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Association between vitamin D, oestradiol and interferon-gamma in female patients with inactive systemic lupus erythematosus: A cross-sectional study

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Abstract

Objectives: To investigate possible associations between 25-hydroxyvitamin D_3 ($25(OH)D_3$), oestradiol (E_2) and IFN-gamma (IFNγ) in female patients with inactive systemic lupus erythematosus (SLE).

Methods: Female patients with inactive SLE and age-matched healthy controls were recruited into this cross-sectional study. Serum concentrations of $25(OH)D_3$, E_2 and IFNγ were measured by radioimmunoassay with gamma-counters and enzyme-linked immunosorbent assay.

Results: 36 patients and 37 controls were enrolled. In patients with SLE, the concentration of $25(OH)D_3$ was lower and E_2 was higher compared with controls. In vitamin D deficient (i.e., $25(OH)D_3 \leq 20$ ng/ml) patients, IFNγ was 150% higher compared with patients with $25(OH)D_3 > 20$ ng/ml and controls. The concentration of E_2 was higher in all patients compared with controls independently of the vitamin D level. A difference was found between patients and controls in the correlation of $25(OH)D_3$ with E_2 and a positive correlation was found between E_2 and IFNγ in all participants.

Conclusions: Our results suggest that E_2 may have a strong modulating effect on vitamin D function which is significant only at low concentration of E_2.

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Introduction

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease and involves the loss of tolerance to nuclear self-antigens, immune complex formation and chronic inflammation.[1] The pathogenesis of SLE consists of a complex of cross-talk mediated by cytokines that orchestrate immune cell interactions.[2] Studies have shown that interferon gamma (INFγ) plays an important role in the development and severity of SLE.[3] In humoral immunity, IFNγ stimulates B-cell activation to induce immunoglobulin secretion, while an in cell-mediated immune response from IFNγ directs differentiation of T cells to a Th1 phenotype.[4–6]

The vitamin D status of an individual depends on access to vitamin D through dietary intake and epidermal synthesis from ultra-violet (UV) light exposure. Importantly, vitamin D reduces the risk for several diseases including severe infections, cancer and autoimmune rheumatic diseases because it regulates both innate and adaptive immunity.[7] Studies have demonstrated vitamin D receptor (VDR) expression in both T- and B-lymphocytes.[8] In addition, 1,25-hydroxy vitamin D_3 (1,25 $[OH]_2D_{3)}$ has been shown *in vitro* to inhibit the action of cytokines produced by Th1 immune cells.[9] Moreover, a direct effect of $1,25(OH)_2D_3$ on B-cell homoeostasis has recently been confirmed.[10] Therefore, vitamin D may have a role in B-cell-related autoimmune disorders such as SLE.

Reports suggest that patients with SLE have alterations in steroid hormone metabolism.[11] It has been demonstrated that oestrogen effects are probably mediated through oestrogen receptors α and β, which are expressed in a wide range of immune cells and are involved in innate and adaptive immune responses.[12] Oestrogens have specific effects on T and B cell maturation, dendritic cells and peripheral blood mononuclear cells.[13] They also cause rapid maturation of B cells in bone marrow where they cause auto-reactive B cell deletion to become less efficient.[14]

In a previous study in females of childbearing age with inactive SLE, an association between vitamin D, oestradiol (E_2), and IFNγ was not completely established.[15] Therefore, the aim of this study was to clarify the relationship further.

Methods

This was a cross-sectional study which conformed to recommendations by the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).[16] Female patients with inactive SLE and age-matched healthy controls were recruited at the Daily Hospital, Division of Rheumatology and Immunology of the Clinical Hospital Centre in Split, Croatia, from June to September 2014. The study complied with the Declaration of Helsinki and was approved by the Ethical Committee of Split University Medical School for clinical studies on human subjects. Written informed consent was obtained from all participants.

Inclusion criteria were: diagnosis of SLE according to American College of Rheumatology (ACR) criteria;[17] generative age (i.e., from puberty to menopause), 24h proteinuria <150 mg/day; maximum dose

of glucocorticoids $\leq$5 mg/day for at least 12 months; Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)[18] scores $\leq$2, for at least one year. Exclusion criteria included: hormonal replacement therapy; immunosuppressive drugs (e.g., azathioprine, methotrexate); anticoagulants; vitamin D supplements; smoking; primary and secondary hyperparathyroidism.

Blood samples were taken from all participants in the morning between 7:00 and 8:00. Antinuclear antibodies (ANA), anti-double-stranded DNA (anti-dsDNA), anti-Smith (anti-Sm), parathyroid hormone (PTH) and complement components C3, and C4, were measured in patients with SLE. Reference values were: ANA- negative U/ml; anti-dsDNA >40 IU/ml; anti-Sm >40AU/ml; complement C3 (0.9–1.8 g/L) and C4 (0.1–0.4 g/L). Complement components were determined using a laser nephelometry (ProSpec nephelometer, Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany).

Circulating levels of 25(OH)D_3 provide a direct reflection of vitamin D status.[19] Serum concentrations of 25(OH)D_3 were measured in samples from all participants using radioimmunoassay (RIA) and a gamma-counter (DIA source Immunoassays, Louvain-la-Neuve, Belgium catalogue number KIP 1961; P. I. Number 1700543/en; Revision nr.:130729/1). Intra-assay variation was 8.7% and inter-assay variation was 7.3%. Vitamin D deficiency was defined as <20 ng/ml, insufficiency was 21–29 ng/ml and normal range was 30–80 ng/ml.[19] Serum concentrations of E_2 were also measured using RIA and gamma-counter and intra-assay variation was 6.3%, and inter-assay variation was 10.3%. Oestrogen status was assessed from samples taken from the 3rd–5th day of the menstrual cycle. Reference values for E_2 were between 0.11–0.65 nmol/L. Levels of IFNγ was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA) which had a detection limit of 0.99 pg/ml.

Statistical analyses

Sample size was estimated using PASS software (Release 11, NCSS, LLC. Kaysville, Utah, USA; 2011). Using a standard deviation (SD) of 2.08 ng/ml for the concentration of 25(OH)D_3 with $\alpha = 0.05$ and 1-β, it was estimated that 36 patients with SLE were required for the study.[15] Statistical analyses were performed using PASS software (Release 11, NCSS, LLC. Kaysville, Utah, USA; 2011) and $P < 0.05$ was taken to indicate statistical significance.

Differences in serum 25(OH)D_3, IFNγ, and E_2 between patients and controls were analysed using the Mann–Whitney U test and area under the curve (AUC) was used as the measure of standardized effect together with absolute and relative median differences. Differences between four groups (i.e., low and high levels of vitamin D in patients and controls) were analysed using the Kruskal–Wallis test. Statistical significance from post-hoc tests were corrected by the Holm-Bonferroni method. Correlations between numeric variables were analysed using Spearman's rank correlation. Cut-off values for serum 25(OH)D_3, IFNγ, and E_2 were determined using Receiver Operating Characteristic (ROC) curve analysis and Youden's index J. The independent association of serum 25(OH)D_3, IFNγ and E_2 levels with SLE was assessed using multivariate binary logistic regression.

A measurable increase in SLE disease activity in one or more organ systems involving new or worse clinical signs and symptoms and/or laboratory measurements was deemed an SLE flair.[20] The moderating effect of serum E_2 on the association of serum 25(OH)D_3 and SLE flair was analysed using "Process" release 2.12 (Andrew

F. Hayes, Ohio State University, 2014).[21] Serum E_2 values defining the region of a significant association between serum 25 $(OH)D_3$ and SLE flair were assessed by the Johnson–Neyman technique as implemented in "Process".[21] Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using a multivariate, binary logistic regression model to determine the disease characteristics associated with vitamin D deficiency, IFNγ and E_2 levels.

Results

In total, 36 female patients with inactive SLE and 37 age-matched healthy controls were included in the study. Demographic and clinical characteristics of the participants are shown in Table 1. The median value of $25(OH)D_3$ was statistically significantly lower in patients with SLE compared with controls ($P = 0.001$), while the median values for IFN-γ and E_2 were statistically significantly higher in patients with SLE compared with controls ($P < 0.001$).

Participants were further divided into two groups based on $25(OH)D_3$ levels (i.e., $\leq$20 ng/ml and >20 ng/ml). In the control group, there were no differences in IFN-γ, E_2, and PTH between participants with $25(OH)D_3$ $\leq$20 ng/ml or >20 ng/ml and so data were combined and compared with the two SLE subgroups (Table 2). Patients in $25(OH)D_3 \leq 20$ ng/ml sub group had statistically significantly higher levels of IFNγ compared with patients in the $25(OH)D_3$ >20 ng/ml sub-group or control group ($P < 0.001$). Both the $\leq$20 ng/ml and >20 ng/ml $25(OH)D_3$ sub-groups of patients had statistically significantly higher levels of E_2 than the control group ($P = 0.009$ and $P = 0.035$, respectively).

For all participants, the correlation between E_2 and IFNγ was positive and

Table 1. Demographic and clinical characteristics in patients with systemic lupus erythematosus (SLE) and controls.

	SLE patients ($n = 36$)	Control group ($n = 37$)	Median difference		Statistical significance*	AUC
			Absolute	Relative		
Age, years	40 (33–43)	39 (32–42)				
Disease duration, years	10 (8–13)	–				
Glucocorticoids, mg	2.3 (0.00–3.63)	–				
25 $(OH)D_3$, ng/ml	16.5 (12.7–20.9)	22.9 (18.1–26.4)	−6.4	28%	$P = 0.001$	0.27
IFN-γ, pg/ml	2.4 (1.0–5.7)	1.0 (1.0–1.0)	2.4		$P < 0.001$	0.77
E_2, nmol/l	0.53 (0.36–0.61)	0.33 (0.29–0.40)	0.20	61%	$P < 0.001$	0.25
PTH, pg/ml	13.9 (12.4–16.5)	15.1 (13.1–16.9)	−1.2	8%	*n.s.*	0.43
ANA, U/ml	26 (72.2)	–				
Anti-dsDNA, IU/ml	44.5 (7.3–245.3)	–				
Anti-dsDNA, >40 IU/ml	19 (52.8)	–				
Anti-Sm, AU/ml	44.5 (7.3–245.3)	–				
Anti-Sm, > 40AU/ml	2 (5.6)	–				
C_3, g/l	0.84 (0.73–0.95)	–				
C_4, g/l	0.13 (0.09–0.20)	–				

Data are presented as median (interquartile range) or *n* (%)

$25(OH)D_3$, 25-hydroxy vitamin D_3; IFN-γ, interferon gamma; E_2, oestradiol; PTH, parathyroid hormone; ANA, antinuclear antibody; anti-dsDNA, anti-double-stranded DNA; anti-Sm, anti-Smith; C_3 and C_4, complement components; AUC = Area under the curve; IU, international units, AU, arbitrary unit; *n.s.*, not statistically significant

*Mann–Whitney U test

Table 2. Distribution of participants according to serum level of 25(OH)D_3

	SLE patients		Controls		
	25(OH)D_3 >20 ng/ml	25(OH)D_3 ≤20 ng/ml	25(OH)D_3 >20 ng/ml	25(OH)D_3 ≤20 ng/ml	Statistical significance*
Patients, *n*	9	27	26	11	$P < 0.001$
IFN-γ (pg/ml)	1.0 (1.0–4.7)	2.5 (1.0–6.8)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	$P < 0.001$
E_2 (nmol/l)	0.58 (0.42–0.63)	0.48 (0.35–0.59)	0.33 (0.29–0.38)	0.34 (0.29–0.55)	$P = 0.001$
PTH (pg/ml)	13.6 (12.9–15.4)	14.3 (12.2–17.2)	15.1 (12.8–16.4)	15.0 (13.2–20.2)	*n.s.*

Data are presented as median (interquartile range).
SLE, systemic lupus erythematosus; 25(OH)D_3, 25-hydroxy vitamin D_3; IFN-γ, interferon gamma; E_2, oestradiol; PTH, parathyroid hormone; *n.s.*, not statistically significant
*Kruskal–Wallis test

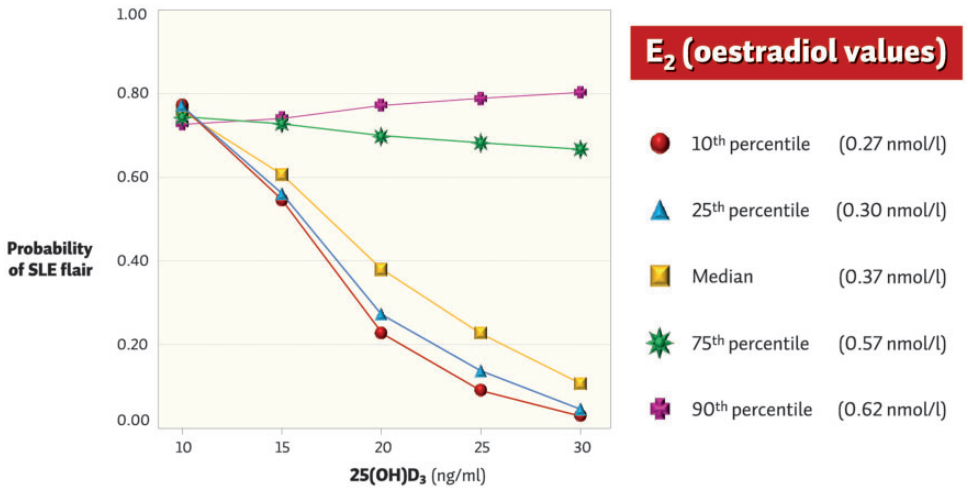

Figure 1. Association of 25(OH)D_3 with systemic lupus erythematosus (SLE) flair at different values of oestradiol (E_2) in female patients (*n*=36) with inactive disease.

statistically significant (Spearman's $\rho = 0.25$; $P < 0.05$; data not shown). In addition, there was a statistically significant ($P = 0.03$) difference between patients and controls in the correlation of 25(OH)D_3 with E_2 (data not shown). These findings suggested an interaction between 25(OH)D_3 and E_2 with regard to SLE flair. A moderator analysis was performed and a statistically significant ($P = 0.03$) interaction between 25(OH)D_3 and E_2 in regard to SLE flair was observed.

At low levels of E_2 (i.e., 0.27 mmol/l; 10th percentile), the association between 25(OH)D_3 and SLE flair was statistically significant ($P = 0.002$) (Figure 1). The higher the 25(OH)D_3, the lower the probability for SLE flair in patients with low E_2 levels. However, at high values of E_2 the association between 25(OH)D_3 and SLE flair was not statistically significant. The Johnson–Neyman technique showed that the association between 25(OH)D_3 and SLE flair was statistically significant

($P < 0.05$) when E_2 values were ≤ 0.473 nmol/l. In our sample, 63% of patients with SLE had $E_2 \leq 0.473$ nmol/L.

The OR for an SLE flair was almost 10 times higher in patients with IFNγ>1.3 pg/ml compared with those with IFNγ $\leq$1.3 pg/ml (95% CI; 2.15, 45.69: $P = 0.003$). The OR for an SLE flair was 7.6 times higher in patients with 25(OH)D_3 $\leq$20 ng/ml compared with those with 25(OH)D_3 >20 ng/ml (95% CI; 2.05, 27.86: $P = 0.002$). In addition, the OR for an SLE flair was 5.4 times higher in patients with E_2>0.345 nmol/L compared with those with $E_2 \leq 0.345$ nmol/L (95% CI; 1.39, 20.87: $P = 0.015$). Therefore, all three targeted parameters were independent of each other and were statistically significantly associated with SLE flair.

Discussion

This study was designed to investigate the association between vitamin D, E_2 and IFN-γ in female patients with inactive SLE. Vitamin D is an important hormone with immunomodulatory properties and has a vital role in many biological and biochemical pathways.[22–24] It has been suggested that UV exposure may catalyse symptom exacerbation or flare events in patients with SLE.[25,26] As a consequence, patients are often advised to adopt sun protective measures and use both physical and chemical barriers routinely ad these measures may result in vitamin D deficiency.[26] Indeed, many patients with SLE have been found to have a deficiency in 25(OH)D_3.[27]

Interest in the association between 25 (OH)D and systemic SLE began in the early 2000's following observations that vitamin D levels were associated with low bone mass in patients with SLE.[28] Lack of sun exposure and use of hydroxychloroquine (HCQ) was thought to be responsible for the low vitamin D levels in these patients.[29] Following these initial findings, several more studies confirmed the association between SLE and 25(OH)D.[27,30,31] Several studies have now shown a positive association between disease activity and vitamin D deficiency in patients with SLE.[32–34] In addition, one study found a negative correlation between the serum concentration of vitamin D and disease activity which suggests a possible protective role for vitamin D in SLE.[33] However, data from a randomized, placebo-controlled, clinical trial involving 90 patients with Vitamin D-deficient SLE showed that vitamin D supplementation did not affect disease activity.[35] Data from this present study, in female patients with inactive SLE showed that their blood levels of vitamin D were lower compared with healthy controls. In accordance with our previous findings, we also found that patients with low levels of vitamin D (i.e., 25(OH)$D_3 \leq 20$ ng/ml) had a 7.6 times higher risk for the development of SLE flair compared with patients with higher levels of vitamin D (i.e., 25(OH)$D_3 > 20$ ng/ml).[15] These results suggest a beneficial effect of vitamin D on disease activity.

The importance of IFNγ in the pathogenesis of lupus was highlighted by a study in lupus mice that found accelerated disease in mice receiving IFNγ or its inducers, while those receiving anti-IFNγ antibodies had a significantly delayed onset of disease.[36] The ability of IFNγ to promote B cell autoantibodies and activate IgG Fc receptors and complement may also contribute to disease severity.[37] This present study showed that levels of IFNγ were higher in patients with inactive SLE compared with controls. In addition, in patients with low levels of vitamin D (i.e., 25(OH)D_3 $\leq$20 ng/ml), IFNγ was 150% higher than patients with higher levels of Vitamin D (i.e., 25(OH)$D_3 > 20$ ng/ml). Furthermore, the risk of an SLE flair was almost 10 times higher in patients with IFNγ >1.3 pg/ml compared with patients

with IFNγ $\leq$1.3 pg/ml. These results emphasize the importance of cytokine balance in SLE.[38] However, studies examining the role of IFNγ in SLE have been contradictory. Some have found a correlation between serum IFN-γ level and disease activity and a correlation between IFNγ expression and severity of lupus nephritis while others show decreased IFNγ levels in lupus nephritis.[39,40] Nevertheless, monoclonal antibodies targeting IFNγ are being investigated for the treatment of SLE.[41]

Oestrogens are considered immunomodulating hormones.[42] One study suggested that E_2 may promote innate immunity by enhancing production of IFNγ from CD11c(+) cells.[43] Others studies have proposed that E_2 has a direct effect on the production of immunoglobulin (Ig) G anti-dsDNA antibodies as well as total IgG in peripheral blood mononuclear cells from patients with SLE.[44,45] However, opinions differ about the influence of disease activity on ovarian function. Some studies have found a relationship between SLE activity and menstrual cycle disturbances, while others have failed to confirm an association.[46–49] In the present study, the risk of a flair was 5.4 times higher in patients with E_2>0.345 nmol/L compared with those with $E_2\leq$0.345 nmol/L. Our results also showed a positive correlation between E_2 and IFNγ and a difference in the correlation between 25(OH)D_3 and E_2 in patients and controls. These findings suggest an interaction between 25(OH)D_3 and E_2 with regard to the probability of SLE reactivation. Interestingly, at low concentrations of E_2, the association between 25 (OH)D_3 and SLE flair was statistically significant but the association was not significant at high concentrations of $E_{2,}$. We hypothesise that the conditional association between 25(OH)D_3 with SLE flair at different values of E_2 suggests a possible pro-inflammatory effect of oestradiol. Furthermore, these findings suggest that vitamin D may lose a protective effect at higher oestrogen levels. Since Vitamin D and oestrogen act via receptors on immune cells,[50,51] including B lymphocytes which are important in the pathogenesis of SLE, we suggest that competition for receptor sites or an altered post-receptor response could explain this proposed effect. In accordance with this suggestion is the observed beneficial effects of anti-oestrogens, such as tamoxifen, in patients with SLE.[52] It is interesting that vitamin D and oestradiol appear to have an interwoven action in SLE. Several studies have shown that the active form of vitamin D, calcitriol, regulates the expression of aromatase.[53–55] In addition, in young women both progesterone oestrogen levels decreased after 4 weeks of vitamin D supplementation .[56]

Our study had several limitations. For example, the sample size was small and the study had a cross-sectional design. More longitudinal research is needed to determine the validity of the cross-sectional associations between 25(OH)D_3 and E_2 observed in this study. In addition, to maintain homeostasis in patients with SLE, an evaluation of hormone and cytokine status should be considered. A better understanding of the hormonal and cytokine status in patients with SLE may lead to future strategies for disease prevention. Furthermore, more studies are required in patients with inactive SLE using high doses of vitamin D for long periods.[57]

In conclusion, a positive correlation was found between E_2 and IFNγ in all participants and a difference was observed between patients and controls in the correlation of 25(OH)D_3 with $E_{2.}$ Furthermore, we observed a significant interaction between vitamin D and E_2 with regard to the probability of SLE flair. Our results suggest that oestrogens have a strong modulatory effect on vitamin D function, and this dual effect has a protective effect on

SLE flair only at low oestrogen levels. Based on our results, we suggest that vitamin D supplementation may be useful in SLE patients.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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