Journal of
PERIODONTAL RESEARCH

J Periodont Res 2013; 48: 74–79

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2012.01505.x

Elevation of vitamin D-binding protein levels in the plasma of patients with generalized aggressive periodontitis

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Zhang X, Meng H, Sun X, Xu L, Zhang L, Shi D, Feng X, Lu R, Chen Z. Elevation of vitamin D-binding protein levels in the plasma of patients with generalized aggressive periodontitis. J Periodont Res 2013; 48: 74–79. © 2012 John Wiley & Sons A/S

Background and Objective: Vitamin D-binding protein (DBP) is a multifunctional and highly expressed plasma protein. Among its diverse roles, including those in the immune and inflammatory responses, it is the primary carrier of vitamin D, which has been implicated in periodontitis. We hypothesized that there is a correlation between systemic DBP levels and generalized aggressive periodontitis (GAgP).

Material and Methods: Forty-four patients with GAgP and 32 healthy controls were recruited. Clinical parameters were examined, including the mean bleeding index, probing depth, attachment loss and percentage of severely affected sites. Blood chemistry analyses were performed for each subject. Plasma levels of DBP, interleukin-6 (IL-6) and procalcitonin (PCT) were measured using ELISAs, and plasma levels of 25-hydroxy-vitamin D_3 ($25(OH)D_3$) were detected using a radioimmunoassay.

Results: Significantly higher levels of plasma DBP, IL-6, PCT and $25(OH)D_3$, as well as leukocyte counts, neutrophil counts and neutrophil percentages were found in patients with GAgP compared with healthy controls ($p < 0.05$ for all). Multiple linear regression analysis showed that the plasma DBP levels were significantly correlated with GAgP, plasma PCT levels and smoking status ($p < 0.05$ for all). In the GAgP group, the plasma DBP levels in smokers were significantly higher than those in nonsmokers ($p < 0.001$).

Conclusion: Elevated plasma vitamin DBP levels are associated with GAgP.

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Key words: generalized aggressive periodontitis; plasma; procalcitonin; vitamin D-binding protein

Accepted for publication June 6, 2012

Generalized aggressive periodontitis (GAgP) is a form of periodontal infection that mainly affects young people. It is characterized by the rapid destruction of periodontal tissues and can lead to early tooth loss (1). The aggressive nature and the early onset of the disease are dependent on many factors, including patient susceptibility to infection (2). It is well recognized that local periodontal infection can elicit a systemic response by the host and lead to increased levels of systemic inflammatory markers, including the leukocyte count, and C-reactive protein and interleukin-6 (IL-6) levels, some of which are predictive markers for systemic diseases (3–5).

Vitamin D-binding protein (DBP), also known as group-specific component, is a plasma α_2-globulin with a

molecular mass of between 52 and 59 kDa. It is expressed and secreted mainly by the liver. DBP is the major carrier of vitamin D and has been shown to have a direct effect on innate cell functions, including activation of macrophages, enhancing the chemotactic activity of C5-derived peptides and associating with the surfaces of immune cells, such as neutrophils (6,7). Furthermore, DBP is implied to be a positive acute-phase reactant produced by the liver. Its hepatic synthesis could be regulated by proinflammatory cytokines, including IL-6, as well as by hormones (8).

Besides, DBP scavenges actins, binds fatty acids and stimulates osteoclasts. It is a highly polymorphic serum protein, and many of the polymorphisms are associated with susceptibility or resistance to a spectrum of diseases, such as osteoporosis, thyroiditis, diabetes, chronic obstructive pulmonary disease, AIDS, multiple sclerosis, sarcoidosis and rheumatic fever (6). Variations of DBP levels in plasma are believed to be valuable markers for some diseases, including liver disease, sepsis, tuberculosis, cystic fibrosis, Type 1 diabetes and, more recently, ST-elevation myocardial infarction (9–14).

Recently, Wu *et al*. compared the proteomic profile of whole unstimulated saliva in patients with GAgP with those of healthy controls and identified 11 differentially expressed proteins. DBP was among the six proteins with elevated levels in patients with GAgP (15). It is worth noting that vitamin D, the major ligand of DBP, which has been shown to have immunomodulatory effects, is proposed to be associated with periodontal health (16). Previous research from our group revealed a relationship between 25-hydroxy-vitamin D_3 ($25(OH)D_3$) and GAgP. Specifically, we reported that the plasma levels of $25(OH)D_3$ were higher in patients with GAgP than in healthy controls, and were positively correlated with an index of gingival bleeding (17). It has also been shown that initial periodontal therapy reduces both local and systemic $25(OH)D_3$ levels (18).

In view of the roles of DBP in the immune system and in the transport of vitamin D, which is important in periodontal health, we hypothesized that there is a correlation between systemic DBP levels and GAgP. Therefore, the goal of the present study was to assess the DBP levels in plasma of patients with GAgP in comparison with healthy controls, and to evaluate their correlations with other inflammatory markers. The findings could provide us with an insight into the potential roles of DBP in periodontitis, and extend our knowledge regarding the association between periodontitis and general health.

Material and methods

Study population

Forty-four patients with GAgP were selected from the outpatient clinic of the Department of Periodontology at the Peking University Hospital of Stomatology, China. The diagnostic criteria for GAgP were defined according to the classification proposed at the International Workshop for the Classification of Periodontal Diseases and Conditions in 1999, specifically: periodontal disease onset before 35 years of age; at least eight teeth (three or more of which were not first molars or incisors) with a probing depth of > 6 mm; and radiographic evidence of alveolar bone loss. Thirty-two periodontally healthy controls were selected from the staff and students at the School of Stomatology. The inclusion criteria for the controls were: no site with a probing depth of > 3 mm or attachment loss; no bone loss on radiographs; and $< 10\%$ of sites with bleeding on probing. Subjects were excluded if they had a concomitant systemic disease, had undergone periodontal therapy within the previous year, had received antibiotics within the previous 3 months, were pregnant or were receiving vitamin D or calcium supplements (e.g. calcium carbonate, calcium lactate or calcium gluconate). Each subject completed a questionnaire at the beginning of the study, and age, height, weight, body mass index (BMI) and smoking status were also recorded. Smokers were defined as subjects who were currently smoking, and nonsmokers were defined as subjects who had never smoked or had ceased smoking. The study was conducted with the informed consent of all subjects, and the protocol was approved by the Ethics Committee of the Peking University Health Science Center.

Clinical examination

A full-mouth periodontal examination of each subject was conducted using a Williams periodontal probe. Probing depth and attachment loss were recorded for each tooth at six sites (mesial, distal and middle sites of both the facial and lingual sides), with the exception of the wisdom teeth. The bleeding index (19) was also recorded for each tooth. Sites with a probing depth of > 6 mm and attachment loss of > 5 mm were defined as sites of severe periodontitis. For each patient, a set of full-mouth peri-apical radiographs was taken.

Blood collection and assessment

Fasting venous blood samples were taken in a standardized manner. For each subject, blood cell analysis was performed using a hematology analyzer (SYSMEX KX-21; Sysmex, Kobe, Japan). The levels of DBP, IL-6 and procalcitonin (PCT) in plasma samples were determined using commercially available ELISA kits (DBP kit: BioSource Systems, Invitrogen, Grand Island, NY, USA; IL-6 kit: R&D Systems, Inc., Minneapolis, MN, USA; and PCT kit: USCNLIFE, Wuhan, HB, China). The assays were performed according to the manufacturers' protocols. The lower limits of detection for these assays were as follows: DBP, 7.81 μg/mL; IL-6, 0.16 pg/mL; and PCT, 3.9 pg/mL. Plasma $25(OH)D_3$ levels were detected using a commercially available radioimmunoassay kit (DiaSorin, Stillwater, MN, USA) with a lower normal limit of 3.75 nmol/L.

Statistical analysis

Variables were tested for normality and equal variances using the Shapiro–Wilk test and Levene's test,

respectively. Continuous normally distributed variables were reported as the mean values plus or minus standard deviation, whereas the median (lower to upper quartile) was used to describe non-normally distributed data. The unequal variance *t*-test (for plasma DBP and PCT levels) and the Mann–Whitney *U*-test (for age, BMI, clinical parameters, blood cell variables and plasma IL-6 and $25(OH)D_3$ levels) were used to identify any differences between groups. Gender and smoking status were compared using the chi-square test and Fisher's exact test, respectively. The Pearson rank correlation test (for normally distributed variables) and Spearman's rank correlation (for non-normally distributed data) were used to measure the association between the variables. Multiple linear regression analysis was performed using the plasma DBP level as the outcome. Statistical analyses were carried out using SPSS software, version 11.5 (SPSS Inc., Chicago, IL, USA), and *p*-values < 0.05 were considered statistically significant.

Results

The demographic and clinical variables of the two groups and analyses of the intergroup differences are shown in Table 1. There were seven current smokers in the GAgP group, whereas none of the healthy controls smoked. Subjects with GAgP had more higher clinical index values than controls ($p < 0.001$). No significant differences were found in terms of age, gender and BMI between the two groups.

The plasma DBP levels in the GAgP group were significantly higher than those in the control group (234.67 ± 49.51 μg/mL vs. 107.01 ± 21.85 μg/mL, respectively; $p < 0.001$). Higher leukocyte counts, neutrophil counts, neutrophil percentages, plasma IL-6 levels, PCT levels and $25(OH)D_3$ levels were found in the GAgP group compared with the control group ($p < 0.05$; Table 2).

The results presented in Table 3 show that the plasma DBP levels were positively correlated with smoking status, neutrophil count and neutrophil percentage, and plasma IL-6 and PCT levels ($p < 0.05$ for all). No association was found between plasma DBP levels and age, gender, BMI or plasma $25(OH)D_3$ levels ($p > 0.05$; Table 3).

A multiple linear regression analysis was used to explore, in greater detail, the relationships between plasma DBP and the variables evaluated. The diagnosis of GAgP, smoking status, mean probing depth, number of severely affected sites, neutrophil percentages, plasma IL-6 levels, PCT levels and $25(OH)D_3$ levels, as well as the potential confounders of age, gender and BMI, were adjusted for in this model. The results indicate that the diagnosis of GAgP, plasma PCT levels and smoking status were significantly correlated with plasma DBP levels ($p < 0.05$; Table 4).

In the GAgP group, the plasma DBP levels in smokers were significantly higher than those in nonsmokers (271.65 ± 11.22 μg/mL vs. 227.68 ± 50.89 μg/mL, respectively, $p < 0.001$; Fig. 1). Among the nonsmokers, the plasma DBP levels in the GAgP group were significantly higher than those in the control group (227.68 ± 50.89 μg/mL vs. 107.01 ± 21.85 μg/mL, respectively, $p < 0.001$; Fig. 1).

Table 1. Demographic and clinical variables

Variable	GAgP group ($n = 44$)	Control group ($n = 32$)	*p* value
Age (years)[a]	27.00 (23.25–30.00)	24.00 (23.00–26.00)	0.064
Gender (*n*; male/female)[b]	20/24	9/23	0.097
BMI (kg/m^2)[a]	20.60 (18.97–22.59)	21.10 (20.00–22.08)	0.777
Smoking status (*n*; smoker/nonsmoker)[c]	7/37	0/32	**0.019**
Bleeding index[a]	3.79 (3.35–4.00)	1.14 (1.10–1.19)	**< 0.001**
Probing depth (mm)[a]	4.73 (4.09–5.37)	1.41 (1.30–1.74)	**< 0.001**
Attachment loss (mm)[a]	4.47 (3.64–5.74)	0	**< 0.001**
Percentage of sites with severe periodontitis %[a]	29.88 (15.55–47.34)	0	**< 0.001**

Data are given as median (lower quartile–upper quartile), or number of subjects.
Between-group comparisons were performed using the [a]Mann–Whitney *U*-test, the [b]chi-square test or [c]Fisher's exact test.
Values in bold indicate a statistically significant difference ($p < 0.05$).
BMI, body mass index; GAgP, generalized aggressive periodontitis.

Discussion

In the present study, we found that the plasma DBP levels in patients with GAgP were significantly higher than those in normal control subjects and that the levels were not only associated with neutrophil counts and percentages but also with plasma IL-6 and PCT levels. These findings support our initial hypothesis and demonstrate a correlation between elevated plasma DBP levels and GAgP.

In addition, the plasma DBP levels were associated with disease severity. These findings are in accordance with those obtained from a small study on chronic periodontitis (20), which focused on localized DBP levels in salivary samples. Their results showed that the DBP levels in the whole saliva of patients with periodontitis were significantly higher than those of either dentulous or edentulous control subjects, and were correlated positively with the gingival index scores (21).

The plasma DBP levels were positively correlated with neutrophil counts and neutrophil percentages, as well as with plasma IL-6 levels; thus, the increased levels of neutrophils and IL-6 might contribute to the elevated plasma concentrations of DBP in patients with GAgP. This is reasonable considering the previous findings that DBP could be secreted by activated neutrophils (22) and that IL-6 could up-regulate the expression of DBP by

Table 2. Comparisons of the evaluated variables between the generalized aggressive periodontitis (GAgP) group and the control group

Variable	GAgP group (n = 44)	Control group (n = 32)	p value
DBP (μg/mL)[b]	234.67 ± 49.51	107.01 ± 21.85	**< 0.001**
Leukocytes ($\times 10^9$/L)[a]	6.15 (5.10–7.05)	5.10 (4.50–6.20)	**0.012**
Neutrophils ($\times 10^9$/L)[a]	4.05 (3.13–4.93)	2.90 (2.50–3.70)	**0.001**
Neutrophils (%)[a]	66.10 (58.98–71.23)	58.40 (53.20–63.38)	**< 0.001**
IL-6 (pg/mL)[a]	2.30 (0.32–4.11)	1.13 (0.07–1.03)	**< 0.001**
PCT (ng/mL)[b]	0.73 ± 0.39	0.35 ± 0.13	**< 0.001**
25(OH)D_3 (nmol/L)[a]	25.50 (15.63–37.50)	15.25 (6–27.25)	**0.006**

Data are given as mean ± standard deviation or median (lower quartile–upper quartile).
Between-group comparisons were made using the [a]Mann–Whitney U-test and the [b]unequal variance t-test.
Values shown in bold indicate a statistically significant difference ($p < 0.05$).
25(OH)D_3, 25-hydroxy-vitamin D_3; DBP, vitamin D-binding protein; IL-6, interleukin-6; PCT, procalcitonin.

Table 3. Correlations between plasma vitamin D-binding protein (DBP) levels and the variables evaluated in the study cohort (n = 76)

Variables	Plasma DBP levels	
	R (Zero-order correlation)	p-value
Demographic parameters		
Age	0.214[a]	0.064
Gender	−0.210[a]	0.081
BMI	0.140[a]	0.227
Smoking status	0.400[a]	**< 0.001**
Clinical parameters		
Mean bleeding index	0.785[a]	**< 0.001**
Mean probing depth	0.713[a]	**< 0.001**
Mean attachment loss	0.756[a]	**< 0.001**
Severely affected sites (%)	0.772[a]	**< 0.001**
Blood cells		
Leukocyte count	0.181[a]	0.121
Neutrophil count	0.303[a]	**0.008**
Neutrophil (%)	0.418[a]	**< 0.001**
Plasma biomarkers		
IL-6	0.404[a]	**< 0.001**
PCT	0.629[b]	**< 0.001**
25(OH)D_3	0.229[a]	0.061

Zero-order correlation analysis was performed using [a]Spearman's rank correlation analyses and [b]Pearson rank correlation analyses.
Bold numbers indicate a statistically significant difference ($p < 0.05$).
25(OH)D_3, 25-hydroxy-vitamin D_3; BMI, body mass index; IL-6, interleukin-6; PCT, procalcitonin.

Table 4. Results of multiple regression of associations between plasma vitamin D-binding protein (DBP) levels and the variables evaluated[a] in the study cohort (n = 76)

	Standard β	t-test	p-value
Diagnosis	0.613	9.06	**< 0.001**
Plasma PCT levels	0.307	4.66	**< 0.001**
Smoking status	0.199	3.46	**0.001**
Whole model	R^2 = 0.814	F = 90.65	**< 0.001**

[a]The variables evaluated in this model included the diagnosis of generalized aggressive periodontitis (GAgP), age, gender, body mass index (BMI), smoking status, mean probing depth, percentage of severely affected sites, neutrophil percentages, plasma interleukin-6 (IL-6) levels, procalcitonin (PCT) levels and 25-hydroxy-vitamin D_3 (25(OH)D_3) levels.
Values shown in bold indicate statistically significant differences ($p < 0.05$).

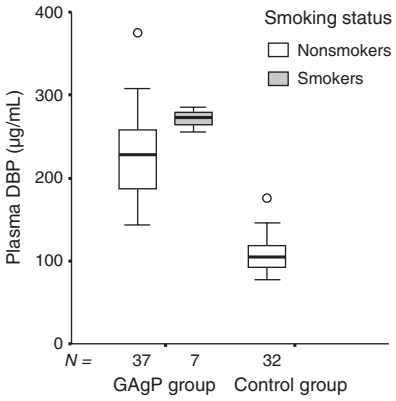

Fig. 1. Box-and-whisker plot illustrating plasma vitamin D-binding protein (DBP) levels in smokers and nonsmokers. In the generalized aggressive periodontitis (GAgP) group, the plasma DBP levels in smokers were significantly higher than those in nonsmokers (271.65 ± 11.22 μg/mL vs. 227.68 ± 50.89 μg/mL, respectively; $p < 0.001$). Among the nonsmokers, the plasma DBP levels in the GAgP group were significantly higher than those in the control group (227.68 ± 50.89 μg/mL vs. 107.01 ± 21.85 μg/mL, respectively, $p < 0.001$).

hepatocytes (8). In addition, the binding of DBP to neutrophil surfaces is considered to be necessary for the co-chemotactic activity of DBP, and these binding sites are reported to be up-regulated when neutrophils are activated by lipopolysaccharide (23,24).

In this study, the plasma 25(OH)D_3 levels in patients with GAgP were significantly higher than those in healthy controls. The plasma DBP levels and the 25(OH)D_3 levels showed a trend to be correlated, which implies a role for DBP as part of the vitamin D axis in periodontitis. The vitamin D axis, which includes vitamin D, vitamin D receptor and DBP, has recently received much attention in systemic diseases, such as lung diseases and diabetes (13,25). Further studies need to be carried out to obtain more details on the roles of the vitamin D axis in periodontitis. One explanation for the weak and nonsignificant correlation between plasma DBP levels and 25(OH)D_3 levels might be the far higher titers of DBP compared with vitamin D metabolites (6). In fact, besides binding vitamin D, DBP still

plays a part in the innate immune response, which is also indicated by our study.

PCT is an established serum marker of infection. It circulates at very low levels normally, but the levels increase dramatically in cases of systemic infection (26–28). The plasma PCT levels in patients with GAgP were found to be significantly higher than in healthy controls. To the best of our knowledge, this is the first report of an association between elevated plasma PCT levels and periodontitis. Although novel, this finding is not surprising considering that periodontitis is a bacterial infection. The high correlations found between plasma DBP levels and plasma PCT levels were, however, somewhat unexpected. Whether there is a direct link between these two proteins, or whether they are related through their individual association to other variables, is unknown and requires further investigation.

In the present study, smoking status was significantly different between patients with GAgP and healthy controls. There were no smokers in the control group. However, the relationship between plasma DBP levels and GAgP in nonsmokers was similar to that noted for the entire study cohort (as shown in Fig. 1). In the adjusted analysis that included smoking status as a covariate, plasma DBP levels were still correlated with the diagnosis of GAgP.

Smoking is believed to have several effects on the immune system and is recognized as one of the most significant risk factors for the development and progression of periodontal disease (29–33). In this study, smoking data were obtained from the questionnaires answered by the participants. From the results, in the patients with GAgP, higher plasma DBP levels were found in smokers compared with nonsmokers. With the limits of the small sample size and possible discrepancies in identifying the participants' true smoking status, we interpret this result to indicate that in subjects with GAgP, smoking might increase the plasma DBP levels. This finding strengthened our belief that DBP has a mechanistic role in the host immune response.

Most of the evidence for the function of DBP in the immune system and host defenses has come from animal studies and *in vitro* cell experiments. There has been relatively little work investigating the role of DBP in inflammatory diseases, despite the wealth of genetic studies. Possible changes in the levels of DBP in local and systemic fluids may provide us with information regarding the involvement of DBP in disease. In this preliminary study, we showed a relationship between elevated plasma DBP levels and GAgP, and revealed a high correlation between plasma DBP and plasma PCT levels. It is still unclear if DBP is simply a marker of the disease or reflective of a role for the protein in pathogenesis. Further studies are warranted to clarify this, as well as the mechanisms involved with the association of DBP with PCT.

Increased levels of serum DBP have been identified as novel indicators of coronary thrombosis in acute myocardial infarction (14). DBP has also been reported to reduce the inhibitory effect of aspirin on thromboxane A2 production when incubated with blood samples from healthy volunteers, and this inhibitory effect is the most important of the antithrombotic functions of aspirin (34). In the present study, we demonstrated a novel association between elevated plasma DBP levels and GAgP, which increases our knowledge on the associations between periodontal disease and general health. Periodontitis might be a risk factor for recurrent vascular events in patients who take aspirin therapy, as patients with periodontitis tend to have higher plasma DBP levels. A longitudinal study regarding how periodontal therapy influences plasma DBP levels will be essential to confirm the relationship between DBP and periodontal inflammation.

Acknowledgements

This work was supported by grants from the National Natural Science Foundations of China (nos 30271411, 30471882 and 30973319); the National Key Project of Scientific and Technical Supporting Programs of China (nos 2002AA217091 and 2007BAZ18B02); and the Clinical Research Fund, Ministry of Health P.R.C.

Competing interests

The authors declare that there are no conflicts of interest in this study.

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