# Vitamin D Antibodies in Systemic Sclerosis Patients: Findings and Clinical Correlations

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**ABSTRACT:** **Background:** Vitamin D is a pivotal factor in calcium homeostasis and exerts immunomodulatory effects. Hypovitamin D has been demonstrated in systemic sclerosis (SSc) patients and may be related to more severe disease of longer duration and with extensive skin involvement.

**Objectives:** To seek anti-vitamin D antibodies in SSc patients, as found by previous research in patients with systemic lupus erythematosus (SLE).

**Methods:** The study included 54 SSc patients and 41 volunteers. Immunoglobulin (Ig) G and IgM autoantibody levels against 25(OH)D and 1,25(OH)D were obtained from patients and controls and were compared. SSc patients were assessed for autoantibody profile and disease severity.

**Results:** Vitamin D antibodies were present in 87% of SSc patients and 42% of controls. Higher levels of anti-25(OH)D IgM antibodies were detected in SSc patients compared to controls (0.48 ± 0.22 vs. 0.29 ± 0.29, respectively, $P = 0.002$); however, IgG levels were lower in the SSc patients. No such discriminative effect was found regarding anti-1,25(OH)D antibodies between SSc and controls. No correlation was found between vitamin D antibodies and other autoantibodies, disease severity, or target organ damage.

**Conclusions:** To the best of our knowledge, this is the first study of these novel anti-vitamin D antibodies in SSc patients and the first time a correlation between IgM 25(OH) vitamin D antibodies and scleroderma has been identified. Further research on the pathophysiological significance and therapeutic potential of vitamin D is required.

*IMAJ* 2015; 17: 80–84

**KEY WORDS:** scleroderma, systemic sclerosis (SSc), vitamin D, vitamin D antibodies, vitamin D deficiency, vitamin D insufficiency, 25-hydroxyvitamin D (25(OH)D), dihydroxyvitamin D (1,25(OH)D)

Vitamin D is a pivotal factor in disorders that involve calcium metabolism, such as osteoporosis and osteomalacia. In vitro studies have demonstrated that vitamin D also exerts immunomodulatory effects. Vitamin D receptors were identified in several types of immune cells and this substance plays a key role both in reaction enhancement of the naïve immune system and in restraint of the type 1 response of the adaptive immune system [1]. The number of vitamin D receptors on CD4 T cells correlates with their immune activity. Adding 1,25(OH) D to CD4 T lymphocytes inhibits proliferation of T helper (Th)-1 cells, but increases the activity of Th-2 cells and related cytokines interleukin (IL)-5 and IL-10 [2]. Vitamin D also affects the naïve immune system via Th-17, which plays a central role in neutrophil activity and inflammatory response. These activities are suppressed by 1,25(OH)D via inhibition of IL-23 and IL-6 production [3].

Systemic sclerosis (SSc) is a chronic connective tissue disease of unknown etiology that causes widespread microvascular damage and excessive deposition of collagen in the skin and internal organs [4]. Clinical associations exist between the pattern of organ involvement (limited vs. systemic disease) and scleroderma-specific autoantibodies, such as antinuclear antibody, anti-centromere antibody and anti-topoisomerase-1 antibody (anti-Scl-70) [5]. However, the etiopathogenesis of the disease and the role of various antibodies in organ involvement and disease manifestations remain unclear.

A high prevalence of vitamin D deficiency was noted among patients with autoimmune diseases [6–8], especially systemic lupus erythematosus (SLE) [9]. Moreover, patients with SLE and severe vitamin D deficiency demonstrated a more severe disease course [10]. These observations led to the hypothesis that vitamin D deficiency may exacerbate autoimmune conditions [6,10]. Vitamin D treatment in animal models of autoimmune diseases, primarily SLE, led to significant improvements [11]. However, similar results have not been fully achieved in humans, and the role of vitamin D treatment in the course of autoimmune diseases has yet to be studied [12].

SSc patients tend to have very low vitamin D levels. This may be attributed to several characteristics of the disease, including disseminated skin involvement and renal injury that may interfere with vitamin D synthesis, as well as vitamin D malabsorption in cases of advanced intestinal disease. Similar to SLE patients, SSc patients with vitamin D deficiency demonstrate a lengthier and more severe disease [13], particularly regarding lung involvement [14,15]. An inverse cor-

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relation was found between the severity of skin involvement, including fibrosis and calcinosis, and vitamin D blood levels [16,17]. However, other studies did not confirm the association between the severity of scleroderma and vitamin D deficiency [18,19]. Another possible explanation for vitamin D deficiency in patients with autoimmune diseases is the presence of neutralizing autoantibodies to vitamin D. Few studies on vitamin D antibodies in these diseases have been conducted. A preliminary study in patients with SLE demonstrated higher levels of anti-vitamin D antibodies compared to those with anti-phospholipid syndrome or pemphigus vulgaris [20].

The above studies, combined with conflicting information regarding the role of vitamin D status in SSc patients, motivated us to examine anti-vitamin D antibodies in SSc patients. We investigated the presence and levels of IgG and IgM antibodies to 25-hydroxyvitamin D (25(OH)D) and dihydroxyvitamin D (1,25(OH)D) in a cohort of SSc patients followed regularly in our outpatient clinic. A complete autoimmune antibody history was obtained and the pattern and severity of organ damage characterized. The main objective was to investigate vitamin D antibodies in a cohort of scleroderma patients. We also tried to detect a correlation between vitamin D antibodies and specific organ involvement and disease severity.

## PATIENTS AND METHODS

The study population comprised 54 consecutive patients diagnosed with SSc (both limited and systemic manifestations) classified according to the American College of Rheumatology (ACR) 1980 criteria [21]. All patients were followed at the Meir Medical Center outpatient clinic. Fifty-five patients with scleroderma enrolled in the study (44 with systemic disease, 11 with limited manifestation). Clinical data included age, disease duration, organ involvement, the presence of autoantibodies, and vitamin D levels prior to treatment initiation. Forty-one volunteers from the hospital staff served as the control group. Controls were questioned about their current health status including smoking, medical history, pregnancy status, regular consumption of medications including oral contraceptives, hormone replacement therapy, and use of nutritional supplements. Blood samples were also tested for vitamin D levels.

### SSC SEVERITY SCORE (SCSS)

A numeric evaluation of disease severity in five organ systems (lung, kidney, skin, gastrointestinal, joints) on a scale ranging from 0 (no injury) to 3 (severe injury) was obtained. Lung injury regarding interstitial lung disease and pulmonary hypertension was estimated. This ranking system correlates with other severity scales, such as that of Medsger et al. [22]. The scores were calculated based on medical record data for the five organ systems, with a range of 0 (healthy) to 15 (severe pan-systemic illness).

### DETECTION OF AUTOANTIBODY LEVELS

- **Anti-vitamin D antibody determination**

For antibody determination, 95-well enzyme-linked immunosorbent assay (ELISA) plates (Maxisorp, Nunc, Denmark) were coated overnight at 4°C with 1,25(OH) vitamin D (Sigma, St. Louis, MO, USA) 5 g/ml and absolute ethanol. The plates were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour at 37°C. Following washing with PBS, the sera from the subjects were added at a dilution of 1:100 in 1% BSA and PBS for 4 hours at room temperature. Immunoglobulin binding was probed by goat anti-human IgG antibodies conjugated to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hr at 37°C. The plates were developed using phosphatase substrate (Sigma) and read at optical densities (OD) of 405 nm using an ELISA reader (Anthos HT2, Anthos Labtech Instruments, Salzburg, Austria).

- **Vitamin D measurement**

The commercial kit, LIAISON 25(OH) vitamin D (DiaSorin Inc., Saluggia, Italy) was used to measure serum concentration of 25(OH) vitamin D. A low 25(OH) vitamin D level was set as < 30 ng/ml, while < 10 ng/ml was considered insufficient.

### STATISTICAL ANALYSIS

Descriptive statistics included counts, mean, median, standard deviation, minimum and maximum values for continuous measurements, and contingency tables with counts and percents for categorical measurements. Comparison of anti-vitamin D levels between the treatment and control group were assessed using the Mann-Whitney test. Receiver operating characteristic (ROC) curve was applied to identify the discriminative ability of anti-vitamin D to identify the best discriminative threshold, which was determined to be 0.3 U.

A logistic regression model including vitamin D metabolite antibodies, age and gender subclasses was applied to identify independent factors that could predict SSc. Statistical analyses were carried out using IBM SPSS software, version 22.0. Significance level was defined as $P = 0.05$.

## RESULTS

### DEMOGRAPHICS AND GENERAL PATIENT PROFILE

The study included 95 subjects (54 SSc patients and 41 healthy controls). The scleroderma patients were significantly older than the control group (52.6 ± 14.7 vs. 35.7 ± 8.5 years, $P < 0.001$) and there were more females in both groups (87.3% and 85.4%, respectively) [Table 1]. Although measurement of vitamin D levels was not part of the current research, we obtained vitamin D levels that were taken prior to beginning vitamin D therapy: 82.1% of the patients had documented vitamin D deficiency (< 30 ng/dl) compared to only 9.8% of controls.

**Table 1.** Demographic characteristics of scleroderma patients versus controls

| | Scleroderma (n=54) | Controls (n=41) | *P* value |
|---|---|---|---|
| Age (years) | 52.6 ± 14.7 | 35.7±8.5 | ‹ 0.001 |
| Female gender | 48 (88.9%) | 35 (85.4%) | 0.757 |
| Comorbidities* | 31 (57.4%) | 6 (14.6%) | ‹ 0.001 |
| Smoker | 5 (9.1%) | 11 (26.8%) | 0.029 |
| Documented vitamin D insufficiency | 82.1% (n=39) | 9.8% (n=41) | |
| Systemic/limited scleroderma | 44/10 (79.6%/20.4%) | Irrelevant | |
| Length of disease (years, range) | 10 ± 6.6 (2–43) | Irrelevant | |
| Scleroderma severity score (range) | 5.56 ± 2.5 (2–11) | Irrelevant | |
| Antibody profile | | Irrelevant | |
| Antinuclear antibody (ANA) | 52 (98.1%) | | |
| Anti-topoisomerase I (Scl-70) | 28 (51.9%) | | |
| Anti-centromere | 10 (18.9%) | | |

Data are presented as n (%) or mean ± SD
*Comorbidities included hypertension, hyperlipidemia, diabetes mellitus, asthma and obesity

**Table 2.** Vitamin D antibody levels in scleroderma patients and controls

| | Scleroderma patients | Controls | *P* value* |
|---|---|---|---|
| Anti 25(OH)D IgG | 0.23 (0.11–1.94) | 0.26 (0.13–1.85) | 0.049 |
| Anti 25(OH)D IgM | 0.48 (0.11–1.10) | 0.29 (0.04–1.61) | 0.002 |
| Anti 1,25(OH)D IgG | 0.31 (0.13–1.34) | 0.25 (0.14–0.63) | 0.887 |
| Anti 1,25(OH)D IgM | 0.21 (0.07–1.46) | 0.27 (0.05–1.79) | 0.367 |

**P* values by Mann-Whitney test

**Table 3.** Risk factor for scleroderma determined by vitamin D antibodies, age and gender calculated by regression model analysis

| Variable | OR | *P* value | 95% CI | |
|---|---|---|---|---|
| | | | Lower limit | Upper limit |
| Anti 25 (OH) vitamin D IgG | 4.8 | 0.034 | 1.1 | 20.3 |
| Anti 25 (OH) vitamin D IgM | 7.5 | 0.003 | 2.0 | 27.5 |
| Anti 1,25(OH) vitamin D IgG | .90 | 0.868 | .30 | 3.0 |
| Anti 1,25(OH) vitamin D IgM | .90 | 0.862 | .30 | 3.0 |
| Age | 1.1 | 0.000 | 1.0 | 1.2 |
| Gender | 1.6 | .5820 | .30 | 9.6 |

Scleroderma Severity Score (ScSS) ranged from 2 to 11 with an average of 5.6 ± 2.5, implying severe injury in at least two organ systems and moderate damage in three organ systems. Half the patients had a score of 6 or less and 75% had a score of less than 8. Severe degrees of involvement were noted in the following systems: muscular (n=5), skin (n=6), gastrointestinal (n=3), and renal (n=2).

### PRESENCE AND SIGNIFICANCE OF VITAMIN D ANTIBODIES

Vitamin D antibodies were present in 87% of SSc patients and 42% of controls. There were significant differences in the levels of anti-25(OH)D antibodies between the scleroderma and control groups. IgM levels were higher in SSc patients compared to controls (0.48 vs. 0.29, $P = 0.02$, Mann-Whitney test). In contrast, IgG antibodies were lower in scleroderma patients (0.23 vs. 0.26, $P = 0.005$, Mann-Whitney test). It should be noted that anti-1,25(OH)D subtype IgG antibodies also demonstrated a higher trend but the difference was not statistically significant ($P = 0.878$) [Table 2].

ROC curve analysis was applied to identify the anti-vitamin D antibody 25(OH) D and 1,25(OH)D thresholds that best discriminate between SSc patients and controls. A cutoff level/threshold of 0.30 U/L had 87.0% sensitivity and 58% specificity, whereas a threshold of 0.20 U/L demonstrated higher sensitivity of 92.6% but lower specificity of only 42.5%. At a cutoff of 0.3, 47 patients (87%) and 17 controls (42%) were positive to the antibodies. A logistic regression model demonstrated that age and anti-25(OH)D IgM and IgG levels were significantly related to scleroderma disease, whereas higher levels of anti-25(OH)D IgM (> 0.3) increased the risk of SSc, with odds ratio (OR) of 7.5 and 95% confidence interval (CI) 2.0–27.5, $P <$ 0.003. Lower levels of anti-1,25(OH)D IgG (< 0.3) increased the risk of SSc (OR 4.79, 95%CI 1.1–20.3, $P < 0.035$). For age, the OR was 1.0 (95%CI 1.0–1.2) [Table 3].

Borderline differences in ScSS were noted between patients with anti-25(OH)D and those without antibodies (66 vs. 5 respectively, $P = 0.114$). For the correlation between ScSS and antibodies, only anti-1,25(OH)D IgM demonstrated a weak, negative correlation with borderline significance ($r = -0.272$, $P = 0.056$). Anti-25(OH)D IgM had a weakly positive correlation with Scleroderma Severity Subscale related to muscle involvement ($r = 0.288$, $P = 0.035$). There was no significant difference in anti-25(OH)D or anti-1,25(OH)D levels in 15 patients with severe involvement of one or more systems compared to subjects with less severe disease. No further association was identified between vitamin D antibodies and targeted system injury or other autoimmune antibody profile subtypes.

More female subjects had anti-25(OH)D values ≥ 0.2 (89.6% vs. 57.1%, $P = 0.055$) compared to males. A higher incidence of anti-25(OH)D (threshold > 0.3) was noted among all female participants in the study (74.7% vs. 46.2%, $P = 0.049$).

## DISCUSSION

In addition to its well-known functions regarding bone metabolism, vitamin D, a fat-soluble vitamin, has significant effects on the immune system. Several in vitro studies have demonstrated its efficacy in preventing and treating autoimmune diseases [11,23,24].

SSc patients have a high prevalence of vitamin D deficiency [17]. Hypovitamin D in SSc patients can be attributed to skin

involvement and renal injury that might interfere with vitamin D synthesis and malabsorption due to advanced intestinal disease. Vitamin D antibodies may also cause vitamin D deficiency in SLE patients, as demonstrated by Carvalho et al. [20] in a preliminary study. However, there is no information regarding vitamin D antibodies in patients with SSc.

The present study presents novel data regarding vitamin D antibodies in SSc patients. A total of 95 subjects, 54 of whom had SSc, were tested for both 25(OH) D and 1,25(OH)D antivitamin D antibodies and two subtypes of antibodies, IgM and IgG. Varying levels of anti-25(OH)D, especially the IgM subclass, differentiated SSc patients from controls. Furthermore, anti-25(OH)D IgM was higher in the SSc group than the controls (0.48 ± 0.22 vs. 0.39 ± 0.33, $P = 0.013$), while IgG was lower (0.27 ± 0.26 vs. 0.34 ± 0.29, $P = 0.026$). There were no statistically significant differences regarding anti-1,25(OH)D antibodies between groups, although a slight trend was noticed.

Few studies on vitamin D antibodies in autoimmune diseases have been conducted, mostly without antibody subtypes or vitamin D metabolite-related analysis [20,25]. Carvalho and team [20] found vitamin D antibodies in a subset of patients with SLE. The results in SSc patients presented here are in concert with those of Carvalho's study, demonstrating the presence of these unique antibodies. Different autoantibodies have been described in various frequencies, but it has not been determined whether they are all pathogenic in SLE. However, the association found by Carvalho et al. between anti-vitamin D antibodies and anti-dsDNA in SLE patients suggests that despite their low frequency in the cohort presented here (4%, n=7), anti-vitamin D antibodies may play a role in SLE pathogenesis. However, this breakthrough study did not perform subclass analyses; therefore, we lack information regarding the role of IgM vitamin D antibodies in SLE.

An Indonesian study by Handono [25] showed a similar trend: 70.63% of SLE patients had low (< 30 ng/ml) vitamin D serum levels. Autoimmune antibodies were frequently present in these patients: anti-dsDNA in 70.30% and anti-vitamin D in 64.81%. Serum levels of 25(OH) vitamin D correlated negatively with anti-dsDNA and anti-vitamin D antibodies ($r = -0.416$, $P = 0.032$, and $r = -0.537$, $P = 0.041$, respectively). The authors postulated that low vitamin D levels in patients with SLE may be caused by anti-vitamin D antibodies.

The present study did not find a statistically significant difference between patients and controls in antibody titers against the active metabolite 1,25(OH) vitamin D. It is important to remember that the major circulating form of vitamin D is 25(OH)D and its level is currently considered the best indicator of vitamin D stores. Since 25(OH)D is the dominant form in circulation, with a longer half-life than that of 1,25(OH)D, it may well be a more immunogenic form.

In this study, anti-vitamin D was detected among both SSc patients and healthy controls. These results are consistent with previous research. Carvalho et al. detected a high frequency of vitamin D antibodies among healthy controls, raising the hypothesis that antibodies develop in individuals who consume high doses of vitamin D. Unfortunately, we do not have data regarding vitamin D intake/exposure among the controls, but since they are relatively young (mean age 35.7 years) and healthy, we assume that most did not consume vitamin D regularly.

The literature lacks information regarding subtype analysis and metabolite dominance of vitamin D antibodies. Based on our literature review, this analysis has not been published for any autoimmune disease.

Unlike previous reports that demonstrated a correlation between anti-vitamin D antibodies and anti-dsDNA antibodies among SLE patients, no such correlation was observed in the current study. Moreover, no other autoantibody levels correlated with anti-vitamin D levels.

This study was limited by a relatively small cohort for an antibody study. The healthy controls were not perfectly matched with the study group. In addition, the lack of a validated, standardized, commercial assay for vitamin D antibodies was a major limitation. Therefore, we determined normal values based on differences between SSc patients and controls. Moreover, a single measurement from one blood sample is another limitation, which is common in antibody studies due to the cost of the assay. In addition, vitamin D measurements were not part of the study. Reliable data on oral intake of milk products, vitamin D supplementation and sun exposure among the healthy controls were lacking.

## CONCLUSIONS

To our knowledge this is the first report of these novel anti-vitamin D antibodies in SSc patients. Our study is also innovative because it includes measurement of antibodies for both vitamin D metabolites and subtype antibodies analysis. We showed that SSc patients have higher levels of IgM anti-25(OH) D antibodies. Our results raise questions about a possible additional immunogenic role of both vitamin D metabolites. The observation that vitamin D antibodies are more frequent in SSc patients can also aid in differentiating scleroderma patients from healthy controls. Further studies with a larger cohort are required to confirm these findings and to examine the utility of these novel anti-vitamin D antibodies as a diagnostic and prognostic marker in SSc patients.

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