

# TNF- $\alpha$ , TNF- $\beta$ and IL-10 gene polymorphism and association with oral lichen planus risk in Saudi patients

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## ABSTRACT

**O**bjectives: Oral lichen planus (OLP) is a chronic inflammatory oral mucosal disease. Cytokines play an important role in the pathogenesis and disease progression of OLP. Various reports have implicated cytokine gene polymorphisms in susceptibility to develop some immune mediated conditions including OLP. The purpose of this study was to investigate the association of tumor necrosis factor (TNF)- $\alpha$ , TNF- $\beta$  and interleukin (IL)-10 gene polymorphisms with the OLP risk. **Material and Methods:** Forty two unrelated patients with OLP and 211 healthy volunteers were genotyped for TNF- $\alpha$  (-308 G/A), TNF- $\beta$  (+252A/G), IL-10 (-1082G/A), IL-10 (-819C/T), and IL-10 (-592C/A) polymorphisms. **Results:** The frequencies of allele A and genotype GA of TNF- $\alpha$  (-308G/A) were significantly higher while allele G and GG genotypes were lower in OLP patients as compared to the controls ( $P < 0.001$ ). The frequency of GA genotype of TNF- $\beta$  (+252A/G) was significantly higher in patients than in controls while the AA genotype was completely absent in OLP patients. These results indicated that allele A and genotype GA of TNF- $\alpha$  (-308G/A) as well as the GA genotype of TNF- $\beta$  (+252A/G) polymorphisms are associated with OLP risk. The frequencies of alleles and genotypes of -1082G/A, -819C/T and -592C/A polymorphisms in IL-10 gene did not differ significantly between OLP patients and controls ( $P > 0.05$ ). However, haplotype ATA extracted from 1082G/A, -819C/T, -592C/A polymorphisms of IL-10 were more prevalent in OLP patients when compared to controls indicating its possible association with OLP susceptibility. **Conclusion:** It is concluded that TNF- $\alpha$  (-308G/A), TNF- $\beta$  (+252A/G) and IL-10 (-1082G/A, -819C/T and -592C/A) polymorphisms are associated with the susceptibility of OLP, thus giving additional support for the genetic basis of this disease.

**Keywords:** Oral lichen planus. Tumor necrosis factors. Interleukin-10. Genetic polymorphism.

## INTRODUCTION

Oral lichen planus (OLP) is a chronic inflammatory oral condition whose etiology is not fully elucidated. It is the prototype of oral lichenoid lesions characterized by T-cell mediated immune responses and abnormal epithelial keratinization cycles<sup>6</sup>. OLP manifests as white striations, white papules, white plaques, erythema, erosions, or blisters predominantly affecting the buccal mucosa, tongue and gingival. The OLP lesions may co-exist with

cutaneous and genital lesions, or may be the only disease manifestations. The prevalence of OLP ranges between less than 1 and 3% of the general population<sup>17</sup>. The prevalence of OLP in Arab countries including Saudi Arabia has been reported ranging from 0.35 to 1.7%<sup>2</sup>.

The OLP lesions are consistently more persistent than the dermal lesions and have been reported to carry a risk of malignant transformation to oral squamous cell carcinoma of 1-2% (reported range of malignant transformation 0-12.5%)<sup>10</sup>.

Clinically, OLP may be divided into three subtypes: reticular, erythematous (atrophic), and erosive<sup>23</sup> and more than one subtype of OLP may be present in an individual patient. The pathogenesis of OLP is very complex and involves possible antigen presentation by the oral keratinocytes that could be either of an exogenous or an endogenous origin<sup>9</sup>. This antigenic trigger is accompanied by a mixed inflammatory response comprising of mainly T-cells, macrophages, and mast cells, as well as the associated cytokines and cytotoxic molecules<sup>9,10</sup>. It is a disease associated with middle aged people and is more common among women<sup>4</sup>.

Cytokines play an important role in the pathogenesis and disease progression of OLP and a strong body of evidence suggests that OLP is a T-cell-mediated disease. The genetic factors that influence the immune function have been suggested to contribute to the OLP<sup>6,12</sup>. The gene polymorphisms of T-helper cell subtype Th1/Th2 cytokines, tumor necrosis factor (TNF)- $\alpha$ , and interleukin-10 (IL-10) have been reported to affect the susceptibility to, and the progression of OLP<sup>3,14</sup>. Recently Chauhan, et al.<sup>8</sup> (2013) suggested that proinflammatory cytokines are an important factor in understanding the disease burden of OLP and their comorbid factors. However, immunogenetic studies of OLP have given controversial results and the precise cause of OLP is still unclear.

TNF- $\alpha$  and TNF- $\beta$  genes are located in tandem on chromosome 6 between the Class I and Class II cluster of the major histocompatibility complex (chromosome 6p21.1–6p21.3). TNF- $\alpha$ -308G/A polymorphism (rs1800629) has been reported to be associated with several autoimmune/inflammatory diseases including OLP<sup>3,8</sup>. The genetic variation at position –308 of the TNF- $\alpha$  gene results in two allelic forms in which the presence of guanine (G) defines the common variant and the presence of adenine (A) defines the less common one. The A-allele of TNF- $\alpha$ -308G/A polymorphism displays increased gene transcription as compared to the common allele G. It has been shown to produce levels of TNF- $\alpha$  transcription 6–7 fold greater<sup>31</sup>. TNF- $\beta$  +252A/G polymorphism (rs909253) has been reported at position +252 within the first intron of the TNF- $\beta$  gene, consisting of guanine (TNF- $\beta$  +252G) on one allele and adenine (TNF- $\beta$  +252A) on the alternate allele<sup>18</sup>. The presence of G at this position defines the mutated allele known as TNF- $\beta$  1 (allele-1) which is a less frequent allele and is associated with higher TNF- $\alpha$  and TNF- $\beta$  production<sup>18</sup>.

Interleukin-10 (IL-10) gene maps to the junction of 1q31-q32. It shifts the Th1/Th2 balance by down regulating the Th1 responses and by suppression of pro-inflammatory cytokines, such as TNF- $\alpha$  and interferon gamma (IFN  $\gamma$ ) secretion<sup>26</sup>. Three

promoter polymorphisms: -1082A/G (rs18000896), -819T/C (rs1800871) and -592A/C (rs1800872) are reportedly involved in the IL-10 transcription rate, thereby directly affecting its production level<sup>19</sup>. The -1082G, -819C and -592C alleles (GCC haplotype) have been associated with elevated levels of IL-10 production<sup>15</sup> while ACC and ATA haplotypes exhibit intermediate and low IL-10 gene transcription respectively<sup>27</sup>. In this study we examined the association of five single nucleotide polymorphisms (SNPs) of TNF- $\alpha$ , TNF- $\beta$ , and IL-10 genes in the etiopathogenesis of OLP in Saudi patients.

## MATERIAL AND METHODS

### Study group

A total of 253 Saudi subjects visiting Prince Sultan Military Medical City (PSMMC), Riyadh, Saudi Arabia were recruited for this study. Forty two (16 male, 26 female) unrelated patients with oral lichen planus, ages ranging from 27 to 72 years, and 211 (140 male, 71 female) unrelated, healthy patients matched voluntary blood donors with ages ranging from 20 to 65 years from the same population were studied for polymorphisms in TNF- $\alpha$ , TNF- $\beta$  and IL-10 genes. Patients with any other inflammatory/autoimmune diseases were excluded from the study. This study was approved by the research and ethical committee of PSMMC and written informed consent was obtained from each subject before recruitment.

OLP was diagnosed according to the clinical manifestations and histopathological criteria of the World Health Organization. All patients were diagnosed through a review of the patient's history, physical examination and histological findings by an oral pathologist. A 4 mm punch biopsy of lesional tissue that extends into the submucosa was performed. Symptoms were bilateral, more or less symmetrical lesions, and a lace-like network of slightly raised gray-white lines (reticular pattern). Erosive, atrophic, bullous, and plaque-type lesions were only accepted as a subtype in the presence of reticular lesions elsewhere in the oral mucosa. Histopathological criterium were the presence of a well-defined band-like zone of cellular infiltration that was confined to the superficial part of the connective tissue, consisting mainly of lymphocytes, signs of "liquefaction degeneration" in the basal cell layer, and the absence of epithelial dysplasia<sup>28</sup>. No patient was suspected to have drug or restoration related lichenoid lesions, and no patient showed histologic signs of dysplasia. In cases where the clinical and histological findings were inconclusive, direct immunofluorescence (DIF) microscopy of perilesional mucosa was used to exclude autoimmune vesiculobullous disorders.

## PCR amplification

Genomic DNA was extracted from the peripheral blood of OLP patients and controls using the QIA amp<sup>®</sup> DNA mini kit (QIAGEN Hilden, North Rhine-Westphalia, Germany). TNF- $\alpha$ , TNF- $\beta$  and IL-10 genes were amplified using amplification refractory mutation systems (ARMS)-PCR methodology<sup>21</sup> to detect polymorphisms at positions -308 of TNF- $\alpha$ , +252 in intron1 of TNF- $\beta$  and at loci -592, -819, and -1082 of IL-10 genes. PCR amplification was carried out using Ready to Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Reactions consisted of 10 denaturation temperature cycles for 15 s at 94°C, annealing for 50 s at 65°C and extension for 40 s at 72°C. Then 25 denaturation cycles of 20 s at 94°C, annealing for 50 s at 59°C and then extension of 50 s at 72°C. Final extension was performed at 72°C for 7 min. A positive control was included in the PCR assay by amplification of the human growth hormone gene. The amplified products for the various samples were separated on the 1.5% agarose gel, stained with ethidium bromide and photographed.

## Statistical analysis

The frequencies of alleles and genotypes were calculated. Hardy-Weinberg equilibrium was determined using Hardy-Weinberg Equilibrium Calculator for 2 Alleles (<http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2alleles.html>). The Bayesian method of the PHASE program (version 2.1) was applied to reconstruct the haplotype (<http://stephenslab.uchicago.edu/software.html>). The differences in allele/genotype frequencies between patients and controls were analyzed by Fisher's exact test using the CalcFisher software<sup>13</sup>. *P* values  $\leq 0.05$  were considered significant. The strength of the association of the disease with respect to a particular allele/genotype is expressed by odd ratio interpreted as *relative risk* (RR) following the Woolf's method as outlined by Schallreuter, et al.<sup>22</sup> (1993). It was calculated only for those alleles/genotypes which were increased or decreased in OLP patients when compared to the control group. The RR was calculated for all the subjects using the formula given below:

$$RR = \frac{(a \times d)}{(b \times c)}$$

a = number of patients with expression of allele or genotype

b = number of patients without expression of allele or genotype

c = number of controls with expression of allele or genotype

d = number of controls without expression of allele or genotype.

Etiologic Fraction (EF) indicates the hypothetical genetic component of the disease. The values 0.0

- 0.99 are of significance. EF was calculated for a positive association only where  $RR > 1$  using the following formula<sup>25</sup>.

$$EF = \frac{(RR-1)f}{RR}, \text{ where } f = \frac{a}{(a+C)}$$

Preventive Fraction (PF) indicates the hypothetical protective effect of one specific allele/ genotype for the disease. PF was calculated for negative association only where  $RR < 1$  using the following formula<sup>25</sup>. Values  $< 1.0$  indicate the protective effect of the allele/ genotype against the manifestation of disease.

$$PF = \frac{(1-RR)f}{RR(1-f)+f}, \text{ where } f = \frac{a}{(a+C)}$$

## RESULTS

Among OLP patients the male to female ratio was 16:26 (1:1.6). There was no significant difference in clinical manifestation or prognosis comparing men to women in our study. Thirty-five patients (83.333%) had lesions on the buccal mucosa, three (7.14 %) each on the tongue and gingival while one patient (2.39%) had lesions on the palate. The majority of patients (85%) had white lichen. In our patients, reticular type OLP was the commonest (80.95%) followed by erosive (23.81%) and then atrophic types (4.76%).

The genotype and allele frequencies of TNF- $\alpha$  (-308G/A) and TNF- $\beta$  (+252A/G) promoter polymorphisms are presented in Table 1. In both the lichen planus patients and control groups the genotype distributions were in Hardy-Weinberg equilibrium. The frequency of the heterozygous genotype GA was significantly higher in OLP patients than in the control ( $P < 0.001$ ) whereas the frequency of homozygous genotypes GG was significantly lower in OLP than controls ( $P < 0.001$ ). The frequency of allele-A was significantly higher in OLP patients than control subjects ( $P < 0.001$ ). On the other hand, allele-G was significantly lower in OLP patients when compared to the control ( $P < 0.001$ ). The difference in frequency of the AA genotype between the two groups was not statistically significant.

The frequency of the GA genotype of TNF- $\beta$  (+252A/G) promoter polymorphism was significantly higher in patients group than in the controls. Homozygous AA genotype was completely absent in OLP patients whereas it was present in 12% of the controls. The frequencies of alleles of TNF- $\beta$  (+252A/G) polymorphism were not significantly different between OLP patients and healthy controls (Table 1). Albeit, the frequencies of allele-G were slightly higher in the OLP patients than they were in the control subjects.

The results of SNPs for IL-10(-1082G/A), IL-10(-592C/A), IL-10(-819C/T), and corresponding alleles and genotypes are summarized in Table 2. The genotype distributions of IL-10 polymorphisms in patient and controls were in Hardy-Weinberg equilibrium. The frequencies of -1082GG and

1082GA genotypes were slightly higher while the frequency of -1082AA genotype was lower in OLP patients when compared to the control subjects, however, the differences were not statistically significant.

Similarly the frequencies of alleles and

**Table 1-** Genotype and allele frequencies of TNF- $\alpha$  and TNF- $\beta$  variants in oral lichen planus patients and matched controls

Genotype/ allele	OLP (N=42)		Control (N=200)		P-value	RR	EF†/ PF
	N	%	N	%			
<b>-308 G/A</b>							
GG	5	11.9	110	55	<0.001*	0.11	0.257
GA	36	85.72	76	38	<0.001*	9.789	0.288†
AA	1	2.38	14	7	0.479	0.324	0.121
G allele	46	54.76	296	74	<0.001*	0.425	0.153
A allele	38	45.24	104	26	<0.001*	2.351	0.153†
<b>+252 A/G</b>							
GG	4	9.52	28	14	0.616	0.647	0.063
GA	38	90.48	148	74	0.025*	6.767	0.173†
AA	0	0	24	12	0.010*	-	-
G allele	46	54.76	204	51	0.55	1.163	0.026†
A allele	38	45.24	196	49	0.55	0.859	0.026

N=number of subjects, \*statistically significant, RR=relative risk, EF=etiologic fraction, PF=preventive fraction

**Table 2-** Genotype and allele frequencies of IL-10 variants in oral lichen planus patients and matched controls

Genotype/ allele	OLP (N=42)		Control (N=186)		P-value	RR	EF*/PF
	N	%	N	%			
<b>-1082G/A</b>							
GG	5	11.9	14	7.53	0.357	1.6	0.098*
GA	33	78.5	140	75.27	0.841	1.2	0.032*
AA	4	9.5	32	12.2	0.251	0.51	0.095
G-allele	43	51.2	168	45.16	0.334	1.3	0.047*
A-allele	41	48.8	204	54.84	0.334	0.78	0.048
<b>-819C/T</b>							
CC	12	28.5	88	41.71	0.122	0.559	0.086
CT	25	59.5	102	48.34	0.239	1.5	0.065*
TT	5	11.9	21	9.95	0.78	1.2	0.032*
C-allele	49	62	278	65.88	0.521	0.846	0.026
T-allele	30	38	144	34.12	0.521	1.182	0.172*
<b>-592C/A</b>							
CC	12	28.5	88	41.71	0.122	0.559	0.086
CA	25	59.5	102	48.34	0.239	1.5	0.065*
AA	5	11.9	21	9.95	0.78	1.2	0.032*
C-allele	49	62	278	65.88	0.521	0.846	0.026
A-allele	30	38	144	34.12	0.521	1.182	0.172*

N=number of subjects, \*statistically significant, RR=relative risk, EF=etiologic fraction, PF=preventive fraction

genotypes of IL-10 (-819C/T) and IL-10 (-592C/A) polymorphisms did not differ significantly between OLP patients and controls ( $P>0.05$ ). Albeit, the frequencies of genotypes -819 CT, -819 TT, 592 CA and 592 AA were higher in OLP patients while -819 CC and -592 CC genotypes showed a reverse pattern with lower frequency in OLP patients when compared to the controls (Table 2). The frequencies

of haplotypes extracted from 1082G/A, -819C/T, -592C/A polymorphisms of IL-10 are shown in Table 3. The haplotype ATA was significantly increased in OLP patients when compared to controls ( $P=0.008$ ).

The frequencies of alleles and genotypes of all five polymorphisms with gender are summarized in Table 4. No statistically significant difference was found in the frequencies of alleles or genotypes

**Table 3-** Haplotype analysis of IL-10 -1082G/A, -819T/C and -592C/A polymorphisms in OLP and control groups

Haplotype	OLP (N=42)		Controls (N=186)		P-value
	N	(Frequency)	N	(Frequency)	
GCC	7	(0.166)	42	(0.225)	0.533
ACC	13	(0.309)	82	(0.440)	0.165
ATA	20	(0.476)	48	(0.258)	0.008*
ACA	2	(0.047)	14	(0.075)	0.742

N=number of subjects, \*statistically significant

**Table 4-** Genotype and allele frequencies in male and female oral lichen planus patients

Genotype/ allele	Male (N=16)		Female (N=26)		P-value
	N	%	N	%	
<b>TNF-<math>\alpha</math> (-308)</b>					
GG	2	12.5	3	11.54	1
GA	14	87.5	22	84.61	1
AA	0	0	1	3.85	0.99
G-allele	18	56.25	28	53.85	0.99
A-allele	14	43.75	24	46.15	0.99
<b>TNF-<math>\beta</math> (+525)</b>					
GG	1	6.25	3	11.54	1
GA	15	93.75	23	88.46	1
AA	0	0	0	0	-
G-allele	17	53.13	29	55.77	0.82
A-allele	15	46.87	23	44.23	0.82
<b>IL-10 (-1082)</b>					
GG	1	6.25	4	15.38	0.63
GA	15	93.75	18	69.23	0.12
AA	0	0	4	15.38	0.27
G-allele	17	53.13	26	50	0.82
A-allele	15	46.87	26	50	0.82
<b>IL-10 (-819)</b>					
CC	6	37.5	6	23.08	0.48
CT	8	50	17	65.38	0.35
TT	2	12.5	3	11.54	1
C-allele	20	62.5	29	55.77	0.65
T-allele	12	37.5	23	44.23	0.65
<b>IL-10 (-592)</b>					
CC	6	37.5	6	23.08	0.48
CA	8	50	17	65.38	0.35
AA	2	12.5	3	11.54	1
C-allele	20	62.5	29	55.77	0.65
A-allele	12	37.5	23	44.23	0.65

of TNF- $\alpha$ , TNF- $\beta$  and IL-10 gene polymorphisms between male and female OLP patients.

## DISCUSSION

In this study 62% of the OLP patients were women. OLP has previously been reported to be more prevalent in females than males in American, British, Chinese, Iranian, Italian, Japanese and Spanish populations<sup>4,5,20</sup>. In our patients OLP was more frequent in the third to fourth decade of life and the median age of the patients group was 42 years. Reticular OLP was the most common (80.95%) with the buccal mucosa as the most affected site (83.33%). These results are compatible with previous studies from various ethnic groups<sup>5,20</sup>. There was no significant difference in the clinical manifestation or prognosis when comparing men to women however, in our study, these observations should be taken with caution as the study's sample size is small.

Our results on TNF- $\alpha$  promoter polymorphism in Saudi OLP patients suggested that genotype GA-positive individuals at position -308 of TNF- $\alpha$  are at higher risk for OLP, whereas GG genotypes might be negatively associated with the disease. Data analysis further demonstrated that allele A (TNF- $\alpha$  2 allele) increases the risk of OLP, whereas allele G (TNF- $\alpha$  1 allele) reduces OLP risk. Similarly higher frequencies of TNF- $\alpha$  308 A allele and -308 GA genotype have been reported in Italian<sup>7</sup>, Indian<sup>8</sup>, and Chinese patients with an OLP<sup>3,12</sup>. Predominance of the AA genotype was also reported in Thai<sup>14</sup> and Brazilian patients with OLP<sup>32</sup>. Further, genetic polymorphisms of cytokine-encoding genes are also known to predispose malignant diseases and have been associated with oral precancerous lesions (OPCLs). TNF- $\alpha$  (-308G/A), IL-6 and TGF- $\beta$  polymorphisms have been associated with the development of OPCLs in Taiwanese patients<sup>11</sup>.

Allele A (TNF- $\alpha$  2 allele) lies on the extended haplotype HLA-A1-B8-DR3-DQ2, which is associated with high TNF- $\alpha$  production. The allele A (TNF- $\alpha$  2 allele) has been demonstrated to be a much stronger transcriptional activator than the common allele G (TNF- $\alpha$  1 allele)<sup>31</sup>. Moreover, TNF- $\alpha$  (-308G/A) promoter polymorphism has a direct effect on TNF- $\alpha$  gene regulation (increased TNF- $\alpha$  expression) and may be responsible for the association of allele A with the high TNF- $\alpha$  phenotype and more severe diseases<sup>31</sup>. Further populations bearing a higher proportion of the allele A (TNF- $\alpha$  2 allele) are reportedly predisposed to several metabolic, degenerative, inflammatory, and autoimmune diseases<sup>1</sup>.

The results of this study also show that the GA genotype of TNF- $\beta$  (+252 A/G) was positively associated with OLP risk, whereas genotype

AA might be protective. This report is the first to show an association between TNF- $\beta$  (+252 A/G) polymorphism and OLP. This +252 A/G polymorphism on intron 1 of TNF- $\beta$  is in linkage disequilibrium with a missense variant (Thr 26 Asn) in exon3<sup>18</sup>. It is also closely linked to TNF- $\alpha$ , which is in linkage disequilibrium with HLA-A, B8, and DR3<sup>30</sup> and has been reported to define a high TNF- $\alpha$  expressing haplotype in addition to a modifying expression of TNF- $\beta$  itself<sup>18</sup>. TNF- $\beta$  (+252A/G) polymorphism has also been associated with numerous autoimmune diseases<sup>1</sup>. High serum and salivary levels of proinflammatory cytokine and TNF have been detected in OLP patients<sup>24</sup>. Thalidomide, a known TNF suppressant has been used to successfully treat OLP showing the implication of high TNF- $\alpha$  expressing genotypes of TNF- $\alpha$  and - $\beta$  polymorphisms in the pathogenesis of OLP<sup>29</sup>.

Our results show no significant differences in the frequencies of alleles and genotypes of IL-10 (1082G/A), IL-10 (-592C/A), IL-10 (-819C/T) polymorphisms between OLP patients and healthy Saudi controls. However, haplotype ATA from 1082G/A, -819C/T, -592C/A of IL-10 had significant association with OLP in Saudi patients. Two other studies dealing with the IL-10 gene polymorphisms in OLP populations could also not find any difference in the frequencies of IL-10 alleles and genotypes between the patients with OLP and healthy controls<sup>3,32</sup>. However, the -1082A/-819T/-592A (ATA) haplotype of the IL-10 polymorphisms, which correlated with a lower serum level of IL-10, has a significant association with OLP in a Chinese cohort with Han ethnicity<sup>3</sup>. Thus, this study together with the study from China supports that the low producer ATA haplotype of IL-10 polymorphisms is linked to the risk of OLP.

Our results also indicated that the frequencies of alleles and genotypes of all the five polymorphisms studied are independent of gender (Table 4). However, these results should be taken with caution as the number of males and females is too low for any significant conclusion.

Besides TNF- $\alpha$  and IL-10, a number of inflammation and immune regulation-related cytokines, including interferon-gamma (IFN- $\gamma$ ), IL-4, and IL-8, have been suggested to have an important role in the pathogenesis of OLP. Abnormal expression patterns of various inflammation-related cytokines, including IL-10, and TNF- $\alpha$ , in lesions, saliva, serum and peripheral blood mononuclear cells from patients with OLP has also been reported, which may reflect the immune dysregulation status of cytokines and emergence as central players in the immunopathogenesis of OLP<sup>16</sup>. Recently Carrozzo<sup>7</sup> (2014) suggested that the pro inflammatory milieu associated with degranulated mast cells might play a role in helping T cells breach the epidermal

basement membrane in OLP lesions.

## CONCLUSION

The polymorphisms in TNF- $\alpha$  (-308), TNF- $\beta$  (+252) and IL-10 (-1082, -819 and -592) gene are associated significantly with the risk of OLP susceptibility. However, further studies are required that use a larger sample size to confirm this association.

## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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