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Evaluating the Expression Levels of Genes (ITGAM and TNFAIP3), and Cytokines (IFN- α and TLR-7) as Markers of Systemic Lupus Erythematosus in Iraqi Patients

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Abstract
Background: Systemic lupus erythematosus (SLE) is characterised by the formation of immune complexes in numerous organs, which can result in the loss of immunological tolerance and a range of clinical symptoms. Due to the clinical heterogeneity of the condition and multitude pathways that can result in its expression, making it necessary to identify potential genetic and secretory indicators of SLE to aid early diagnosis in asymptomatic or susceptible patients. Here, the demographics, the clinical manifestations, cytokines interferon α (IFN-α) and Toll-like receptor 7 (TLR-7) as well as expression levels of human integrin alpha M chain (*ITGAM*) and tumour necrosis factor (TNF) alpha induced protein 3 (*TNFAIP3)* genes were investigated in SLE patients in comparison with healthy subjects. **Patients and Methods:** Sixty patients clinically diagnosed with SLE, along with 60 age-matched healthy individuals (control) were recruited in the study. Serum levels of cytokines (IFN-α and TLR-7) as well as expression levels of *ITGAM* and *TNFAIP3* genes were measured using enzyme-linked immunosorbent assay (ELISA) techniques and real-time qualitative polymerase chain reaction (RT qPCR) respectively. **Results**: The results obtained showed significant overexpression of IFN-α in SLE patients compared to healthy subjects (i.e. 344.79±30.72 U/mL, SLE vs. 234.88±28.55 U/mL, healthy subjects) ($p = 0.01$). Similarly, the SLE patients had significantly higher mean TLR-7 levels compared to healthy subjects (i.e. 86.48±57.41 U/mL, SLE vs. 426.66±23.20 U/mL, healthy subjects) ($p = 0.001$). The gene expression analysis showed upregulation of *ITGAM* and *TNFAIP3* in SLE patients by 15.48 folds and 43.08 folds respectively. Also, receiver operating characteristic analysis showed that *ITGAM* had specificity of 100% with the sensitivity of 98% (AUC = 1.00), while *TNFAIP3* had the specificity of 98% with the sensitivity of 93% (AUC = 0.97). **Conclusion:** Cytokines IFN-α and TLR-7 as well as genes *ITGAM* and *TNFAIP3* proved as diagnostic markers of SLE as well as putative role in disease management.

Keywords: Lupus; Interferons; Genetic Markers; Toll-like Receptors; Cytokine Markers.

Introduction

SLE is an autoimmune disorder causing immune complexes to accumulate in multiple organs, leading to various symptoms . SLE's cause is unknown, but genetics, environment, gender, and immunological factors may contribute. It may cause permanent organ damage in patients . SLE affects 400,000 people annually worldwide, with an incidence rate of 5.14 (1.4 - 15.13) per 100,000 person-years.. Early detection of SLE can reduce flare-ups, increase healthcare usage, and lower financial burden . Criteria have been developed to diagnose SLE and distinguish it from SLE mimics. The European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) 2019 criteria aim to improve SLE classification. A weighted approach for each item in the criteria and a positive antinuclear antibody (ANA) (titre ⩾1:80) as the entry criterion was adopted . SLE diagnosis criteria include seven clinical domains and three immunological domains. SLEDAI is a validated model used to monitor disease progression and response to treatment . Some researchers have raised concerns about the specificity of anti-double stranded DNA and the reliance on a single autoantibody, ANA, for SLE diagnosis. Scientists have identified five genes that are consistently dysregulated in SLE patients through replicated observational genome-wide association study (GWAS). These genes are the human integrin alpha M chain (ITGAM), B-lymphocyte kinase genes (BLK), B- cell scaffold protein with ankyrin repeats 1 (BANK1), plant homeodomain (PHD) and ring finger domains 1 (PHRF1), Phox domains containing serine/ threonine kinase (PXK), and (TNFAIP3) . SLE cytokine imbalances cause inflammation, organ damage, and immunological failure via overexpression of IFN-α and IFN-γ, leading to overproduction of inflammatory proteins. Other key players in SLE include cytokines such as interleukins (IL-6, IL-1), and TNF-α, as well as immunomodulatory cytokines like IL-10 and transforming growth factor-β (TGF-β). IL-21 and IL-17 are relevant in autoimmunity, and new study on IL-2 has refocused attention on this cytokine in SLE studies (Fava and Petri, 2019). More

research on diagnostic and prognosis markers were needed to predict disease development, identify early stages, and forecast flare-ups. Creating biomarkers that detect subsets of patients is the ultimate goal for matching therapies with the right targets in clinical trials .Here, the expression levels of two SLE-associated genes ITGAM (encoding the human integrin alpha M chain, which is essential for mediating cell adhesion to integrin ligands, phagocytosis, and inflammatory cytokine production in macrophages) and TNFAIP3 (encoding TNF-induced protein 3, a potent suppressor of NF-kB pathways for immune homeostasis) as well as two SLE-associated cytokines, IFN-α and (TLR-7) were assessed in patients with SLE in comparison to healthy participants. The results of this investigation may point to important indicators for the non-invasive early diagnosis of SLE in individuals who may be at risk. Thus, the aim of the Study is identifying potential genetic and secretory indicators of (SLE) to aid early diagnosis in susceptible patients or to prevent the development of disease.

Patients and Methods

The study was conducted on clinically diagnosed SLE patients visiting clinics in Iraq. The study included patients ranging from 16 to 65 years old with SLE for one to fifteen years. The study included healthy individuals of the same age as controls. It was conducted from November 2022 to August 2023 with all subjects being Arab Iraqis. Ethical approval was obtained from the research and ethics committee of the College of Medicine, University of Kerbala in Iraq (No: 345:1-1-2022). Both groups consented after being informed about the study and its aims. Excluded from the study were participants with a history of connective tissue diseases, SLE, autoimmune diseases, inflammation, pregnancy, malignant tumours, and neurological disorders.

Data was collected through questionnaire interviews and venous blood samples were drawn from healthy volunteers and SLE patients to measure cytokine levels and isolate PBMCs. All protocols were followed to ensure health and safety. Plasma cytokine levels were measured in triplicate using ELISA kits. Cytokine assays were calibrated with World Health Organization (WHO) standards. Viable cell count was done using trypan blue stain in a haemocytometer chamber. The sample had 5×106 cells/mL with viability >95%. Blood samples were diluted with phosphate buffered saline (PBS) and mixed with Ficoll-Hypaque gradient to isolate peripheral blood mononuclear cells (PBMCs). RNA was extracted from the cells for gene expression analysis using RT-qPCR. Primers were designed using Primer 3plus and synthesized by Alpha DNA Ltd. (Canada). Gene expression was evaluated using SYBR green amplification and double delta Ct analysis. The reference gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the fold change in gene expression was measured as the ratio of the test group to the control group. A fold change of 1 indicates no change, numbers between 1 and 0 indicate decreased gene expression, and numbers greater than 1 indicate increased gene expression. Thresholds were established to standardize target gene expression and acquire precise Ct values. Thermal cycling settings are summarized in Table 1.

Table 1: The primer sequences for Genes of Interest and House Keeping Gene (GAPDH)

Primer	**Sequence** *(5′→3′ direction)*	**Primer size** *(bp)*	**Product size** *(bp)*	**Temp.** *(°C)*
ITGAM				
Forward	GCTGATGCCCAATAAAGATG	20	139	58
Reverse	TGTACACTTGAATGCCTTGT	20		
TNFAIP3				
Forward	GGTTGCTGTCATATTTGCTC	20	194	58
Reverse	TCGTCTGGGAAAAACTTAGG	20		
GAPDH				
Forward	GAAATCCCATCACCATCTTCCAGG	24	160	58
Reverse	GAGCCCCAGCCTTCTCCATG	20		

ITGAM: integrin-alpha M chain, TNFAIP3: tumour necrosis factor (TNF) alpha induced protein 3, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

SPSS-25 was used for data analysis. Simple statistical measures and tests were used to present and compare the data. The statistical significance was set at $p < 0.05$.

Results

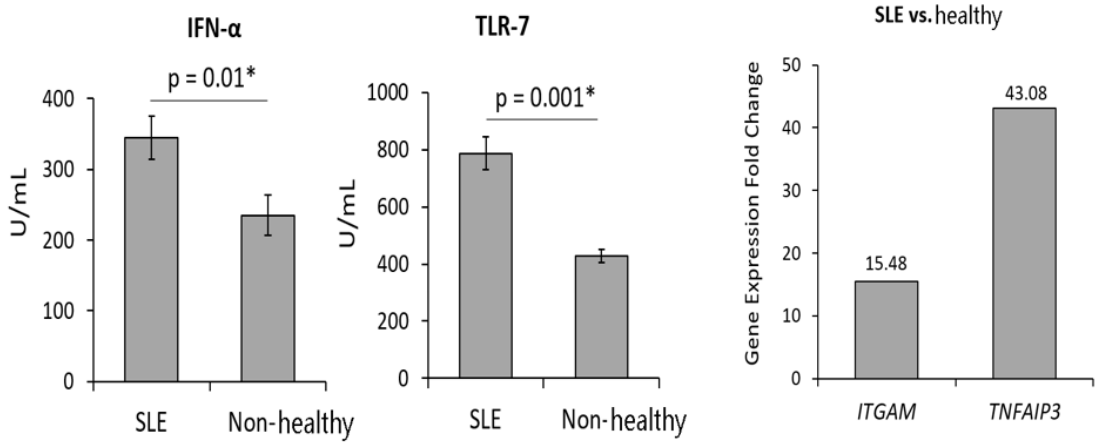
In this study, a total of 60 SLE patients and 60 healthy participants were recruited. The mean age of the SLE patients was 39.50 years which was not statistically different from that of the control group was 39.30 years ($p = 0.194$). Of the SLE patients, 73.3% had a family history of SLE. There was a statistically significant difference ($p = 0.01$) in the family history between the study groups in Table 2.

Table 2: Demography and Some Clinical Features of the Study Subjects

	SLE			**Healthy**			**p - value**
	All *(n = 60)*	**Female** *(n = 36)*	**Male** *(n = 24)*	**All** *(n = 60)*	**Female** *(n = 28)*	**Male** *(n = 32)*	*(SLE vs. healthy-subjects)*
Age, *years* (mean *(SEM)*)	41.53 *(1.71)*	40.21 *(1.83)*	39.50 *(2.04)*	39.30 (1.55)	37.38 *(1.52)*	41.09 *(1.47)*	0.194
Marital Status, *n (%)*							
Single	17 *(28.3)*	6 (16.7)	11 *(45.83)*	8 *(13.3)*	3 *(10.71)*	5 *(15.6)*	0.04
Married	43 *(71.7)*	30 *(83.3)*	13 *(54.17)*	52 *(86.7)*	25 *(89.29)*	9 27 *(84.4)*	
Family History of SLE, *n (%)*							
Yes	44 *(73.3)*	29 *(80.6)*	15 *(62.5)*	0 *(0.0)*	0 *(0.0)*	0 *(0.0)*	
No	16 *(26.7)*	7 *(19.4)*	9 *(37.5)*	60 *(100.0)*	28 *(100.0)*	32 *(100.0)*	
ANA, *IU/mL* (*mean (SEM)*)	8.79 *(2.04)*	10.08 *(1.24)*	7.53 *(2.82)*	0.91 *(0.03)*	0.93 *(0.04)*	0.90 *(0.03)*	0.01
SLE Disease Severity, *n (%)*							
Mild	36 *(60.0)*	21 *(58.3)*	15 *(62.5)*	-	-	-	
Moderate	19 *(31.7)*	11 *(30.6)*	8 *(33.3)*	-	-	-	
Severe	5 *(8.3)*	4 *(11.1)*	1 *(4.2)*	-	-	-	

SLE; systemic lupus erythematosus, SEM; standard error of mean, ANA; anti-nuclear autoantibodies
*Statistically significant at $p < 0.05$

The serum levels of cytokines IFN-α and TLR-7 were measured in SLE patients and healthy subjects. SLE patients showed significantly higher levels of both cytokines. ROC curves showed that TLR-7 had higher specificity (93%) and sensitivity (50%) than IFN-α (83% and 40%, respectively). The data is graphically presented in Figures 1 and 2.



(A)
SLE: systemic lupus erythematous, IFN-a: Interferon-a, TLR-7: toll-like receptor 7

(B)
SLE; systemic lupus erythematous, ITGAM: integrin-alpha M chain, TNFAIP3: tumour necrosis factor (TNF) alpha induced protein 3

Figure 1.(A). Mean Levels of Serum IFN-α and TLR-7 Cytokines, (B) Expression Levels of *ITGAM* and *TNFAIP3* Genes.

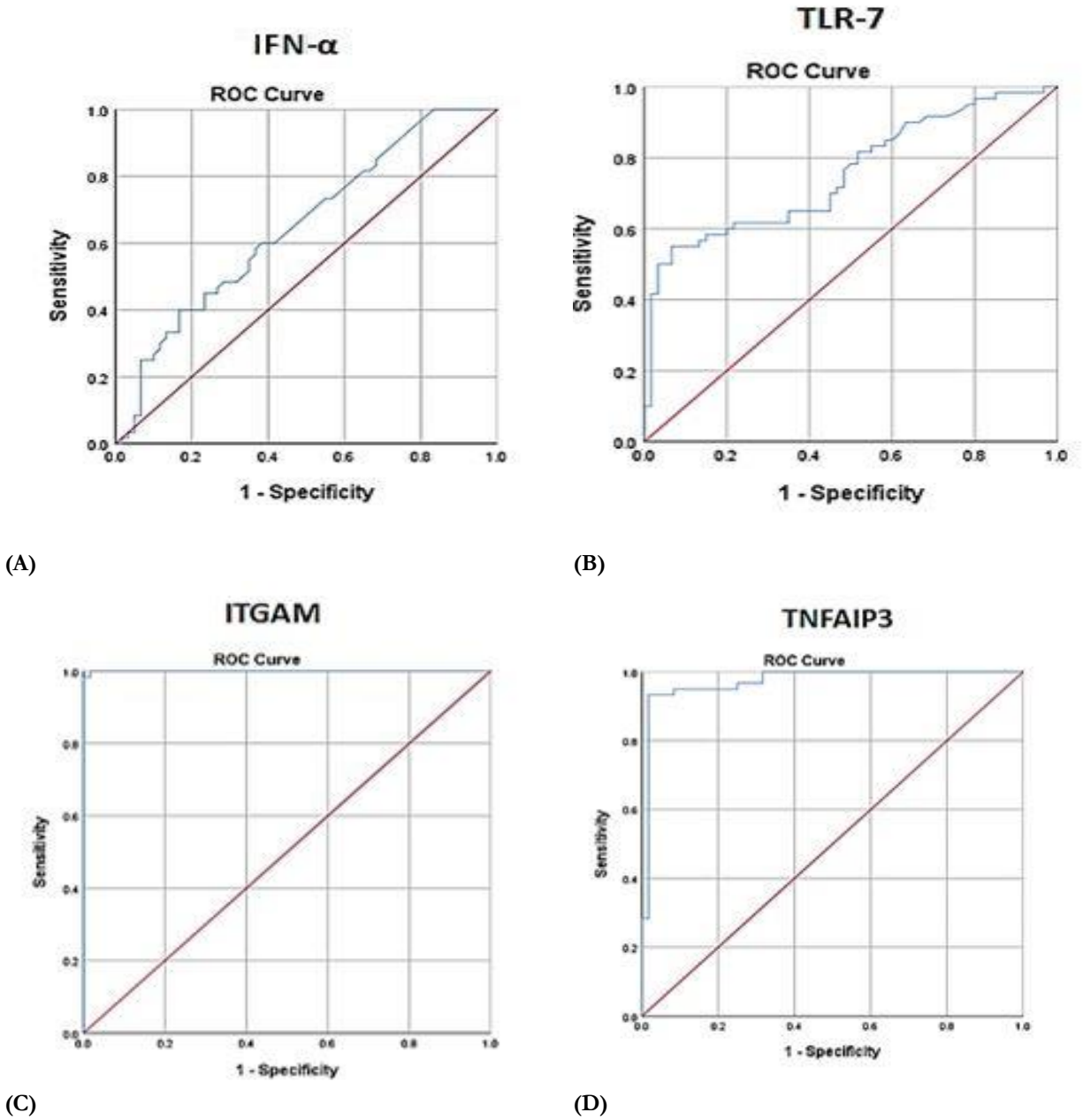


Figure 2: Receiver operating characteristic (ROC) Curve for the Measured (A) INF-α (B) TLR-7 Cytokine Levels (C) *ITGAM* (D) *TNFAIP3* Gene Expression Levels.

In SLE patients, the gene expression analysis showed a significant increase in ITGAM (15.48-fold) and TNFAIP3 (43.08-fold) compared to the healthy group. The ROC curves data revealed that ITGAM had 100% specificity and 98% sensitivity (AUC = 1.00), while TNFAIP3 had 98% specificity and 93% sensitivity (AUC = 0.97) as shown in Table 3.

Table 3: Receiver Operating Characteristic Curve Data for Cytokines INF-α and TLR-7 as well as *ITGAM* and *TNFAIP3* Genes

	Cytokines		Genes	
Parameters	**IFN-α**	**TLR-7**	***ITGAM***	***TNFAIP3***
AUC	0.65	0.75	1	0.97
Interpretation	Fair	Good	Excellent	Excellent
p-value	0.004	0.001	0.004	0.001
Best Cut-off	513.59	569.27	3.507	12.735
Sensitivity (%)	40	50	98	93
Specificity (%)	83	93	100	98

AUC: area under the curve, IFN-α : interferon α, TLR-7: toll-like receptor 7, ITGAM: integrin-alpha M chain, TNFAIP3: tumour necrosis factor alpha induced protein 3.

Table 4 shows the correlation between cytokine expression levels and gene expression levels of the study subjects. IFN-α had moderate positive correlation with TLR-7 and relatively weak positive correlation with ITGAM. TLR-7 had a moderate positive correlation with ITGAM and a strong positive correlation with TNFAIP3. ITGAM and TNFAIP3 showed a strong positive correlation.

Table 4: Pearson's Correlation Matrix Indicating the Association between Cytokine Expression Levels and Gene Expression Levels

Variables	TLR-7	*ITGAM*	*TNFAIP3*
IFN-α	0.345* *(p = 0.017)*	0.245* *(p = 0.024)*	0.151 *(p = 0.099)*
TLR-7		0.352* *(p = 0.013)*	0.633** *(p < 0.001)*
ITGAM			0.824** *(p < 0.001)*

IFN-α: interferon α, TLR-7: toll-like receptor 7, ITGAM: integrin-alpha M chain, TNFAIP3: tumour necrosis factor alpha induced protein 3

**Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05 level (2-tailed)

Discussion

While SLE can be broadly described as autoimmune disease that affects multiple organs and systems, its aetiology and pathophysiology do not conform to this simple approach, they are multifactorial involving the combination of immunological, genetic as well as environmental factors resulting to immunological dysfunction that is caused by abnormalities in the cytokine production . For these reasons, SLE has a complicated diagnosis and disease severity classification index that may be challenging to execute effectively in clinical settings. The disease has clinical heterogeneity, with multiple pathways that may lead to its expression and as a result, researchers have over the years made it necessary to identify putative genetic and secretory indicators of SLE in an effort to help patients who are asymptomatic or subclinical discover the illness early on . In line with this, the demographics, the clinical manifestations, cytokines (IFN-α and TLR-7) as well as expression levels of *ITGAM* and *TNFAIP3* genes were investigated in SLE patients in comparison with healthy subjects.

Both of the SLE patients and the healthy individuals recruited for the study were Iraqi Arabs, that was crucial, in order to accomplish homogeneity in the study population. Furthermore, there was no statistically significant difference ($p < 0.05$) in the mean and age range comparisons between the study groups. However, like previous studies have observed, there was a preponderance of women (60%) in this study. Interestingly, the results showed a significant difference ($p < 0.05$) in the marriage status between the SLE patients and the healthy group. Interestingly, contrary to the findings of Qu et al., which indicated that differences in marital status between SLE and healthy groups were not statistically significant (i.e., $p = 0.058$, p-value set at > 0.05) , a marginal significance ($p = 0.04$) between these groups was observed - a finding that could highlight the complicity of marital status in SLE and hence, it may play a part in the disease assessment in future investigations. Numerous studies have thoroughly assessed the contribution of a family history of SLE on the susceptibility to develop SLE. Cooper et al. (2002) recognised history of SLE in a parent or sibling as risk factor for the development of lupus or other systemic autoimmune illnesses . Studies that have been conducted more recently have reported similar outcomes . Here, it was noted that none of the healthy respondents had a family history of the condition, compared to 73% of SLE patients who had one. Furthermore, a meta-analysis revealed that familial history negatively affects the clinical symptoms and laboratory abnormalities in individuals with SLE .

Cytokines, in general, play a significant role as intercellular messengers and coordinate immune cell interactions throughout immunological responses. When the synthesis of cytokines is dysregulated, it leads to tissue inflammation and organ damage as well as immunological failure. Certain cytokines are unique to SLE and are implicated in both local inflammation and systemic immunological dysfunction . Furthermore, since the 1970s, interferons have been linked to the pathophysiology of lupus ; this have been confirmed by more recent studies . Immune complexes with nucleic acid signalling through toll-like receptors (TLRs) 7 and 9 cause the generation of type I interferon . In this study, an overexpression of IFN-α and TLR-7 were observed in the SLE patients. Damiati et al evaluated global expression of cytokines in SLE patients and reported IFN-α and TLR-7 among the highly overexpressed cytokines . Moreover, TLR-7 ligands have been reported induce higher IFN-α production in Caucasian females with SLE , as well as Mayan female SLE patients in Mexico . Additionally, it has been demonstrated that IFN-α production mediates the enhanced retention of TLR-7 in the lysosomes of palasmacytoid dendritic cells and consequently, in experimental lupus, the suppression of TLR-7 and TLR-9 attenuates lung injury and glomerulonephritis .

Beyond imbalances in soluble mediators, genetic factors contribute to immune dysfunction and since the 1990s, SLE susceptibility genes have been pursued using genome-wide family-based linkage studies . Custom-designed dense marker sets have become available recently as a result of improved understanding of the structure of the human genome, which made high throughput genotyping relatively inexpensive and efficient . In recent years, the design of genome-wide association studies (GWAS), has also been applied, as it has the capacity to screen hundreds of thousands of single nucleotide polymorphisms (SNPs) throughout the genome without requiring prior knowledge of candidate regions or genes. Today, numerous SLE-associated genes have been found and reported based on data from different GWAS . Replicated observational studies show that *ITGAM* and *TNFAIP3* are consistently upregulated in SLE patients . Similarly, a significant upregulation of these two in SLE patients relative their healthy counterparts by 15 and 43 folds respectively was observed. The findings suggest that the

expression of these genes may confer genetic susceptibility to SLE. Moreover, several polymorphism studies have associated variants of these genes with increased risk of SLE in North Indian , Egyptian and South-western Chinese populations.
To further investigate the pathogenesis of SLE, interaction between cytokines and genes was studied. With the exception of *TNFAIP3*, which showed no statistical correlation with IFN-α, positive correlation was observed between the genes and cytokines analysed in this study. Similar correlations between genes and cytokines in SLE have been reported in previous studies . This highlights the causative role of these genetic factors on the dysregulation of cytokine production in SLE, and by extension, their role in the pathogenesis of the disease, which makes them candidate biomarker genes for early detection of SLE or assessment of susceptibility to the disease.
It is challenging to identify the ideal biomarker for SLE since it must fulfil specific criteria: It must: (1) accurately reflect the underlying pathophysiology or treatment target; (2) possess validity, reliability, high predictive values, sensitivity, and specificity; (3) be able to track SLE activity or flare-ups; (4) be able to measure it in tissues, cells, or fluids with reliability; (5) be stable, repeatable, easily detected, and inexpensive enough to be readily available in the majority of laboratories . In this study, while the AUC for IFN-α and TLR-7 were respectively fair and good, with below par sensitivity and relatively high specificity, the excellent AUC coupled with high (>90%) sensitivity and specificity were observed for the genes *ITGAM* and *TNFAIP3*. This suggests that TNF-α is an important and potentially effective approach for the diagnosis of SLE, making them promising markers for monitoring the course of SLE, specifically, in Iraqi patients.
In conclusion, the findings have highlighted in addition to female predominance, the possible role of marital status in SLE. Most importantly however, is the strong candidacy of cytokines IFN-α and TLR-7 as well as genes *ITGAM* and *TNFAIP3* as diagnostic markers of SLE. However, scaled-up longitudinal multicentre investigations involving large population from different race and ethnicities would validate these assertions. Lastly, this study sheds light on the pathogenesis of SLE and offers guidance to clinicians regarding the management of treatment protocols based on patient gene expression profiles and cytokines, as well as potential diagnostic and therapeutic implications.

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Conflict of interest
The authors declare no conflict of interest.

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