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## The genetic influence of VEGFA single nucleotide polymorphism (SNP) (rs1570360) on the risk, disease activity, and severity in systemic lupus erythematosus patients.

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## The Genetic Influence of VEGFA Single Nucleotide Polymorphism (SNP) (rs1570360) on the risk, Disease Activity, and Severity in Systemic Lupus Erythematosus Patients

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### ABSTRACT

**Background:** SLE is a chronic autoimmune illness in which numerous variables, including epigenetic, genetic, and environmental factors, interact to determine disease risk. The role of the VEGF single nucleotide polymorphism (SNP) in SLE patients was assessed in the current study.

**Aim of The Work:** TO determine whether the vascular endothelial growth factor (VEGF) 1154G/A single nucleotide polymorphism (SNP) is associated with systemic lupus erythematosus (SLE) risk & disease activity.

**Patients and Methods:** The study included 50 SLE patients and 50 healthy controls. Full clinical assessment and laboratory investigations were done for the SLE patients. For both patients and controls, the (VEGF) 1154G/A SNP was detected using real-time PCR. Systemic Lupus International Collaborating Clinics Damage Index (SLICC DI) and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) were assessed for SLE patients.

**Results:** the minor A allele of VEGFA (1154G/A) SNP was significantly higher in SLE patients with lower SLICC when compared to the healthy subjects (p-value 0.033).

**Conclusion:** According to the findings, the A allele of VEGFA (rs1570360) SNP may protect against SLE and be linked to a better clinical outcome.

**Keywords:** VEGF; RT-PCR; gene polymorphism.

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### INTRODUCTION

Rheumatic disorders are a wide and diverse category of illnesses with an autoimmune component <sup>1</sup>. SLE is a debilitating multisystem rheumatic illness with wide epidemiological heterogeneity<sup>2</sup> and is characterized by immune system dysfunction and phenotypic variability.<sup>3</sup> As a result, understanding the molecular pathways involved in the pathophysiology of SLE manifestations could be important for developing more effective treatment and prophylactic interventions.<sup>4</sup>

SLE is a chronic autoimmune disease caused by a lack of immunological tolerance and the development of autoantibodies on a long-term basis <sup>5</sup> and it is a chronic autoimmune illness in which numerous variables, including epigenetic, genetic, and environmental factors, interact to determine disease risk.<sup>6</sup> The existence of multiple autoantibodies, which resulted in the development and deposition of immunological complexes, could be regarded as disease markers.<sup>7</sup> Despite the fact that the death rate among SLE patients has dropped significantly, the disease still has a significant physical and psychological impact.<sup>8</sup> When vascular

endothelial cells are damaged and activated, the pathogenesis of SLE may begin. SLE manifests in Egypt in a wide range of clinical and immunological forms.<sup>9</sup>

Despite the diversity of appearances, the pathophysiology of rheumatic diseases is strikingly similar in many ways. With the help of inflammatory cytokines and growth factors, as well as the participation of vascular endothelium, inflammatory processes proliferate through many types of immune system cells and tissues.<sup>10</sup> One factor of them is Vascular endothelial growth factor (VEGF) which is still being studied for its impact on the genesis and progression of rheumatic illnesses.<sup>11</sup> However, in SLE patients, its serum level was noticeably elevated and may be used as a possible indicator of disease activity. VEGF may have a role in the pathogenesis of SLE, and as its concentration appears to be a measure of activity, it may be useful for disease monitoring and therapy planning.<sup>12</sup>

In individuals with knee osteoarthritis, serum and synovial Vascular Endothelial Growth Factor (VEGF) levels were linked to clinical, functional, radiographic, and ultrasound (U/S) severity.<sup>13</sup> The VEGF family includes placental growth factor, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and VEGF-F.<sup>14</sup> On chromosome 6p12, the VEGF gene is present. It has eight exons. The most potent angiogenic factors include VEGF. It promotes endothelial cell migration and proliferation, which results in neovascularization and a rise in vascular permeability, and hence increases angiogenesis.<sup>15</sup> VEGF, a member of the platelet-derived growth factor family and a crucial regulator of angiogenesis and vascular remodeling, is expressed in inflammatory cells such monocytes, lymphocytes, neutrophils, and eosinophils as well as tissues rich in blood vessels like lung tissue.<sup>16</sup> Additionally, since inflammatory cells secrete VEGF, the inflammatory component in many disorders may be represented by it.<sup>17</sup>

In autoimmune illnesses, angiogenesis is a symptom of inflammatory activation, indicating histopathologic processes such as endothelial cell proliferation and migration, as well as subsequent vascular remodeling.<sup>18</sup>

Angiogenesis is a significant pathogenic mechanism in autoimmune disorders, which are characterized by immune-mediated tissue destruction. The angiogenesis modulator VEGF is highly raised in numerous autoimmune disorders, including systemic sclerosis, rheumatoid arthritis (RA), and SLE.<sup>19</sup>

In people with systemic sclerosis, increased levels of the potent angiogenic molecule VEGF or decreased levels of its natural inhibitors may contribute to the development of vasculopathy.<sup>20</sup> The relationship between SLE and a VEGF gene polymorphism is rarely studied.<sup>21</sup>

The aim of the present study was to evaluate whether vascular endothelial growth factor (VEGF) 1154G/A single nucleotide polymorphism (SNP) is associated with systemic lupus erythematosus (SLE) risk & disease activity.

## PATIENTS AND METHODS

50 SLE patients and 50 healthy participants (normal controls) were recruited from Rheumatology and Rehabilitation Department at Beni-Suef University Hospital. All patients had previously been identified as having SLE according to European League Against Rheumatism/ American College of Rheumatology (EULAR/ACR) classification criteria (2019).<sup>22</sup> Any patient who had known to have underlying chronic diseases other than SLE was excluded.

All study participants had comprehensive clinical examinations and full history taking. Complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), liver, and renal function tests were done for all individuals. On the other hand, complement 3 (C3), complement 4 (C4), anti-double-stranded deoxyribonucleic acid (anti-dsDNA), Anti-nuclear antibodies (ANA), urine analysis, and 24-hour urinary proteins were done for SLE patients only. Patients with SLE underwent renal biopsy when necessary, and the findings were categorized in accordance with the International Society of Nephrology/Renal Pathology Society (ISN/RPS) lupus nephritis diagnostic system.<sup>23</sup>

The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to assess SLE disease activity<sup>24</sup> while the Systemic Lupus International Collaborating Clinics Damage Index (SLICC DI) was used to assess the extent of the damage.<sup>25</sup>

The research was approved by the Beni-Suef University Ethical Committee (FWA#: FWA00015574). Before being included in the study, all the patients gave their informed consent.

Detection of VEGFA SNP (rs1570360) by Real-time PCR: 3 ml of venous blood was drawn from each participant. The samples were kept at -20° C in the same vacutainer (EDTA tube) for DNA extraction. Using the QIAamp DNA Mini Kit, DNA extraction was conducted according to the manufacturer's procedure (cat. No 51104, QIAGEN).

The following primers were used:  
AGCCCGGGCCCCGAGCCGCGGTGGA[A/G]  
GGGCTGAGGCTCGCCTGTCCCCGCC. RT-PCR was used to detect the (VEGF) 1154G/A (rs1570360) SNP using the TaqMan allelic discrimination approach. Step-one real-time PCR of Applied Biosystems (USA) was used to execute the PCR reaction.

Statistical analysis: The Statistical Package for the Social Sciences (SPSS) version 22 was used. For numerical data, means, standard deviations (SD), median, and range were used. Qualitative data was represented using frequency and percentages. Pearson's Chi-square or Fisher's exact tests were used to determine the results. Quantitative data from two groups was compared using a student t-test or Mann-Whitney test. The Bonferroni method was used to correct the p-value when multiple comparisons were made. The odds ratio (OR) and its 95 percent confidence interval (CI) were obtained for risk estimation using logistic regression.

## RESULTS

SLE patients had a mean age of 39.2±9.4 years, with 35(70%) females and 15(30%) males, while the control group had a mean age of 40.1± 13 years, with 36 (72%) females and 14(28%) males. All clinical findings and laboratory investigations of SLE patients are summarized in table-1. The number of patients with renal disorder was 23 (46%), class 2 were 10 patients (20.0%), class 3 were 3 patients (6.0%) and class 4 were 10 patients (20.0%).

Clinical Findings	Mean±SD / Number (%)	Lab. investigations	Mean±SD / Number (%)
Disease duration (months)	45.8± 47.2	Hb(gm/dl)	10.6±1.8
Fever	9 (18%)	TLC (10 <sup>3</sup> /uL)	5.96±2.76
Malar rash	42 (64%)	Platelets(10 <sup>3</sup> /uL)	263.0±107.8
Discoid rash	2 (4%)	ESR (mm/hr)	66.1±33.3
Photosensitivity	38 (76%)	Creatinine(mg/dl)	1.02±0.22
Oral ulcers	29 (58%)	24 hrs urinary protein (mg/L)	1567.7±2557.1
Alopecia	15 (30%)	C3 (mg/dl)	83±42.8
Arthritis/arthralgia	28 (56%)	C4 (mg/dl)	15.7±10.8
Renal disorders	23(46%)	Positive ANA	50 (100%)
Hematologic disorders	17(34%)	Anti-dsDNA (+ve)	27 (54 %)
Serositis	17(34%)	SLEDAI	18±7.5
Neurologic	6 (12%)	Positive SLICC	13 (26%)
Vasculitis	4 (8%)	Positive CRP	15 (30%)
Myositis	0 (0 %)	Urea (mg/dl)	46.4 ± 27.1

**Table 1:** Clinical Findings & Laboratory investigations of SLE patients.

Abbreviations: anti- CCP: anti-cyclic citrullinated peptide, ESR; erythrocyte sedimentation rate, TLC: total leukocytic count, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus, Hb: hemoglobin, C: complement, CRP: C-reactive protein, ANA: antinuclear antibodies, SLICC: Systemic Lupus International Collaborating Clinics Damage Index, SLEDAI: systemic lupus erythematosus disease activity index, dsDNA: double-stranded deoxyribonucleic acid.

Regarding the genetic effect of VEGFA (rs1570360) SNP on SLE patients; no statistically significant correlation was detected between VEGF -1154 SNP genotype frequency distribution under the codominant, dominant and recessive models (Table-2). However, our study revealed that the A allele was predominant in the healthy group compared to the patients with p-value 0.033 (Table-3).

On comparing the homozygous mutant type (AA) in cases and controls, it was 34 % in SLE patients compared to 54 % in controls. With an odds ratio of 2.193, the difference was not statistically significant (P= 0.063 and 95 % CI:0.959-5.014). While the heterozygous genotype (GA) was more prevalent in cases (8%) in comparison to controls (4%). The difference was not statistically significant P= 0.685 with an odds ratio 0.690 (95 % CI: 0.116 - 4.127) as shown in Table-2.

		Patients (SLE) N (%)	Control N (%)	OR	95% CI	p-value
Codominant	GG	29 (58%)	21 (42%)	Reference	-	-
	GA	4 (8%)	2 (4 %)	0.690	0.116-4.127	0.685
	AA	17 (34%)	27 (54 %)	2.193	0.959-5.014	0.063
Dominant	GG	29 (58.0%)	21(42%)	0.524	0.237-1.160	0.330
	GA+AA	21(42.0%)	29 (58.0%)			
Recessive	AA	17 (34.0%)	27(54.0%)	0.439	0.196-0.984	0.132
	GA+GG	33 (66.0%)	23(46.0%)			

**Table 2:** Risk of SLE in different genetic models of VEGF (1154G/A).

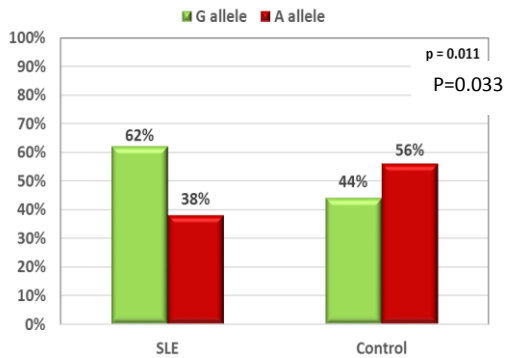
VEGF alleles	Groups		p-value	Odds ratio	95% CI
	SLE patients	Control			
A allele (% within the group)	38.0%	56%	0.011 (Corrected <b>0.033</b> )	0.482	0.274-0.847
G allele (% within the group)	62.0%	44.0%			

**Table 3:** Allele frequency distribution of VEGF in SLE and control groups.

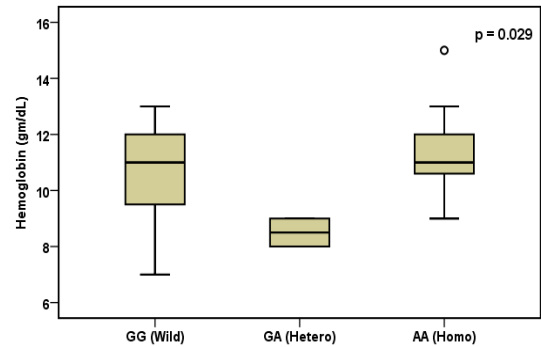
There was a statistically significant difference between the frequencies of the A allele, which was present in 38 percent of SLE patients and 56 percent of controls, and the G allele, which was present in 62 percent of SLE

patients and 44 percent of controls (p-value 0.033, odds ratio 0.482 and 95 % CI: 0.274 - 0.847) as demonstrated in Figure-1 and Table-3.

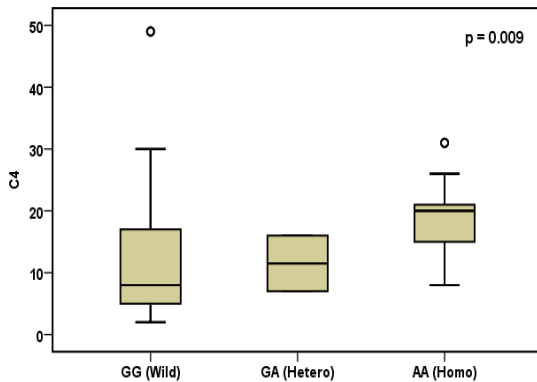
Regarding the distribution of genotypes according to age of the patients (P = 0.395), duration of the disease (p = 0.240), creatinine (P = 0.800), PLT (P=0.129), 24 hours urinary protein (P=0.062), SLEDAI (P=0.922) and C3 (P=0.202), there was no statistically significant difference between the patients. On the other hand, there was statistically significant difference on comparing different genotypes of VEGF -1154 SNP (G/A) on SLE patients in relation to ESR, Hb, TLC and C4 (p-value 0.007, 0.029, 0.031 and 0.009 respectively) as shown in Table-4 and Figures (2,3,4&5).



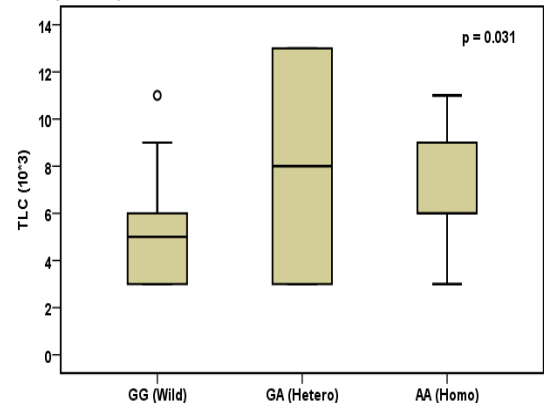
**Fig. 1:** VEGF (1154G/A) alleles in the SLE and control groups



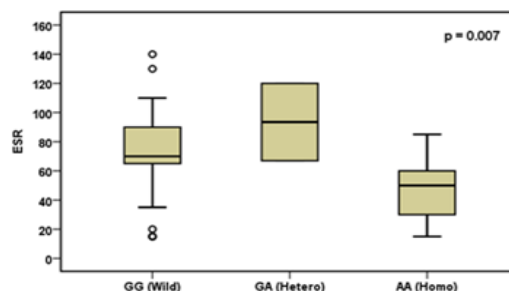
**Fig. 2:** VEGF genotypes (1154G/A SNP) regarding Hb in SLE cases



**Fig. 3:** VEGF genotypes (1154G/A SNP) regarding C4 in SLE cases



**Fig. 4:** VEGF genotypes (1154G/A SNP) regarding TLC in SLE cases



**Figure (5):** VEGF (1154G/A) SNP genotypes in relation to ESR in SLE cases

Comparing the different genotypes (GG, GA, and AA) of the VEGFA (1154G/A) SNP in relation to SLICC, fever, vasculitis, and serositis revealed a statistically significant difference (p-value 0.024, 0.023, 0.004 and 0.047 respectively) while there were no significant differences in the presence of malar rash, discoid rash, arthritis, renal illness, hematological diseases, alopecia, myositis, or neurological disorders (p-value > 0.05) as demonstrated in Tables (5,6 &7).

Variables	VEGF(1154G/A) Genotypes	N	Mean	SD	Median	Minimum	Maximum	P-value
Age	GG (Wild)	29	29.4	9.0	30.0	17.0	51.0	0.395
	GA (hetero)	4	23.5	9.7	19.0	18.0	38.0	
	AA (homo)	17	30.2	10.1	27.0	18.0	47.0	
Disease duration	GG (Wild)	29	39.7	37.0	48.0	1.0	120.0	0.240
	GA (hetero)	4	24.5	27.1	24.5	1.0	48.0	
	AA (homo)	17	61.4	62.2	36.0	6.0	240.0	
ESR	GG (Wild)	29	73.1	34.5	70.0	15.0	140.0	<b>0.007*</b>
	GA (hetero)	4	93.5	30.6	93.5	67.0	120.0	
	AA (homo)	17	47.6	22.1	50.0	15.0	85.0	
Hb	GG (Wild)	29	10.5	1.8	11.0	7.0	13.0	<b>0.029*</b>
	GA (hetero)	4	8.5	0.6	8.5	8.0	9.0	
	AA (homo)	17	11.3	1.5	11.0	9.0	15.0	
TLC	GG (Wild)	29	5.07	2.05	5.00	3.00	11.00	<b>0.031*</b>
	GA (hetero)	4	8.00	5.77	8.00	3.00	13.00	
	AA (homo)	17	7.00	2.47	6.00	3.00	11.00	
PLT	GG (Wild)	29	248.9	101.7	240.0	31.0	555.0	0.129
	GA (hetero)	4	387.5	150.7	387.5	257.0	518.0	
	AA (homo)	17	258.3	94.8	252.0	25.0	420.0	
Creatinine	GG (Wild)	29	1.03	0.19	1.00	0.80	2.00	0.800
	GA (hetero)	4	1.00	0.00	1.00	1.00	1.00	
	AA (homo)	17	1.01	0.29	1.00	0.60	2.00	
Urea	GG (Wild)	29	43.4	16.3	40.0	19.0	100.0	0.234
	GA (hetero)	4	33.5	5.2	33.5	29.0	38.0	
	AA (homo)	17	54.6	40.5	38.0	19.0	140.0	
Protein 24 hours	GG (Wild)	19	1091.2	1057.2	900.0	120.0	3102.0	0.062
	GA (hetero)	2	11200.0	0.0	11200.0	11200.0	11200.0	
	AA (homo)	14	838.4	612.3	640.0	240.0	2011.0	
C3	GG (Wild)	29	82.9	48.2	89.0	14.0	194.0	0.202
	GA (hetero)	4	51.0	27.7	51.0	27.0	75.0	
	AA (homo)	17	90.6	33.2	89.0	50.0	160.0	
C4	GG (Wild)	29	13.9	12.7	8.0	2.0	49.0	<b>0.009*</b>
	GA (hetero)	4	11.5	5.2	11.5	7.0	16.0	
	AA (homo)	17	19.8	6.5	20.0	8.0	31.0	
SLEDAI	GG (Wild)	29	18.0	7.7	15.0	10.0	38.0	0.922
	GA (hetero)	4	16.5	2.9	16.5	14.0	19.0	
	AA (homo)	17	18.2	8.2	18.0	4.0	32.0	

**Table 4:** Relation between VEGF (1154G/A) SNP genotypes in SLE & different variables.

\*p <0.05 is significant

Parameters	Status	Number (N) / %	GG (Wild)	GA (hetero)	AA (homo)	Total	p-value
SLICC	-ve	N	19	2	16	37	<b>0.024</b>
		% within SLICC	51.4%	5.4%	43.2%	100.0%	
	+ve	N	10	2	1	13	
		% within SLICC	76.9%	15.4%	7.7%	100.0%	
Fever	No	N	20	4	17	41	<b>0.023</b>
		% within Fever	48.8%	9.8%	41.5%	100.0%	
	Yes	N	9	0	0	9	
		% within Fever	100.0%	0.0%	0.0%	100.0%	
	Total	N	29	4	17	50	
		% within Fever	58.0%	8.0%	34.0%	100.0%	
Vasculitis	No	N	29	2	15	46	<b>0.004</b>
		% within Vasculitis	63.0%	4.3%	32.6%	100.0%	
	Yes	N	0	2	2	4	
		% within Vasculitis	0.0%	50.0%	50.0%	100.0%	
Serositis	Total	N	29	4	17	50	<b>0.047</b>
		% within Vasculitis	58.0%	8.0%	34.0%	100.0%	
	No	N	16	2	15	33	
		% within Serositis	48.5%	6.1%	45.5%	100.0%	
	Yes	N	13	2	2	17	
		% within Serositis	76.5%	11.8%	11.8%	100.0%	
Total	N	29	4	17	50		
	% within	58.0%	8.0%	34.0%	100.0%		

Renal biopsy	Non-Lupus nephritis	Serositis				0.419		
		N	18	2	7		27	
		% within Renal biopsy	66.7%	7.4%	25.9%	100.0%		
	Lupus nephritis	N	11	2	10	23		
		% within Renal biopsy	47.8%	8.7%	43.5%	100.0%		
	Total	N	29	4	17	50		
		% within Renal biopsy	58.0%	8.0%	34.0%	100.0%		
CRP	-ve	N	7	0	8	15		
		% within CRP	46.7%	0.0%	53.3%	100.0%		
	+ve	N	22	4	9	35		
		% within CRP	62.9%	11.4%	25.7%	100.0%	0.129	
	Total	N	29	4	17	50		
		% within CRP	58.0%	8.0%	34.0%	100.0%		
DNA	-ve	N	14	2	7	23		
		% within DNA	60.9%	8.7%	30.4%	100.0%		
	+ve	N	15	2	10	27		
		% within DNA	55.6%	7.4%	37.0%	100.0%	0.908	
		Total	N	29	4	17	50	
			% within DNA	58.0%	8.0%	34.0%	100.0%	

**Table 5:** VEGF (1154G/A) polymorphism genotypes in relation to different parameters.

Parameters	Status	Number (N) / %	GG (Wild)	GA (hetero)	AA (homo)	Total	p-value
Malar rash	No	N	3	0	5	8	0.209
		% within Malar rash	37.5%	0.0%	62.5%	100.0%	
	Yes	N	26	4	12	42	
Oral ulcers		% within oral ulcers	61.9%	9.5%	28.6%	100.0%	0.457
	No	N	14	2	5	21	
	Yes	N	15	2	12	29	
Alopecia		% within alopecia	51.7%	6.9%	41.4%	100.0%	0.209
	No	N	23	2	10	35	
	Yes	N	6	2	7	15	
Neurological disorders		% within neurological disorders	40.0%	13.3%	46.7%	100.0%	0.217
	No	N	27	4	13	44	
	Yes	N	2	0	4	6	
Discoid rash		% within discoid rash	33.3%	0.0%	66.7%	100.0%	--
	No	N	27	4	17	48	
	Yes	N	2	0	0	2	
Arthritis		% within arthritis	100.0%	0.0%	0.0%	100.0%	0.911
	No	N	12	2	8	22	
	Yes	N	17	2	9	28	
Hematological diseases		% within hematological diseases	60.7%	7.1%	32.1%	100.0%	0.733
	No	N	19	2	12	33	
	Yes	N	10	2	5	17	
Myositis		% within myositis	58.8%	11.8%	29.4%	100.0%	--
	No	N	29	4	17	50	

**Table 6:** VEGF (1154G/A) polymorphism genotypes in relation to different parameters.

## DISCUSSION

SLE is an autoimmune disorder caused by defects in the congenital and adaptive immunological system, including clonal proliferation & amplification of autoreactive lymphocytes, development of autoantibodies, and elevated levels of several cytokines and other pro-inflammatory mediators. SLE develops as a result of a vicious cycle including autoantigen exposure followed by production of autoantibody, persistent inflammation, and tissue destruction and is influenced by genetic and environmental variables.<sup>26</sup>

VEGF gene polymorphism can impact VEGF activation, and some studies have linked VEGF gene polymorphism to SLE risk.<sup>27</sup> Therefore, our goal was to assess the genetic impact of the VEGFA (rs1570360) SNP on the risk of SLE incidence.

Regarding how VEGFA -1154 SNP affects SLE patients genetically, our results revealed no significant correlation between the SNP and risk of SLE in any of the three genetic models: codominant ( $p=0.685$  &  $0.63$ ), dominant and recessive ( $p=0.33$ , and  $p=0.132$ ; respectively). However, the analysis of the allele frequency distribution of the A and G alleles in SLE patients in comparison to the control group revealed a significant increase ( $p$ -value  $0.033$ ) in the percentage of the A allele compared to the G allele in the control group which may indicate that the minor A allele may have a protective role in the risk of SLE.

Our results regarding the frequency distribution of the genotype of VEGFA-1154 SNP in SLE are in accordance with the results of other studies done by Soltani et al.<sup>8</sup> Also, the results of the present study could be supported by Saravani et al who reported that genotypes of VEGF were observed to be SLE-protective.<sup>28</sup>

The present study demonstrated the absence of a significant correlation between the VEGF (rs699947) SNP and the activity of the disease in SLE patients as defined by the SLEDAI ( $p=0.922$ ). We examined the association between the VEGFA -1154 SNP and SLE disease severity as indicated by the SLICC damage score. Our results implicated that the presence of A allele might be linked to a less severe status in SLE patients ( $p$ -value  $0.024$ ).

There is a strong correlation between disease activity and the fall in C3 and C4 levels, which may not be true in all patients.<sup>29</sup> In the current study, we detected a significant difference in the mean C4 values in SLE, where the presence of the minor A allele is significantly associated with higher mean C4 levels compared to the G allele ( $p$ -value  $0.009$ ). While in the case of mean C3 levels in SLE in relation to the SNP, although the presence of the minor A allele is linked to greater mean C3 levels, however, the difference failed to be statistically significant ( $p$ -value  $0.202$ ).

On the other hand, Wenzhuang and his colleagues in their meta-analysis study proved that there was no correlation between the risk of SLE and the VEGF

gene polymorphism. It should be confirmed, however, by additional research.<sup>27</sup>

Sample size, different methods of analysis, and ethnic differences could explain the discrepancies between our results which reveal that the minor A allele of the VEGFA-1154 SNP has a protective role in SLE and that its presence in SLE patients may be linked to less serious illness and the findings of other studies.

The small sample size of this study is one of its limitations, reducing its statistical power. In addition, detection of one SNP (VEGFA 1154G/A) only was investigated.

## CONCLUSION

The presence of the A allele of the gene of VEGFA-1154 is greater in normal individuals compared to SLE patients, suggesting that its presence may give a protection against SLE. The presence of the A allele is associated with decreased ESR, SLICC/ACR damage scores, fever, vasculitis and higher C4 suggesting that it may be linked to reduced disease activity and severity.

Conflict of interest : none

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