [119,120].

Practice points

- There is increasing evidence that in the evaluation of the vitamin D status, the determination of the serum concentration of DBP should be taken into account. Only in this way, patients could more reliably be classified in those with or without vitamin D deficiency.
- The determination of DBP in other body fluids seems promising with the current available analytical techniques.
- DBP has much more properties than its sterol-binding capacity.
- Based on the current evidence, immunotherapy with DBP-MAF has no place in the treatment of cancer and HIV.

Research agenda

- The lack of an international DBP standard remains a concern for immunoassay manufacturers.
- With the introduction of new applications such as the omics family, future studies will probably further unravel the role of DBP in human pathologies.
- Investigations on the field of bone metabolism and immunology should be encouraged.

Conflict of interest

None.

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