Vitamin D Binding Protein Isoforms and Vitamin D Levels in Diabetes Patients

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ABSTRACT
Vitamin D binding protein (DBP) is the primary transport protein for the multiple forms of vitamin D in the body. Variations in the structure of DBP can affect the binding affinity with vitamin D, which can result in a vitamin D deficiency. Vitamin D deficiency is seen in various autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, and diabetes mellitus type 1 (DM1). The increasing prevalence of autoimmune disorders highlights the importance of identifying possible associations with deficient vitamin D serum levels. The objective of this research was to examine the relationship between the serum concentration of 25-hydroxyvitamin D and the concentration of the specific DBP isoforms in diabetic individuals. Vitamin D concentrations were measured using an EIA method, DBP concentrations were measured using an ELISA test, and the likely DBP isoform was determined using SNP TaqMan® analysis. Diabetic participants were compared to control participants. Allele frequencies were consistent with the standard European Ancestry reference population. A Mann Whitney U test revealed no significant difference among the DBP isoform values between the diabetic group and control population. Linear regression showed no correlation between DBP levels and vitamin D levels (R²=0.3402). There was no observed dosage effect in individuals having one or two copies of the mutant allele to the levels of DBP and vitamin D. DBP isoforms and concentrations of DBP had no effect on vitamin D concentrations in our DM1 testing population.

ABBREVIATIONS: DBP - Vitamin D binding protein, DM1 - Diabetes mellitus type 1

INDEX TERMS: Diabetes Mellitus, Type 1 Diabetes Mellitus, Vitamin D, Vitamin D Deficiency, Vitamin D-Binding Protein

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and susceptibility to DM1.\textsuperscript{3,4} Studies with non-obese diabetic mice have correlated vitamin D deficiency and the occurrence of similar symptoms of DM1.\textsuperscript{5}

Vitamin D has multiple effects on the immune system and the inflammatory process.\textsuperscript{6,7} Vitamin D inhibits T-cell and B-cell proliferation and down regulates inflammatory cytokines (IL-2, IL-6, and IFN-γ). Vitamin D plays a role in preventing autoimmunity and preserving self-tolerance by stimulating T-regulatory cells.\textsuperscript{8} Vitamin D deficient DM1 individuals do not fully benefit from the positive effects of vitamin D and the decreased level could be a contributing factor to an overactive immune response.

Vitamin D Binding Protein (DBP) transports vitamin D to the liver where it is metabolized to 25-hydroxyvitamin D. The 25-hydroxyvitamin D later becomes the active form of vitamin D (1,25 dihydroxyvitamin D) in the kidneys.\textsuperscript{9} 25-hydroxyvitamin D (abbrev. vitamin D) is the major circulating form of vitamin D, the most stable, and is the form routinely measured by clinicians to assess vitamin D status.\textsuperscript{10} Aside from transporting vitamin D and the catabolism of vitamin D, DBP has additional roles in the body, such as activating macrophages, stimulating osteoclasts, and acting as an actin scavenger.\textsuperscript{11} DBP has multiple binding sites on the protein such that the binding of vitamin D to the protein does not limit its other functions.\textsuperscript{12} Variations in the structure of DBP are caused by single nucleotide polymorphisms (SNPs) specifically rs7041 and rs4588 (Table 1). These variations in the protein are entitled Gc1F, Gc1S and Gc2 based on the haplotype of the patient. It has been established that Gc1F has the highest binding affinity for vitamin D and Gc2 has the lowest binding affinity for vitamin D.\textsuperscript{13} The physical alteration of the protein changes the binding affinity to vitamin D, which can lead to decreased vitamin D reaching the cells and tissues.\textsuperscript{14}

As described above, vitamin D plays several important roles for human biology. However, if defects in the carrier protein are resulting in decreased vitamin D being present for the tissues, perhaps some of these diseases are induced due to the lack of functional DBP delivering vitamin D and not due to decreased vitamin D availability.

**METHODS AND MATERIALS**

**Sample Population**
The sample population consisted of individual volunteer participants who lived along the Utah Wasatch Front. Two comparison groups were established from these participants. The first group was control participants (n=31) vs. DM1 participants (n=31) and the second group was categorized by the genetic haplotypes that most often cause the DBP isoform (Gc1F, Gc1S and Gc2) (Table 1).

**Table 1. Haplotypes and Protein Isoform Categorization**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Gc1F</th>
<th>Gc1S</th>
<th>Gc2</th>
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<td>SNP</td>
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</tr>
<tr>
<td></td>
<td>TG</td>
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**Testing Procedure**

Blood from participants was collected via standard venipuncture under informed consent after the nature of the experiment was explained. Two tubes, one Serum Separator Tube and one K\textsubscript{2}-EDTA Vacutainer®, were drawn from each participant. Samples were centrifuged at 1200 xg for twelve minutes within two hours of collection to prevent degradation of the white blood cells. Following the centrifugation, the plasma was aliquoted without disturbing theuffy coat. The plasma and serum were frozen at -20°C for vitamin D quantification and Vitamin D Binding Protein quantification, respectively. Vitamin D Binding Protein quantitation was carried out using the Abcam® GC-Globulin (Vitamin D Binding Protein) Human
ELISA kit. The 96-well plate was read using the BioTek® EPOCH 2 Spectrophotometer according to the specification of the Abcam® GC-Globulin (Vitamin D Binding Protein) Human ELISA kit. 25-hydroxyvitamin D concentrations were measured using the Immunodiagnostic System® Enzyme Immunoassay (EIA) method.

The buffy coat from the K$_2$-EDTA tube was removed after centrifugation to increase the yield of deoxyribonucleic acid (DNA). The buffy coat was stored in a microfuge tube at -20°C. DNA was then extracted from the buffy coat using the QIAamp® DNA Blood Mini Kit protocol. The resulting DNA was analyzed by the University of Utah Genomics Core Facility to confirm the presence of rs7041 and rs4588 SNPs using SNP TaqMan® analysis. Results from the SNP TaqMan® analysis were used to determine the most probable Vitamin D Binding Protein isoforms and the SNP genotype call plots were manually curated for any sign of abnormal clustering. During testing, the corresponding diabetic or control category of each sample remained anonymous to all researchers for the duration of all testing.

**Statistical Tests**

Hardy-Weinberg Equilibrium was established using the allele frequencies of each SNP. The Shaprio-Wilk test was used to assess normality. Kruskal-Wallis rank sum test was used to compare the variances of the three groups to avoid multiple pairwise comparisons. Following the Kruskal-Wallis rank sum test, the Mann Whitney U test was performed to compare the mean 25-hydroxyvitamin D level and the mean DPB concentrations. The Mann Whitney U test was also used to compare the Control and Diabetic groups (Table 2). Linear regression was used to test the dosage effect of the mutant allele in the three different isoforms and a separate linear regression was performed between the DBP concentration and the 25-hydroxyvitamin D concentration. A Chi-squared test compared the SNPs of our sample population (rs4588 and rs7041) to the HapMap CEPH (Utah residents with ancestry from northern and western Europe) population. An alpha level of 0.025 was used for statistical testing. All statistical analysis was carried out using R-studio stats software.

**RESULTS**

**Participant Demographics**

There was a population of sixty-two participants, 31 diabetics and 31 control patients, male (58%) and 26 female (42%). The mean age of all participants was 29.1 (SD 12.8), male participant mean age was 24.9 (SD 11.3) years and female participant mean age was 35 (SD 12.6) years. The self-reported ethnicity of our population was 96.8% Caucasian and 3.2% Hispanic. The gender, age, and self-reported ethnicity of the test and control groups were similar. A questionnaire was used to exclude participants with a preexisting condition that could affect their vitamin D status. Things considered to affect vitamin D status were, sun exposure, vitamin D supplementation, other autoimmune disorders, gastrointestinal disease, pregnancy, age, and the use of some prescription medication. Any participant who had a preexisting condition that affected their vitamin D status was not considered for the study. Because age affects vitamin D metabolism, only participants from ages 6-67 were considered.

**Vitamin D levels and DBP levels of Diabetic Group**

There were 18 male and 13 female diabetic participants. The mean age of this group was 29.2 (SD 12.9) with a participant age range from 8 to 56 years. The mean male age was 25.1 (SD 11.2) and the mean female age was 35 (SD 13.3).

The mean vitamin D level for the group was 31 ng/dL (SD 12.8) with a mean 26 ng/dL (SD 5.1) in the males and a mean of 36 (SD 17.6) in the females. The mean DBP level for the group was 1583 µg/L (SD 484.9) with a mean 1594 µg/L (SD 470.6) in the males and a mean of 1568 µg/L (SD 517.2) in the females.

**Vitamin D levels and DBP levels of Control Group**

There were 18 male and 13 female control participants. The average age of this group was 29.1 (SD 12.9) years with a participant age range from 9 to 53 years. The mean male age was 24.8 (SD 11.7) years and the mean female age was 35 (SD 12.5) years.

The mean vitamin D level for the group was 32 µg/dL (SD 15.7) with a mean 32 (SD 18.5) in the males and a mean of 32 (SD 11.7) in the females.
The mean DBP level for the group was 1963 µg/L (SD 1151.2) with a mean 1954 µg/L (SD 1425.7) in the males and a mean of 1917 µg/L (SD 669.7) in the females.

**Comparison of Vitamin D and DBP (Diabetic vs Control)**

The Shapiro-Wilk test established that the mean concentration of DBP and vitamin D was not normally distributed. The mean concentrations of DBP and 25-hydroxyvitamin D showed no statistical difference among the comparison groups (Table 2). The linear regression of the DBP concentration and the 25-hydroxyvitamin D concentrations yielded a $R^2$ of 0.3404, showing no correlation.

**Comparison of Vitamin D and DBP (Isoforms Gc1F, Gc1S and Gc2)**

Based on the haplotypes the participants were categorized into the most probable phenotype of DBP, (Gc1F=35/60, Gc1S=19/60 and Gc2=6/60) (Table 1). A Chi-squared test showed that all alleles are in equal distribution according to the Utah residents with ancestry from northern and western Europe, CEPH, using the HapMap statistical data (rs4588 $p=0.3949$ and rs7041 $p=0.6903$). When comparing the variances of the DBP concentrations between the three isoforms there was a statistical difference amongst the groups (Gc1F, Gc1S and Gc2 $p$-value = 0.00509). There was no statistical significant difference amongst the vitamin D concentration and the three isoform groups (Gc1F, Gc1S and Gc2 $p$-value = 0.3708). There was no dosage effect of the mutant allele on the concentrations of DBP ($R^2=0.0017$) (Figure 1). There was no significant difference between the data when differentiated by age or sex so those variables were not considered separately.

**DISCUSSION**

Based on our review of previous studies, we anticipated a correlation between the participants DM1 status and their vitamin D levels. However, we found that in our population there was no significant difference in the vitamin D status between control and DM1 participants (Table 2). While we found the complete lack of association surprising, it is possible that other factors such as the sample size or a physiological compensation in DM1 individuals over time could explain this. Furthermore, we also hypothesized that the likely DBP of a participant would influence their vitamin D status. Analysis of the associated SNPs of rs7041 and rs4588 to identify the most probable DBP isoform present showed there was not a significant difference between the particular DBP of the participant and their vitamin D status. Poor linear association was found between the overall DBP concentration and the 25-hydroxyvitamin D concentration. DBP has multiple roles in the body and is seen in excess compared to vitamin D. This lack of association is expected and has been seen in other studies. Linear regression of the three DBP isoform variants against the DBP concentrations showed a minor decreasing trend of the overall DBP concentration and the DBP isoform (Figure 1). The dosage effect of the SNPs on the vitamin D binding protein and the vitamin D concentrations was statistically insignificant due to the limited population size. Analysis of a larger sample size would help determine the presence of a dosage effect based on the mutant haplotype and would be an area for further investigation.

**Figure 1.** Regression Analysis of Vitamin D Binding Protein and Isoform Type: This graphical representation of the concentrations of the DBP in each isoform category plotted against a dosage number. Gc1F is represented by 0 because it is the most common and contains the least amount of the mutant alleles. Gc1S is represented by 1 because the alleles that code for this protein contain the second most amount of mutant alleles and Gc2 is represented by 2 containing the most amount of mutant alleles.
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Table 2. Comparisons of 25-hydroxyvitamin D and Vitamin D Binding Protein: This table is a summary of the results comparing the two main population groups using the Mann Whitney U test, “Control vs. Diabetic” and “GC1F, GC1S and GC2”. (NA: Non parametric ANOVA showed no significant difference amongst the group as a whole so no further statistical testing was performed, NS: Not significant, n: population size)

### 25-hydroxyvitamin D Concentrations comparisons (ng/dL)

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<th>Diabetic n=31</th>
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<td>GC1F v. GC2²</td>
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### Vitamin D Binding Protein Concentration Comparisons (µg/L)

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<th>Diabetic n=31</th>
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As previously stated, DM1 is an autoimmune disorder that leads to the destruction of the pancreatic β cells. At the time of DM1 diagnosis 10%-30% of pancreatic β cells remain. After complete destruction of the β cells, the targeted autoimmune response diminishes. We hypothesize that this reduced autoimmune action in type one diabetics is why it is difficult to find an association between DBP and vitamin D levels. Vitamin D status has been evaluated at the time of DM1 diagnosis, however, the DBP levels and DBP associated SNPs have not. Further investigation is needed to evaluate this hypothesis. It would be beneficial to conduct this research at the time of DM1 diagnosis for a more complete analysis of the effect of vitamin D concentrations on the autoimmune destruction of the pancreatic β cells.

Based on the results of this study it has been shown that in the Utah Wasatch Front Diabetic population there is a lack of association between the vitamin D concentration, DBP isoform and DBP concentration when compared to the control group. It is stated that the data might better correlate if testing were performed at the time of DM1 diagnosis. It is further stated that studying a larger sample size would allow a better analysis of the dosage effect of the alleles of the vitamin D binding protein compared to the vitamin D concentrations. This information could be compared to multiple populations that suffer vitamin D deficiency. The information would help identify groups who are at greater risk of developing vitamin D deficiency prior to secondary symptoms arising.

REFERENCES

3. Zipitis CS, Akobeng AK. Vitamin D supplementation in early


