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ORIGINAL ARTICLE

Vitamin D status and *CYP27B1*-1260 promoter polymorphism in Tunisian patients with systemic lupus erythematosus

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Abstract

Aim: An association between serum vitamin D (Vit D) levels and systemic lupus erythematosus (SLE) has been reported by several studies that suggested the involvement of genetically determined characteristics of enzymes of vitamin D metabolism. Our study aimed to evaluate the relationship between 25 hydroxyvitamin D (25[OH] D) level, the most representative metabolite of VitD status, and polymorphism of the cytochrome P450, *CYP27B1* gene, which influence vitamin D metabolism, and serum levels, in SLE Tunisian patients.

Material and Methods: A cross-sectional study has been conducted in SLE patients (supplemented and not supplemented patients), matched to healthy controls by age and gender. The 25[OH]D serum level was measured by chemiluminescence assay and *CYP27B1*-1260 genetic polymorphism was carried out using PCR-RFLP methods. Statistical analysis was made using Shesis and SPSS.20 Software.

Results: Controls and Vit D not supplemented patients' groups presented the highest percentage of hypovitaminosis D. A significant difference in the mean level of circulating 25[OH]D between Vit D supplemented SLE patients and controls was observed (23.91 ng/ml and 7.18 ng/ml, respectively $p = 3.4\ 10^5$). Our results showed a correlation of high 25[OH]D level with complement component 3 levels and prednisolone drug. Moreover, the analysis of *CYP27B1*-1260 polymorphism in SLE patients and controls revealed a nonsignificant allelic or genotypic association.

Conclusion: Despite the sunny climate, the high prevalence of Vit D deficiency is common in Tunisia. This hypovitaminosis D feature may affect the Vit D levels in our SLE patients but a direct association with the disease or with the genetically determined features remains unclear. More studies are needed to establish thresholds and susceptibility genes according to the characteristics of each population.

1 | INTRODUCTION

Vitamin D (Vit D) has a pluripotent effect and regulates various processes, such as the promotion of apoptosis; induction of cellular differentiation with the simultaneous, inhibition of proliferation; inflammation; angiogenesis; invasion; and metastasis(Arnson et al., 2007; Holick, 2007). Indeed, Vit D boosts innate immunity and suppresses adaptive immunity; it indirectly affects T-cell polarization and shifts the immune response toward tolerance (Adorini & Penna, 2008), and affects antibody production (Cutolo et al., 2011).

The bio-active form of Vit D, named calcitriol (1-α, 25 dihydroxy vitamin D3), is responsible for gene regulation. Calcitriol is formed by the addition of a hydroxyl group to 25-hydroxyvitamin D (25[OH]D), which is the circulating measurable form, determining the vitamin D status (Peelen et al., 2011). This reaction is the result of the enzymatic activity of 25-hydroxyvitamin D3-1α-hydroxylase (CYP27B1) (DeLuca, 2004), which is encoded by *CYP27B1* gene, located on 12q14.1 (Genetic Home References) and consists of nine exons (Kong et al., 1999). This enzyme is classified in the family of mitochondrial cytochrome P450 enzymes (Fu et al., 1997). The translation product is a protein containing 508 amino acids with an N-terminal mitochondrial signal sequence and a heme-binding site (Fu et al., 1997).

Vit D deficiency or insufficiency has been reported as a possible environmental risk factor for various autoimmune diseases, especially systemic lupus erythematosus (SLE) (Colotta et al., 2017; Shoenfeld et al., 2018). SLE is a multi-system autoimmune disease that affects young women (Pons-Estel et al., 2010). Therapeutic management depends on the type and severity of organ involvement and includes nonsteroidal anti-inflammatory drugs, hydroxychloroquine, corticosteroids, and immunosuppressive agents (Rahman & Isenberg, 2008).

A meta-analysis study demonstrated an inverse correlation between 25[OH]D levels and disease activity of SLE (Petri et al., 2013; Sahebari et al., 2014), albeit inconsistently. Indeed, the direct relationship between them has not been established which suggests the impact of the genetically determined features of several key cytochromes P450 (CYP) enzymes of vitamin D metabolism. The activity of CYP27B1 is altered by nonsynonymous single nucleotide polymorphisms (nsSNPs). These alterations affect the synthesis of the active form of the hormone, the calcitriol, resulting in Vit D insufficiency. Rs10877012 and rs4646536 are the two most frequently studied SNPs in *CYP27B1* gene because of their relatively high minor allele frequencies (MAF) (de Souza et al., 2014). While rs4646536 is a 6th intronic variant (de Souza et al., 2014) and was not found to be associated with Vit D deficiency in many studies (Bailey et al., 2007; Orton et al., 2008), rs10877012 is located in the promoter region and may impact the transcription and the translation processes. The reference allele of rs10877012 is G and the alternative allele is T (position 1260 of *CYP27B1* gene). The MAF of this genetic variant oscillates around 27.83% (TopMed study, global population) (de Souza et al., 2014). Several reasons argue for the possible role of this variant in SLE. The association between its functional impact and SLE disease is still not very clear, however, the indirect impact was observed on the level of 25[OH]D circulating in the blood and affected the microenvironment through antigen-presenting, dendrite, and regulatory immune cells (McGrath et al., 2010; Vidigal et al., 2017).

Since the available data on vitamin D metabolism-related parameters in North Africa SLE patients remains rare, the objectives of our study were (1) to determine the prevalence of 25[OH]D deficiency and (2) to analyze the polymorphism of *CYP27B1* gene (rs10877012) in Tunisian Vit D supplemented or not SLE patients.

2 | MATERIAL AND METHODS

2.1 | Study design

Our case-control study involved adult groups collected from the same population living on the east coast of Southern Tunisia and characterized by a Mediterranean climate. This study enrolled 106 patients hospitalized between 2017 and 2019 at the Internal Medicine Department, Hedi Chaker University Hospital, Sfax, Tunisia, and who met at least four of the American College of Rheumatology revised criteria for SLE (Hochberg, 1997). SLE patients were selected based on inclusion and exclusion criteria (see Table S1). The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and Systemic Lupus International Collaborating Clinics (SLICC) damage index was used to assess disease activity and cumulative damage, respectively, in each patient SLICC classification criteria improved the clinical relevance of the ACR criteria, incorporated findings on the immunology of SLE. Baseline demographic, clinical, and biological data are collected at the time of recruitment including age, gender, skin type, photosensitivity, malar or discoid rashes, oral or nasal ulcers, arthritis, serositis (pleuritis or pericarditis), nephritis, and neurological disorders (seizures or psychosis). Biological features involve results of blood cell count (anemia, leukopenia, lymphopenia, or thrombocytopenia), screening for antinuclear antibodies (ANA). A positive titer of ANA (>1:160) is followed by auto-antibodies typing (anti-dsDNA, anti-Sm, anti-RNP, anti-Ro/SS-A, and/or anti- La/SS-B). Data about the use of oral vitamin D

supplementation, and type of treatment received by the patients (hydroxychloroquine, corticosteroids, and immunosuppressant drugs) were also collected.

The healthy control group consisted of 196 healthy volunteers matched and recruited according to the patient's' acquaintances.

The present study was approved by the Research Ethics Committee of the Habib Bourguiba University Hospital of Sfax (protocol number 4/12). All participants provided written consent before the study. Serum samples were collected for the measurement of 25[OH]D and blood samples for DNA extraction.

2.2 | Serological study

For 25[OH]D measurements, 59 enrolled SLE patients were selected in the same period based on the status of the disease. Vit D supplementation use was assessed by self-reported medication history of this patient's group within the last year and defined them as two subgroups: 11 not supplemented (newly diagnosed SLE patients) *group a* and 48 Vit D supplemented SLE patients *group b*. The serum samples were separated from whole blood and were kept at −80°C until biochemical analysis. Serum 25[OH]D levels were measured in duplicate by chemiluminescence assay (Elecsys® Vitamin D total, Cobas, Roche®) according to the manufacturer's instructions. 25[OH]D levels ≥20 ng/ml were considered normal. 25[OH]D levels <20 ng/ml were considered as indicative of vitamin D deficiency and <10 ng/ml as indicative of vitamin D insufficiency.

2.3 | Genetic study

Genomic DNA was extracted from whole blood samples of all participants included in our study, using a standard proteinase K digestion and phenol/chloroform extraction procedure. Rs10877012 (*CYP27B1*#NG_007076.1) polymorphism was genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The sequence of the primers examining the polymorphism was as follows: forward primer 5-GCCTGTAGTGCCTTGAGAGG-3 and reverse primer 5-CAGTGGGGAATGAGGGAGTA-3 (Invitrogen®) (Falleti et al., 2013). The PCR amplification was carried out in a volume of 25 µl including 1x buffer, 2 mM MgCl2, 0.2–0.4 µmol of each primer, 0.12 mMdNTP (Invitrogen), 1U Taq polymerase (Invitrogen), and 50 ηg of DNA template. Enzymatic digestion was performed in a total of 10 µl mixture reaction containing 1x buffer, 0.1x BSA, and 2U *PfeI* restriction enzyme (Thermo Fisher®), selected using the NEBcutter software (http://nc2.neb.com/NEBcutter2/).

2.4 | Statistical analysis

A case-control analysis was performed using SHESIS software (http://analysis.bio-x.cn) for the genetic study. Hardy–Weinberg equilibrium (HWE) was assessed in controls using a χ^2 test with one degree of freedom. A threshold was regarded to indicate a deviation from HWE. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for each allele using 2 × 2 contingency tables to estimate the magnitude of the association.

Comparison of Vitamin D level between the different groups and the correlation study between variables were analyzed using the SPSS (Statistical Package for Social Sciences, Inc) version 20.0. The Kruskal–Wallis, a nonparametrical test, was used to compare the data from subgroups of patients and the control group. The correlation between 25(OH)D levels and the variables used to assess lupus nephritis, disease activity, and autoantibodies positivity was calculated using Pearson's correlation coefficient. Results were considered significant at $p < 0.05$ and correlations were considered clinically significant if they exceeded 0.5.

3 | RESULTS

3.1 | Description of the study cohort

In the genetic study, our cohort is composed of 106 SLE patients and 196 healthy subjects. The demographic characteristics of the study and control participants were collected (data not shown). No significant differences were observed in the demographic characteristics and the gender repartition of patients and control groups (90% women vs 10% men).

The serologic study included a subgroup of 59 SLE patients and the total healthy subjects 'group. All demographic, clinical, biological, and therapeutic data are mentioned in Table 1.

3.2 | Vitamin D levels in the population study

Our results show that the mean serum value of 25[OH]D in total participants was 10.28 ng/ml (minimum: 2.5 and maximum:70.5 ng/ml). A significant difference was observed between men and women (19.24 ng/ml and 9.88 ng/ml; respectively, $p = 3.8\ 10^4$). This significant difference was maintained only in the control group (16.2 ng/ml and 7.18 ng/ml; respectively, $p = 0.002$).

SLE patients presented a significantly higher mean level of 25[OH]D, compared to controls (20.39 ng/ml and 7.18 ng/ml respectively, $p = 1.79\ 10^8$, $t = -5.427$) (Figure 1). The

TABLE 1 Demographic, clinical, and laboratory features of SLE patients of the serological study

Patient features	n = 59
Demographic profiles[a,a]	
Females	52 (88.13%)
Mean age [min-max]	37.8 [21–63]
Criteria for diagnostic classification	
Photosensitivity	24 (40.67)
Malar rash	26 (44.06)
Oral ulcers	10 (16.94)
Arthritis	58 (98.3)
Hemolytic anemia	39 (66.1)
Nephritis	25 (42.17)
Pleurisy	11 (18.64)
Raynaud syndrome	4 (6.77)
Immunologic disorders[a,a]	
Anti-dsDNA	36 (61.01)
Anti-Sm	11 (18.64)
Anti-Nuc	24 (40.67)
Anti-Ro/SSA	25 (42.17)
Anti-La/SSB	12 (20.33)
Anti-RNP	20 (33.89)
SLEDAI	1 (0–2)
Hypo-C31	31 (52.54)
Hypo-C41	26 (44.06)
Hypo-CH50l	14 (23.72)
Treatments*	
Untreated (*group a*)	11 (18.64)
Steroids/prednisone	35 (59.32)
Chloroquine/hydroxychloroquine	35 (59.32)
Azathioprine1	6 (10.16)
Calcium carbonate	29 (49.15)

Abbreviations: ANA, antinuclear antibodies; SLEDAI, systemic lupus erythematosus disease activity index.

[a]Data expressed as n, number of individuals (%).

*Result in the time of blood collection.

prevalence of Vit D insufficiency (25[OH]D serum concentration <10 ng/ml) was lower in SLE patients (40%) than in the control participants (81%) ($p = 0.001$, $t = -3.35$). Moreover 15% of both SLE patients and controls had serum concentrations of 25[OH]D ranging from 10 ng/ml to 20 ng/ml.

Regarding SLE subgroups, there was a significant difference between the mean serum value of 25[OH]D in *group a (*newly diagnosed SLE patients) and *group b* (Vit D supplemented SLE patients*)* (10.94 vs 23.91 ng/ml, $p = 0.005$) and between *group b* and controls (23.91 vs 7.18 ng/ml, $p < 0.001$) (Figure 2). However, no significant difference was observed between the mean serum values of 25[OH]D in *group a* and controls.

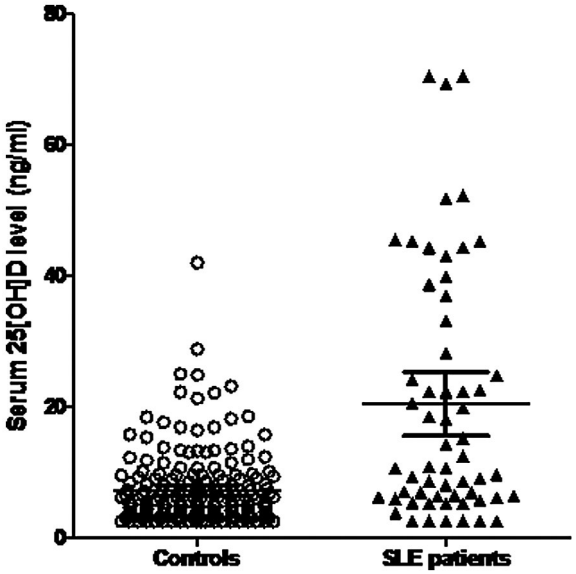

FIGURE 1 Serum (media, Range) 25[OH]D levels among SLE patients and healthy controls

3.3 | Vitamin D and its association with SLE disease activity

Considering the clinical and immunological data in SLE patients, no association was observed, except for the C3 level. Indeed, a high 25[OH]D level was negatively correlated with a complement inhibition C3 (hypo C3) ($p = 0.02$, CI 95% [0.016–0.023]).

Regarding the Vit D supplemented patients' group, 25[OH]D serum concentrations were higher in SLE patients treated with prednisone than in untreated patients ($p < 0.001$). Moreover, the oral administration of a high dose of Vit D increased significantly the 25[OH]D serum concentrations in SLE patients ($p = 0.02$) (Table 2).

While vitamin D serum levels were associated neither with the use of anti-inflammatory/immunosuppressive medications nor with calcium supplement, it is interesting to note that, within patients' group treated with hydroxychloroquine, a significantly higher mean value of 25[OH]D was revealed in patients receiving a dosage that exceeds 200 mg per day (for 200 mg/day, 25[OH]D =17.69 ± 2.57 and for 400 mg/days, 25[OH]D =34.17 ± 5.93 ng/ml, $p = 0.027$, CI 95% [−31.31~-1.61]).

3.4 | CYP27B1 promoter polymorphism (-1260) G/T genotype study

The rs 10877012 polymorphism frequencies were in accordance with the Hardy–Weinberg equilibrium in our SLE patients as well as in the control group (data not shown).

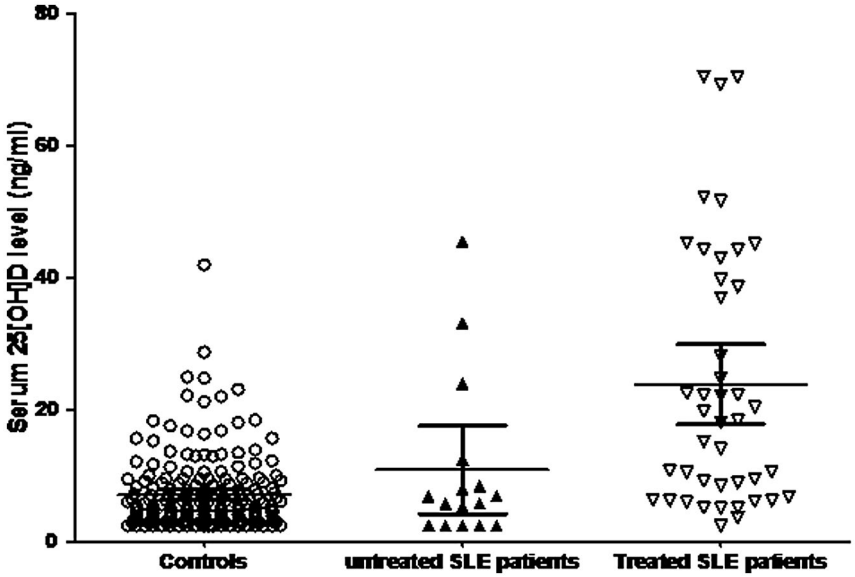

FIGURE 2 Serum (media, Range) 25[OH] D levels among not supplemented and Vit D supplemented SLE patients and healthy controls

TABLE 2 Associations between 25(OH)D serum concentration and the use of medications

			VitD supplemented SLE patients	
Treatment			**Treated patient**	**Untreated patient**
Prednisolone		n (%)	35 (92)	3 (8)
		25(OH)D mean ± SD (ng/ml)	26.57 ± 3.47	4.07 ± 2.35
		p values 95% CI	<0.001 [10.31–28.13]	
		High oral administration dose (2000 IU/day)	**Low oral administration dose (1000 IU/day)**	
Vitamin D supplement	n (%)	39 (81)	9 (19)	
	25(OH)D mean ± SD (ng/ml)	26.5 ± 3.54	12.57 ± 4.4	
	p values 95% CI	0.024 [2.01–25.84]		

TABLE 3 Distribution of the 1a-hydroxylase promoter polymorphism. *CYP27B1*-1260 in SLE patients compared to control individuals

rs10877012 (Allele)		
	G (freq)	**T (freq)**
Case	173 (0.816)	39 (0.184)
Control	183 (0.832)	37 (0.168)
Pearson's *p* value = 0.666754		
rs10877012 (Genotype)		
	GG (freq)	**GT (freq)**	**TT (freq)**
Case	70 (0.660)	33 (0.311)	3 (0.028)
Control	74 (0.673)	35 (0.318)	1 (0.009)
Pearson's *p* value = 0.578037			

There was no significant difference in the distribution of alleles and genotypes at the polymorphic site according to gender. Table 3 demonstrates the absence of a significant association between CYP27B1-1260 polymorphism and SLE disease status. The distribution of both alleles and genotypes frequencies do not show any association with the most common related clinical features (renal involvement, anemia, and malar rash). However, SLE patients carrying the G allele correlate with the presence of anti-SSA, anti-PMSCL, and ACLg Abs ($p = 0.031$, $p = 0.045$, and $p = 0.01$, respectively). The TT genotype was significantly associated with anti-Scl70 and ACLm ($p = 0.001$, and $p = 0.044$, respectively).

Interestingly, although not statistically significant, a higher 25[OH]D serum concentrations were observed among SLE patients carrying T allele of CYP27B1-1260 as compared with patients carrying G allele of CYP27B1-1260 (31.25 ± 7.72 ng/ml and 19.05 ± 2.86 ng/ml, respectively).

4 | DISCUSSION

Vitamin D deficiency is increasingly considered a global problem. Many researchers suggested that this deficiency may be an independent susceptibility factor for developing autoimmune diseases due to its involvement in the regulation of the immune system. Through the detection of bio-active 1,25(OH)2D is hard to perform due to the short half-life of this molecule (i.e., 4–6 h), the majority of serological studies were based at levels of 25[OH]D. Previous observations have found the low levels of 25[OH]D in SLE patients and demonstrated its relation with disease activity and severity (Kumar, 1986).

Our current study evaluated 25[OH]D serum levels in newly diagnosed, not supplemented, SLE patients, compared to Vit D supplemented SLE patients and to healthy control individuals in the Tunisian population.

Although our country is considered a sunny country, our present results indicate a high prevalence of vitamin D deficiency in our control group, especially in women. This result corroborates with a previous study that reported in healthy Tunisian adults, an overall prevalence of Vit D deficiency of 47.6%, with a higher prevalence in women ($p < 0.001$) (Meddeb et al., 2005). Indeed, the female gender is a classical risk factor for hypovitaminosis D in the general population (Carnevale et al. 2001; Mithal et al., 2009) and patients with SLE (Wright et al., 2009). This might be explained by androgen-related differences in Vit D binding protein levels, in the precursor production by the skin, or in the 25-hydroxylation by the liver or can mainly be attributed to lifestyle, particularly, outdoor activities or clothing lower body surface area (Pazaitou-Panayiotou et al., 2012). This notable deficiency has been reported among pregnant women in temperate regions, and in sunny countries (Ergur et al., 2009; Maghbooli et al., 2007; Sachan et al., 2005). Recently, a Tunisian study (Naifar et al., 2020) reported that healthy controls present a low Vit D mean level (7.88 ± 6.08 ng/ml) without considering them in hypovitaminosis D (Naifar et al., 2020). Our result, comforted by this study, supported that Tunisia, like the Middle East North African countries (MENA), was characterized by the highest prevalent Vit D deficiency (Amr et al., 2012; Hoteit et al., 2014; Prentice et al., 2009). Low Vit D concentrations were reported, also, among Saudi Arabians (Elsammak et al., 2011; Sadat-Ali et al., 2009), Oman (Al-Kindi, 2011), United Arab Emirates (Dawodu et al., 2011), and Qatar (Bener et al., 2009; Mahdy et al., 2010).

Among our SLE patients, only 37% have sufficient Vit D levels. A high prevalence of Vit D deficiency was revealed in the newly diagnosed SLE patients which is in agreement with Kamen et al. (2006) noting a similar level with a trend toward lower 25[OH]D levels in newly diagnosed cases (124 patients of Caucasian race) compared with controls. As well, Chen et al. (2007) reported the same observation in 12 newly diagnosed SLE patients, indicating that the deficiency may be present at the onset of lupus and possibly before.

However, SLE is not usually correlated with hypovitaminosis D; other studies showed variations in the Vit D level among lupus patients (Bogaczewicz et al., 2012; Damanhouri, 2009; Handono et al., 2013; McGhie et al., 2014). These variations may be derived from differences in duration of disease, latitude, season, and ethnicity (Iruretagoyena et al., 2015). Indeed, an extremely complicated and multifactorial interaction among various genetic, environmental, hormonal, and immunological factors is probably involved and SLE patients showed multiple additional risk factors for the induction of Vit D deficiency, which in turn seems to further influence the disease severity. Particularly, the reduced sun exposure due to photosensitivity, the use of photo-protection, the alteration of renal Vit D metabolism, as well as dark skin are all further explanations for Vit D insufficiency (Azrielant & Shoenfeld, 2016).

Regarding related SLE serological features, high titers of anti-dsDNA, and low complement levels, useful tools to monitor SLE disease activity, are associated with the disease (Giles & Boackle, 2013). Our results showed an exclusive significant association between Vit D level and hypo C3. This observation sustains previous studies supporting a crucial role of the complement system in SLE but disagrees with others demonstrating the absence of any association between serum levels of 25[OH]D and the presence or absence of the main disease manifestations. Indeed, SLEDAI scores did not differ significantly before and after Vit D supplementation in SLE patients, this result was supported by Karimzadeh et al. (2017).

Many drugs commonly used in disease management, such as glucocorticoids and hydroxychloroquine, might interfere

with Vit D metabolism and determine changes in the serum levels of 25[OH]D (Chaiamnuay et al., 2013; Huisman et al., 2001; O'Regan et al., 1979). This goes along with our results showing a sole association with prednisolone drug. We reported also that the administration of a high dose of vitamin D3 can be an effective treatment protocol to correct Vit D insufficiency in SLE patients. This observation corroborates with other studies (Close et al., 2013).

Genetic predisposition may also affect Vit D blood concentrations and it has to be accounted for in the Vit D deficiency studies. Genetic susceptibility to Vit D deficiency could be an additional possible explanation. As already mentioned, the main action of CYP27B1 is the formation of the bio-active form of Vit D from 25[OH]D. Therefore, the *CYP27B1*-1260 promoter polymorphism is regarded to be more important than polymorphisms within VDR genes at least in the development of endocrine autoimmune disorders (Bailey et al., 2007; Lopez et al., 2004). It is worth mentioning that numerous studies have linked this rs10877012 polymorphism to 25[OH]D levels. Moreover other studies suggested that the *CYP27B1*-1260 GG genotype constitutes a pathogenic cofactor in immune dysfunction by impairing intracellular 1,25(OH)2D3 levels in mononuclear and T cells. However, the impact of this genotype on *CYP27B1* expression is not fully known and the measurement of 25[OH]D levels is only a downstream effect of the potential impact (Vidigal et al., 2017). Despite these arguments, we did not reveal any significant association with SLE outcome nor with different clinical or serological parameters. Additionally, the genetic variation of CYP27B1 was not significantly associated with the response to Vit D_3 supplementation, which is in contradiction with the results of other studies (Hu et al., 2019).

According to our knowledge, our study presented one of the first examinations of the CYP27B1-rs10877012 genetic association with SLE in the Tunisian population. The allelic frequencies of rs10877012-T observed in all participants (SLE patients and controls) were higher compared to the data from the 1000 Genome project, focusing on the African population and European subpopulations (Tomei et al., 2020). These findings could be explained by the important genetic diversity of our Tunisian population. Future studies across North Africa may increase our understanding of the historical demographic factors influencing the genetic background in the region (Cherni et al., 2016).

Our study is among the rare association studies focusing on 25[OH]D levels, Vit D pathway gene, and SLE risk in the North of Africa population. However, because of the presence of some limitations, we cannot establish a causal relationship between Vit D serum concentration and disease activity in SLE patients. Some parameters related to Vit D status were not properly assessed; the season, skin pigmentation as a surrogate of sun exposure, and the duration and timing of sun exposure were not determined. These factors varied from day to day depending on weather conditions, clothing, and occupational tasks, and most of the participants were unable to properly respond to this query. The lack of estimation of Vit D intake using a food frequency questionnaire is another limitation of the study. However, it is well recognized that the estimation of Vit D dietary intake using dietary questionnaires is less accurate than the measure of circulating Vit D. It is also possible that the sample size or gender specificity may have affected our results.

In conclusion, although our study revealed a high frequency of Vit D deficiency and insufficiency among patients with SLE compared to healthy controls, there was not any significant correlation between its serum levels with severity of the disease, except for C3 levels. However, the correction of Vit D status may be beneficial in controlling inflammation and disease activity. Prospective studies are needed to investigate and establish a potential causal relationship between Vit D status and disease activity in SLE.

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CONFLICT OF INTEREST

None of the authors has any potential financial conflict of interest related to this manuscript. The authors alone are responsible for the content and writing of the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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