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Impaired Vitamin D Metabolism in Systemic Sclerosis

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ABSTRACT

Objective: *To evaluate vitamin D status, receptor, and metabolic enzymes in systemic sclerosis and their impact on disease phenotype.* ***Methods:*** *sixteen scleroderma patients and twelve controls were evaluated. Vitamin D status was assessed by measuring 25(OH)D serum levels. The expression of mRNA for VDR (vitamin D receptor), hydroxylation enzymes (CYP27B1, CYP24A1) on peripheral blood mononuclear cells (PBMCs) was evaluated by real-time PCR.* ***Results:*** *There was no statistical difference in vitamin D levels between scleroderma patients and the control group (p=0.647). Though, patients with low 25(OH) levels had more active and severe disease with lung involvement (p=0.031), pulmonary hypertension (p=0.046), and poor quality of life (p=0.006). VDR mRNA expression was higher in systemic sclerosis (p=0.014) and associated with CRP (p=0.046) and decreased DLCO (p=0.033). PBMCs of scleroderma patients expressed lower CYP27B1 mRNA levels (p=0.021).* ***Conclusions:*** *Defects in the vitamin D axis were associated with more severe disease in SSc patients. Taking together our results suggest that scleroderma patients might benefit from targeting the vitamin D axis with active metabolites.*

Keywords: Systemic sclerosis, Vitamin D metabolism, Disease phenotype

INTRODUCTION

Vitamin D has pleiotropic effects that go beyond its traditional role in calcium homeostasis, related to Vitamin D Receptor (VDR) ubiquitous distribution. Hundreds of genes controlled by vitamin D directly or indirectly influence cell cycle, proliferation, differentiation, and apoptosis [1]. A large number of studies showed that vitamin D deficiency might be a risk factor for a wide spectrum of acute and chronic illnesses [2].

Vitamin D, to become biologically active, needs two important hydroxylation steps: the first in the liver by the 25-hydroxylases (CYP27A1) and the second in the kidneys, where 25(OH)D is converted by 1-α-hydroxylase (CYP27B1) to its active form 1α,25-dihydroxyvitamin D (1,25(OH)2D) [3,4]. Moreover, vitamin D, in an autocrine manner, can regulate its expression through 24-hydroxylase, CYP24A1 [5]. It has been shown that resting monocytes, dendritic cells, activated T and B lymphocytes express VDR, CYP27B1, and CYP24A1 [6,7].

Special attention has been given to vitamin D's effects on the innate immune system and endothelial dysfunction. Vitamin D seems to influence Toll-Like Receptors (TLR) stimulation, interferon (IFN) response, and antimicrobial peptides production such as cathelicidin (hCAP18/LL37) [8-11]. 1α,25(OH)2D3 can also inhibit ET-1-dependent DNA synthesis and cell proliferation in aortic vascular smooth muscle cells [12]. An increase in angiogenic properties of progenitor endothelial cells has also been reported for vitamin D [13].

Systemic Sclerosis (SSc) is an autoimmune disease in which endothelial dysfunction and immune activation lead

to excessive accumulation of extracellular matrix in the skin and internal organs [14,15]. In SSc patients, vitamin D deficiency was identified to be frequent and associated with disease activity or phenotype characteristics such as pulmonary hypertension, lung involvement, extensive cutaneous forms [16-22]. A recent systematic review confirmed a higher prevalence of vitamin D deficiency and insufficiency in SSc patients compared to controls and inversely correlated with the severity of SSc [23]. It is still unclear if the high prevalence of vitamin D deficiency in SSc patients is related to diminished synthesis due to skin involvement or to other metabolic abnormalities. Intervention for low serum vitamin D levels in SSc pathogenesis remains controversial, as well as the significance of vitamin D supplementation in such patients [24].

Based on the data presented, we evaluated a group of SSc patients for the pathological significance of vitamin D insufficiency/deficiency. We also investigated the relationship between 25(OH)D level and *CYP27A1*, *CYP27B1*, and *VDR* genes expression to identify potential metabolic abnormalities.

MATERIALS AND METHODS

Patients

Sixteen SSc patients and twelve matched controls were included in the study. The patients were selected according to the American College of Rheumatology criteria for systemic sclerosis [25]. The patients were evaluated for the pattern of disease, Rodnan score, musculoarticular, gastrointestinal, cardiovascular, pulmonary, and renal involvement, inflammatory markers, autoantibodies (antinuclear, anticentromere, antiscleroderma70), complement (serum C3), nailfold capillaroscopy. All patients had routine osteoporosis assessments by dual absorptiometry (DXA). Several scores and questionnaires were completed: European Disease Activity Score according to the European Scleroderma Study Group guidelines, MEDSGER severity score, and Health Assessment Questionnaire-Disability Index (HAQ-DI) [26,27]. The subjects' written consent was obtained according to the Declaration of Helsinki.

Cells Isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from 20 ml heparinized venous blood by centrifugation over Ficoll-Paque (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). Cell number and viability were evaluated by light microscopy using trypan blue dye.

Quantification of mRNA Specific to *VDR, CYP27B1, CYP24A1* by real-time RT-PCR for mRNA

Total RNA was isolated from PBMCs using Quick-RNA Mini-Prep kit (Zymo Research, Freiburg, Germany). For each subject, 1 µg of total RNA was reverse-transcribed with MMuLV enzyme (Fermentas, Thermo Fisher Scientific, Vilnius, Lithuania), using Oligo (dT) primers (Fermentas, Thermo Fisher Scientific, Vilnius, Lithuania) in a final volume of 40 µl. Synthesized cDNA (50 ng) was used in each PCR reaction. α-actin was used as an endogen control. Gene mRNAs quantification was performed with Maxima® SYBR Green qPCR Master Mix (2X) (Fer-mentas, Thermo Fisher Scientific, Vilnius, Lithuania) and the primers were used at a concentration of 250 nM. For each primer pair, a dilution curve of a positive cDNA sample was used to calculate the efficiency (E) of the amplification. The relative expression level of each target gene-specific mRNA was normalized to α-actin mRNA level. The formula used for the calculation of gene expression level was calculated using the formula: relative expression=(1+Etarget) (-Ct target) × 10000/(1+Eref)(-Ct ref).

Measurement of Serum 25(OH) Vitamin D Level

Peripheral blood was collected in anticoagulant-free tubes and centrifuged for 5 min at 2000 rpm. 25(OH)D level was measured in the sera by an ELISA kit (DIA-Source® 25-Hydroxyvitamin D Total ELISA assay (Nivelles, Belgium)) according to the manufacturer's protocol. 25(OH)D levels were considered normal for values ≥ 30 ng/ml, insufficient for values between (20-30) ng/ml, and deficient for values under 20 ng/ml.

Statistical Analysis

All calculations were performed with IBM SPSS Statistics 20.0. All data are presented as mean ± Standard Deviation (SD), median (95% confidence interval), or percentages. Student t-tests or Mann-Whitney tests were used to evaluate significant differences between SSc patients and controls for the nominal variable. The chi-square test was used to compare categorical variables. Pearson's bivariate correlation or Spearman's rank correlation coefficient were used

to evaluate the association between evaluated variables. The values of $r>0.5$ and $p<0.05$ were considered statistically significant.

RESULT

Demographic and Clinical Characteristics of SSc Patients

Patients' data are presented in Table 1.

Table 1 Clinical and para-clinical data of systemic sclerosis patients

Characteristics	**% Patients (No.=16)**
Female/Male	93.75/6.25
Pattern of scleroderma diffuse/limited	81.25/18.75
Active digital ulcers	37.5
Synovitis	37.5
Digital contractures	31.25
Muscle weakness	93.75
Gastrointestinal involvement	
esophagus/stomach/bowel	81.25/43.75/12.50
Pulmonary Arterial Hypertension (PAH)	37.5
Pulmonary fibrosis	43.75
Autoantibodies	
Anti-Centromere Antibodies (ACA)	25
anti-Scl70 antibodies	68.75
Nailfold capilaroscopy pattern (early/activel/late)	12.5/43.75/37.5
Osteoporosis	43.75
Therapy	
Corticotherapy	43.75
Immunosuppresion	62.5
Characteristics	**median (95% CI[a])**
Disease duration (years)	6.5 (4.47-12.85)
Rodnan score	9.5 (7.16-12.96)
Activity score	4.5 (3.14-5.36)
Medsger score	6.50 (5.62-8.76)
Estimated sPAP[b] (mmHg)	27.0 (25.46-43.23)
DLCO[c] (% predicted)	67.4 (46.53-68.83)
HAQ[d]	1.0 (0.72-1.38)

[a]: Confidence Interval, [b]: systolic pulmonary arterial presure, [c]: diffusing capacity for carbon monoxide, [d]: health assessment questionnaire

Vitamin D Status and Metabolic Particularities in Systemic Sclerosis

SSc patients and controls had similar sera level of 25(OH)D (20.71 ± 10.52 ng/ml for SSc patients and 17.67 ± 7.42 ng/ml for controls, $p=0.647$) (Figure 1A).

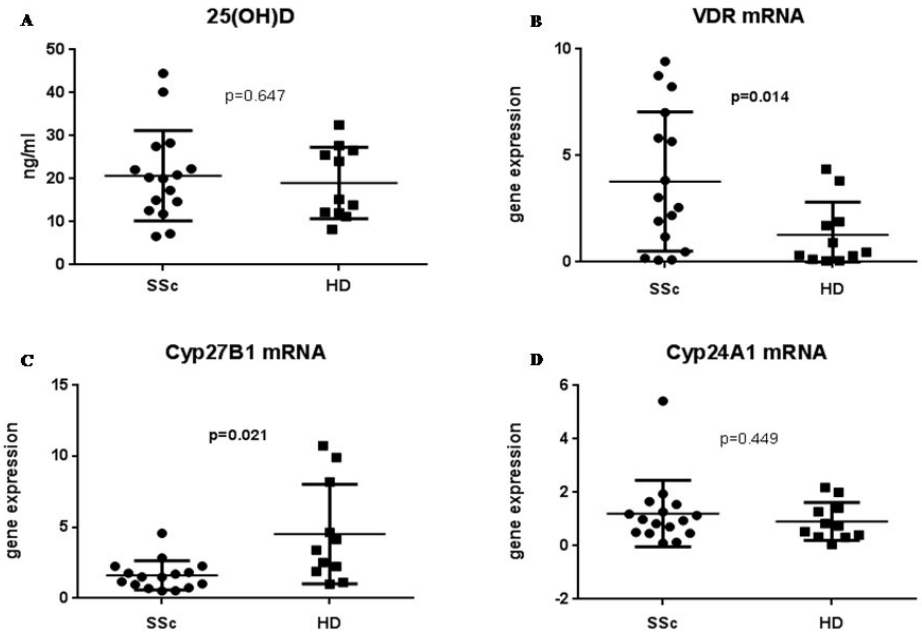

Figure 1 A): Comparison of 25(OH)D levels, B): VDR mRNA expression; C): CYP27B1; D): CYP24A1 between SSc patients and controls

The VDR mRNA was significantly higher in SSc PBMCs than in control PBMCs (p=0.014) (Figure 1B). When SSc patients were grouped based on serum 25(OH)D level (SSc group with 25(OH)D level ≤ 20 ng/ml (deficiency) and SSc group with 25(OH)D >20 ng/ml level (insufficiency), VDR mRNA was more expressed on SSc PBMCs with vitamin D deficiency compared to those with vitamin D insufficiency. VDR mRNA was more expressed in PBMCs SSc compared to PBMCs HD (p=0.014) (Table 2).

Table 2 Differences between SSc patients and controls grouped based on vitamin D level regarding *VDR, CYP27B1, CYP24A1* mRNA expression. Data are presented as mean values (standard deviation-SD) for each parameter and p values for differences

Group	Subgroup	Statistic	25(OH)D (IU)	*VDR* mRNA (AU)	*CYP27B1* mRNA (AU)	*CYP24A1* mRNA (AU)
SSc	Global	mean (SD)	20.71 (10.52)	3.78 (3.27)	1.62 (1.04)	1.21 (1.24)
	Insufficiency	mean (SD)	23.06 (3.42)	2.60 (3.34)	1.34 (0.79)	1.34 (1.84)
	Deficiency	mean (SD)	12.18 (4.01)	4.78 (3.47)	1.71 (1.35)	0.96 (0.55)
	Insufficiency *vs.* Deficiency	p	<0.001	0.254	0.539	0.612
Controls	Global	mean (SD)	17.67 (7.42)	1.23 (1.61)	4.16 (3.47)	1 (0.69)
	Insufficiency	mean (SD)	25.98 (1.54)	0.39 (0.36)	4.42 (4.34)	1.00 (0.79)
	Deficiency	mean (SD)	12.14 (2.40)	1.79 (1.91)	3.99 (3.20)	1.00 (0.68)
	Insufficiency *vs.* Deficiency	p	<0.001	0.136	0.873	0.996
SSc *vs.* Controls	Global	p	0.647	0.014	0.021	0.449
	Insufficiency	p	0.085	0.132	0.251	0.685
	Deficiency	p	0.983	0.080	0.152	0.896

SSc PBMCs expressed significantly lower levels of CYP27B1 mRNA (Figure 1C) when compared to HD PBMCs (p=0.021).

CYP24A1 mRNA (Figure 1D) was comparable between groups (p=0.449).

In SSc patients, no correlation between serum vitamin D levels and the expression levels in PBMCs of *VDR*, *CYP27B1*, or *CYP24A1* mRNAs was identified. In contrast, in HD PBMCs a significant correlations between the expression levels of *VDR* and *CYP24A1* mRNAs (r=0.673, p=0.033), between *VDR* and *CYP27B1* mRNAs (r=0.682; p=0.021) and between *CYP27B1* and *CYP24A1* mRNAs (r=0.891, p=0.001) were identified. No statistically significant differences in *VDR, CYP27B1, CYP24A1* mRNA expression were identified between deficiency and insufficiency SSc subgroups (Table 2).

Relationship between Vitamin D and Features of Systemic Sclerosis

SSc patients with deficiency had more active (p=0.002) and severe (p=0.006) diseases than those with insufficiency. They also had significantly decreased DLCO levels (p=0.017) (Table 3).

Table 3 Impact of vitamin D level on disease phenotype; the median, 95% Confidence Interval (CI) as well as p-values are presented for each SSc group and for those features that were found significantly different

Compared Groups	Statistics	Activity score	Rodnan score	Medsger score	DLCO[a] (% predicted)	Estimated sPAP[b] (mmHG)
SSc deficiency	median	5.50	10.50	9.50	41.00	39.00
	(95% CI)	(4.26-7.12)	(7.05-17.70)	(6.74-11.51)	(27.42-63.56)	(24.91-59.21)
SSc insufficiency	median	2.75	7.00	5.00	72.55	25.00
	(95% CI)	(1.72-3.91)	(5.00-10.50)	(4.09-6.41)	(60.32-79.43)	(21.43-31.82)
	p-value	0.0022	0.096	0.006	0.017	0.074
[a]: diffusing capacity for carbon monoxide, [b]: systolic pulmonary arterial presure						

Vitamin D level was negatively correlated with disease activity (p<0.001), Medsger severity score (p=0.001), diagnosis of pulmonary hypertension (p=0.046), poor quality of life (p=0.006), the presence of osteoporosis (p=0.042); positive correlations were found with DLCO values (p=0.031).

The expression level of VDR mRNA in PBMCs was associated with inflammatory markers (p=0.046) and impaired lung function (p=0.033). For CYP27B1 mRNA a trend for a negative correlation with pulmonary arterial hypertension was found (p=0.062).

DISCUSSION

Several studies have evaluated vitamin D status in scleroderma patients, most of them reporting low levels, but only a few studies included a control group [16-22]. Low vitamin D levels in SSc patients were universal and independent of geographic origin or vitamin D supplementation [23]. However, there are no previous studies reporting vitamin D metabolism particularities in SSc patients.

Our study showed that most of the scleroderma patients have low levels of vitamin D (35% classified as deficiency, 35% as insufficiency). Still, there was no significant difference in the mean 25(OH) levels compared to the control group (p=0.67).

To evaluate if vitamin D metabolism is impaired in SSc patients, we measured the expression of mRNA for *VDR* and *CYP27B1* and *CYP24A1* in SSc PBMCs by comparison with control PBMCs. Our results showed that *CYP27B1 i*s expressed at a very low level while *VDR* mRNA is highly expressed in SSc PBMCs. The reduced expression level of *CYP27B1* suggests that SSc PBMCs may be unable to convert 25(OH)D to the active form. The active form of vitamin D can regulate its expression by suppressing *CYP27B1* and inducing *CYP24A1* [28]. In our case, the downregulation of *CYP27B1* may not be induced by an active form of vitamin D, since the circulating level of 25(OH)D was low. Moreover, SSc PBMCs did not express high levels of *CYP24A1* mRNA, thus confirming this hypothesis. Low 25(OH) D levels combined with low *CYP27B1* expression may have therapeutical implications suggesting that inadequate levels of vitamin D in SSc patients should be treated with an active vitamin D metabolite.

Vitamin D deficiency in SSc is potentially related to several factors. Dermal fibrous thickening with capillary damage could lead to reduced synthesis of pre-vitamin D3 from 7-dehydrocholesterol by ultraviolet-B radiation in the epidermis; gastrointestinal involvement and malabsorption could play an additional role; moreover, many patients with SSc experience a remarkable impairment in physical functioning, and are prone to a sedentary lifestyle and hence diminished sunlight exposure [18]. In our group there was a trend of correlation of vitamin D levels with Rodnan score, suggesting that the extent of cutaneous involvement might be a cause of inadequate D levels but not the only one.

Our study demonstrated that both vitamin D and VDR mRNA expression negatively correlated with pulmonary arterial hypertension. Vitamin D deficiency in pulmonary arterial hypertension is not well described, although studies on small groups report benefits [29]. Active forms of vitamin D may reduce the expression of VEGF and also endothelin [30,31].

For studied SSc patients strong correlations were found with DLCO values for both 25(OH)D level and VDR mRNA expression. This might be about calcitriol interference with this fibrotic process, acting on the TGFβ-pathway by regulating Smad-dependent transcription [32]. Vitamin D can inhibit fibroblast proliferation and myofibroblast transdifferentiation induced by TGFβ1 [33]. Moreover, vitamin D diminishes the expression of collagen I and III and fibronectin on myofibroblasts induced by *TGFβ1* [34]. 1,25(OH)2D pre-vents the upregulation of *col1a1* mRNA, and as a consequence, the synthesis of extracellular matrix proteins (i.e., actin, fibronectin, collagen) and the formation of stress fibres by fibroblasts [32]. Hence, calcitriol lessens the epithelial-to-mesenchymal transition of SSc-lung epithelial cells, stimulated by TGFβ [34]. Another pathway involved in tissue fibrosis is The Renin-Angiotensin-Aldosterone System (RAAS). In experimental animal models, 1,25(OH)2D can interfere also with the RAAS pathway, enhancing the expression of angiotensin-converting enzyme 2 and promoting the conversion of angiotensin II into angiotensin 1-7 with chemotactic roles [35].

Probably, in SSc patients impaired VDR signalling with reduced levels of its ligand vitamin D3 may contribute to hyper-active TGFβ signalling and aberrant fibroblast activation in SSc.

The increased expression of VDR mRNA that we identified in SSc PBMCs, in the context of reduced sera level of vitamin D may suggest activation of lymphocytes since the highest expression of VDR was reported in activated T cells [36]. Dysregulation of vitamin D processing by the immune cells of SSc patients is also supported by the lack of any correlation between VDR mRNA and CYP27B1 mRNA in SSc patients, which was identified for controls. These data also support the hypothesis that VDR upregulation might be a sign of cell activation, which in the absence of its ligand is not able to exert its effects.

CONCLUSION

Low vitamin D status, increased VDR expression and decreased expression of activating enzyme (*CYP27B1*) in SSc patients suggest defects in the local production of and response to active vitamin D. SSc patients with inadequate levels of vitamin D have more active and severe disease (pulmonary hypertension, lung involvement) and poor quality of life. Low levels of 25(OH) associated with decreased CYP27B1 mRNA expression may suggest the use of active vitamin D metabolites for supplementation. Deficiencies in vitamin D metabolism are accompanied by dysregulation of mRNA levels of some IFN signature genes and cathelicidin in PBMCs. Their defects are associated with a more severe form of the disease (lung involvement, joint contracture, inflammation, advanced peripheral damage). Further research would be needed to investigate the causes of low vitamin D status in SSc, its relevance to disease progression, its influence on immune and vascular abnormalities, and the potential effects of supplementation in the treatment.

DECLARATIONS

Supplementary Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization: Laura Groseanu, and Violeta Bojinca; Methodology: Laura Groseanu and Florian Berghea;

Software: Florian Berghea; Validation: Laura Groseanu, Violeta Bojinca, Andra Balanescu; Formal analysis: Daniela Opris-Belinski; Investigation: Laura Groseanu, Violeta Bojinca; Resources: Laura Groseanu; Data curation: Florian Berghea; Writing-original draft preparation: Laura Groseanu; Writing-review and editing: Violeta Bojinca; Visualization: Florian Berghea, Daniela Opris-Belinski; Supervision: Violeta Bojinca, Florian Berghea; Project administration: Florian Berghea; Funding acquisition: Andra Balanescu. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the Declaration of Helsinki.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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