Original Research Article

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Association of interleukin-36α gene expression in Egyptian patients with systemic lupus erythematosus with organ involvement and disease activity

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Abstract

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease characterized by a wide spectrum of clinical manifestations with varying severity. The dysregulation of cytokines contributes strongly to disease pathogenesis. Recently, it has been shown that the imbalanced antagonist/agonist profile of interleukin (IL)-36 cytokines, might play a role in autoimmune disorders. Our aim is to investigate mRNA expression of *Interleukin-36α* (IL-36α) in the peripheral blood of Egyptian systemic lupus erythematosus (SLE) patients and its association with disease activity and organs involvement. We assessed the relative expression of *IL-36α* mRNA by real time polymerase chain reaction and the comparative CT method on the peripheral blood of 49 SLE patients, and 40 healthy controls. Patients were subjected to thorough history and clinical examination, in addition to routine hematological, biochemical, and serological studies. Disease activity was evaluated using the SLE Disease Activity Index (SLEDAI). The relative expression of *IL-36α* mRNA was significantly higher in SLE patients compared to healthy controls (4.3±2.9 vs 1, respectively, $P<0.0001$). Fold change of *IL-36α* expression was significantly increased in patients with moderate and high disease activity (SLEDAI>5) than patients with mild disease activity (SLEDAI≤5) (4.3±1.2 vs 2.7±2.0, respectively, $P=0.006$) and positively correlated with SLEDAI ($r=0.505$, $P=0.001$). Regarding major organ involvement, the mean±SD expression of *IL-36α* in patients with arthritis and mucocutaneous involvement was significantly higher compared to those without organ involvement (4.2±1.5 vs 2.8±1.8, $P=0.039$ and 4.1±1.4 vs 2.8±2.1, $P=0.041$, respectively). In conclusion, the *IL-36α* is significantly more expressed in SLE patients compared to healthy controls and correlated with SLE disease activity and arthritis. *IL-36α* expression could be a useful biomarker for SLE disease activity in Egyptian patients with SLE.

Keywords: Systemic Lupus Erythematosus; Biomarker; *IL-36α* expression; RT-PCR; SLEDAI

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Introduction

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease that predominantly affects young women. It is characterized by a wide spectrum of clinical manifestations with varying severity, and alternating courses of flares and remissions.[1,2] The dysregulation of cytokines, which is the key player in the immune system, contributes strongly to disease pathogenesis[3,4] Recently, it has been shown that the imbalanced antagonist/agonist profile of interleukin (IL)-36 cytokines, might play a role in autoimmune disorders.[5]

IL-36 is a member of IL-1 family of cytokines, which includes 11 members, 7 agonists and 4 antagonists. The IL-1 family had a significant role in both the innate and acquired immunity by either promoting the resolution of infection or favoring inflammation through their binding to the receptors and co-receptors of the IL-1R family. The expression and activity of these cytokines and receptors are finely regulated; however, an overexpression or uncontrolled activation can initiate or enhance a pathologic inflammatory response.[6,7] IL-36 family comprises three agonist members, IL-36α, IL-36β and IL-36γ and a natural antagonist, IL-36 receptor antagonist (IL-36Ra).[8] IL-36 cytokines have prompted great interest in research field of immunology and autoimmune diseases. Previous studies have suggested that IL-36 cytokines may be crucial mediators at the interface between innate and adaptive immunity[5] and may play an important role in the pathogenesis of autoimmune diseases, such as psoriasis, rheumatoid arthritis (RA) and primary Sjogren's syndrome.[9-11]

Recently, several studies have reported elevated plasma levels of *IL-36α* and IL-36γ in patients with SLE[12,13] and showed correlation with disease activities. However, there is inconsistency in the expression of *IL-36α* despite high serum level in patients with SLE.[13] Therefore, we conducted this research to investigate expression of *IL-36α* mRNA on whole blood samples from Egyptian patients with SLE in comparison to healthy controls and their association with disease activity, and major organs involvement.

Materials and Methods

Study design and participants

This is a case-control comparative study of *IL-36α* mRNA expression among Egyptian patients with SLE in comparison with age- and gender-matched healthy controls and its relation to disease activity, and major organs involvement. It was carried out at physical medicine, rheumatology and rehabilitation, nephrology, medical biochemistry and molecular biology departments, as well as oncology diagnostic unit lab, faculty of medicine, Suez Canal university hospital, Ismailia, Egypt. The study was conducted according to the 1964 Helsinki declaration and its later amendments. All participants provided informed written consent. The study was approved by the institutional research ethics committee of faculty of medicine, Suez Canal university, Ismailia, Egypt (approval number: 4482).

Forty-nine Egyptian SLE patients fulfilling the 2012 Systemic Lupus International Collaborating Clinic Classification Criteria[14], and 40 age- and gender-matched healthy controls were recruited for the study. The healthy controls were recruited from relatives and friends accompanying the patients, lab technicians, nurses, and administrative workers who were free of any rheumatic diseases. Patients were excluded if they had other autoimmune diseases such as overlap, mixed connective tissue disease, dermatomyositis, polymyositis, sarcoidosis, and systemic sclerosis.

Clinical assessment

All patients underwent comprehensive medical history and physical examination to collect socio-demographic and clinical data such as age, gender, disease duration, systemic involvement, and medications. SLE disease activity was assessed by the SLE Disease Activity Index-2K (SLEDAI-2K).[15] Activity categories have been classified according to SLEDAI scores: no activity (SLEDAI = 0), mild activity (SLEDAI = 1-5), moderate activity (SLEDAI = 6-10), high activity

(SLEDAI = 11-19), and very high activity (SLEDAI 20).[16]

Routine laboratory assessment

Peripheral blood samples were collected from all patients and controls. Routine laboratory assessment included complete blood count (CBC) (Sysmex XT 5 parts differential cell counter, Germany), and erythrocyte sedimentation rate (ESR) by Westergren method. C-reactive protein (CRP), liver and renal function tests, serum complement C3 and C4, by (cobas c 501, Roche diagnostics, Germany). Estimated glomerular filtration rate (eGFR) was calculated according to 4-variable equation.[16] SLE patients were tested for antinuclear antibodies (ANA) by (Bio-Rad, HEp-2 cells immunofluorescence assay, California, USA), and the dsDNA antibodies by EIA (Bio-Rad, California, USA). Renal biopsy was classified according to the 2003 International Society of Nephrology (ISN)/Renal Pathology Society (RPS) classification.

mRNA IL-36α gene expression:

Purification of total cellular RNA, from fresh whole blood samples collected in EDTA coated tubes, was performed using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany, cat. No. 52304) and in accordance with the manufacturer's instructions. NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA) was used to assess the quantity and quality of RNA and cDNA. Reverse transcription was done with High Capacity cDNA Reverse Transcription Kit (Invitrogen/Life Technologies, USA, cat. No. Archive) using a thermocycler (Robocycler Gradient 96, BIOMETRA®, LA, USA). The relative expression level of *IL-36α* cytokine was determined by StepOne™ Real-Time PCR System (Applied Biosystem) by using Maxima SYBR Green qPCR master mix (Thermofisher Scientific, USA, cat. No. K0251) and genes' specific primer assays (willowfort, UK) that was designed by us and evaluated using online Bioinformatics tools. The 25µl total volume for each reaction included 8.5µl RT product (diluted to reach concentration of 50 ng of cDNA), 12.5µl SYBR green, 0.05 Rox solution, 2µl of forward primer (willowfort, UK), 2µl of reverse primer (willowfort, UK), for *IL-36α* gene; Forward primer sequence 5'-GACACACCTCAGCAGGGGAGCATTCAGG-3' and Reverse 5'- AACAGCATAGTTAACCCAAAGTC AGTAG-3', and for *GAPDH* gene; Forward primer sequence 5'-CTCCTCACAGTTGCCATGTA-3' and Reverse 5'-GTTGAGCACAGGGTACTTTATTG-3'. The reactions were incubated in a 48-well optical plate at 95°C for 15 min for initial denaturation step, followed by 40 cycles of denaturation at 95°C for 15s, annealing at 56°C for 30s and extension at 72°C for 30s. The fluorescence signal was acquired at 72°C. Each PCR run was followed by melting curve analysis to exclude primer dimer. *GAPDH* was used as a reference gene. The relative expression of *IL-36α* was calculated following the $2^{-\Delta\Delta CT}$ Livak method.[17,18] By using this method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene (*GAPDH*) and relative to the healthy controls. For the control sample, ΔΔCT equals zero and 2^0 equals one, so that the fold change in gene expression relative to the control equals one.[17,18] For the patient samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression relative to the healthy controls.[17,19,18,20.]

Statistical analysis

Collected data were analyzed using Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM Corporation, Armonk, NY, USA) and GraphPad software version 8 (San Diego, California, USA). Categorical variables were presented as frequencies and percentages (%). Quantitative variables were tested for normality with the Shapiro–Wilk and was presented as mean ± SD for normally distributed variables and as median (interquartile range-IQR) for non-normally distributed variables. The difference in *IL-36α* expression levels between groups was tested by independent t-test. Pearson's correlation coefficient was calculated to assess the correlation between *IL-36α* expression level and disease characteristics in studied groups. All tests were two-tailed and $P<0.05$ was considered significant. Moreover, the predictive performance of *IL-36α* expression for disease activity in SLE patients was evaluated using the Receiver operator characteristic (ROC) analysis. Areas under the curve (AUC) and its

95% confidence intervals were calculated for all coordinate points of *IL-36α*, while its optimal cut-off value was identified based on Yuden index. Sensitivity (SN), specificity (SP), negative predictive value (NPV), and positive predictive value (PPV) were calculated for the optimal cut-off value.

Results

Participants' demographic, clinical, and serological characteristics

Demographic, clinical and laboratory data of the 49 SLE patients are presented in table 1. Forty-six SLE patients were females (93.8%) with a mean (SD) age of 31.7 (9.5) years. The median (IQR) of the disease duration was 4 (1-10) years. There were 40 age- and gender-matched healthy controls. Among them 33 were females (90%) and their mean (SD) age of 37 (7.8) years.

The most frequent organs involvement of SLE patients were arthritis, mucocutaneous followed by kidney involvement and hematological affections [30/49 (61.2%), 28/49 (57.1%), and 12/49 (24.5%), respectively]. Renal biopsies reports were available for about 12 patients. Class IV lupus nephritis was the most common pathological class reported in 7/12 patients (58.3%).

ANA was positive in 46 patients (93.8%), while 34 patients (69.4%) were positive for anti-dsDNA. Thrombocytopenia (Platelets <100,000 cells/ul) was found in 12 patients (24.5%). Current proteinuria >0.5 gm/day was present in 10 patients (20.4%) and estimated glomerular filtration rate (eGFR ≤ 30) was noted in 5 patients. Low C3 was found in 19 patients (38.7%) and low C4 in 18 patients (36.7%).

The median (IQR) of SLEDAI was 6 (2-16). Twenty-six SLE patients out of 49 (53.1%) had active disease with SLEDAI >5. Regarding medications, 39 SLE patients (79.6%) were on steroids, hydroxychloroquine (83.6%), average dose 400mg/day, azathioprine (55.1%), dose range (100-150 mg/day), and mycophenolate mofetil (34.7%).

Table 1. Sociodemographic, clinical, and serological characteristics of SLE patients.

Characteristics	SLE Patients n = 49
Age, years, mean ± SD	31.7±9.5
Females, n (%)	46 (93.8)
Disease duration, years, median (IQR)	4 (1-10)
Clinical manifestations, n (%)	
Arthritis	30 (61.2)
Mucocutaneous involvement	28 (57.1)
Renal disease	12 (24.5)
Current proteinuria ≥ 500mg/day, n (%)	10 (20.4)
Lupus nephritis class, 12 patients, n (%)	
Class II	1/12 (8.3)
Class III	2/12 (16.7)
Class IV	7/12 (58.3)
Class V	2 /12(16.7)
eGFR — ml/minute /1.73 m^2	
eGFR ≥ 90 ml per minute per 1.73 m^2	34 (69.3)
60 ≥ eGFR < 90ml per minute per 1.73 m^2	10 (20.4)
eGFR ≤ 30 ml per minute per 1.73 m^2	5 (10.3)
Hematological affection	12 (24.5)
Neuropsychiatry affection	3 (6.1)
Serositis	2 (4.1)

Table 1. (Continued).

Characteristics	SLE Patients n = 49
Immunological features, n (%)	
ANA positive, n (%)	46 (93.8)
Positive anti-dsDNA, n (%)	34 (69.4)
Positive anti-SSA, n (%)	3 (6.1)
Positive anti-SSB, n (%)	2 (4.1)
Low C3	19 (38.7)
Low C4	18 (36.7)
Disease activity	
SLEDAI, median (IQR)	6 (2-16)
SLEDAI > 5, n (%)	26 (53.1)
Medications, n (%)	
Steroids, n (%)	39 (79.6)
Hydroxychloroquine, n (%)	41 (83.6%)
Azathioprine, n (%)	27 (55.1)
Mycophenolate Mofetil, n (%)	7 (34.7)
Cyclosporin A, n (%)	1 (2.0)
Cyclophosphamide, n (%)	7 (14.3)

SLE, Systemic Lupus Erythematosus; SD, standard deviation; IQR, interquartile range; eGFR, estimated glomerular filtration rate; ANA, Anti-Nuclear Antibodies; C3, complement 3; C4, complement 4; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

mRNA expression of IL-36α

The mean ± SD of mRNA expression of *IL-36α* was significantly elevated in SLE patients in comparison with healthy controls [4.3±2.9 vs 1, *P*<0.0001] (Figure 1). Furthermore, mRNA expression of *IL-36α* was significantly elevated in patients with SLEDAI >5 compared to those with SLEDAI ≤ 5 [4.3±1.2 versus 2.7±2.0, *P*=0.006] (Figure 2). Also, a significant moderate positive correlation was noted between *IL-36α* expression and SLEDAI (r =0.505, *P*= 0.001). The ROC curve analysis (Figure 3) showed that *IL-36α* expression level significantly predicted high SLE disease activity (SLEDAI>11), with an AUC of 0.73 (95% CI: 0.57-0.86; *p*=0.004). An expression level greater than 4.4 folds showed the best performance with 90.9% sensitivity, 57.1% specificity, 45.5% positive predictive value "PPV", and 94.1% negative predictive value "NPV".

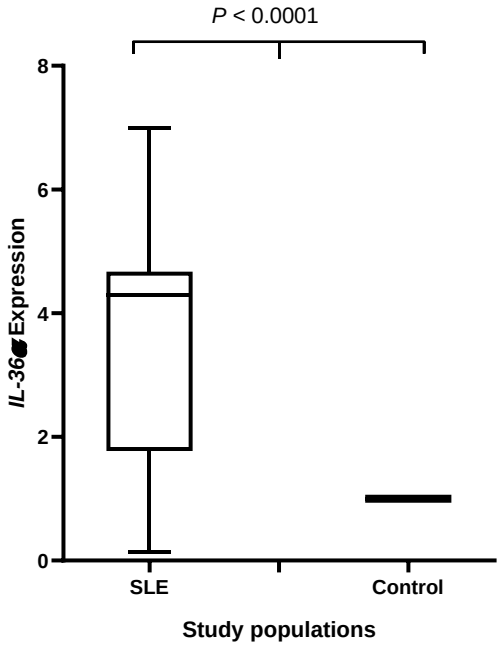

Figure 1. Relative expression of *IL-36α* in the study groups. The mean fold change of *IL-36*α was significantly higher in SLE patients compared to healthy control (4.3 ± 2.9 vs 1, respectively, Mann-Whitney U test, *P value* <0.0001).

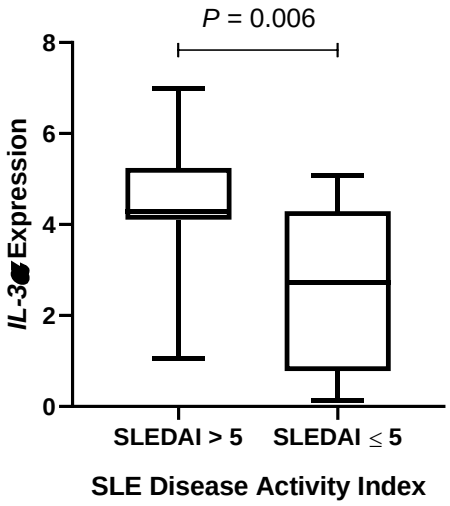

Figure 2. Relative expression of *IL-36α* in SLE patients according to disease activity. SLE patients with moderate-high SLE disease activity (SLEDAI>5) had significantly higher expression of *IL-36α* compared to those in remission or with mild SLE disease activities (SLEDAI ≤5) (4.3 ± 1.2 vs 2.7 ± 2.0, *P value* =0.006).

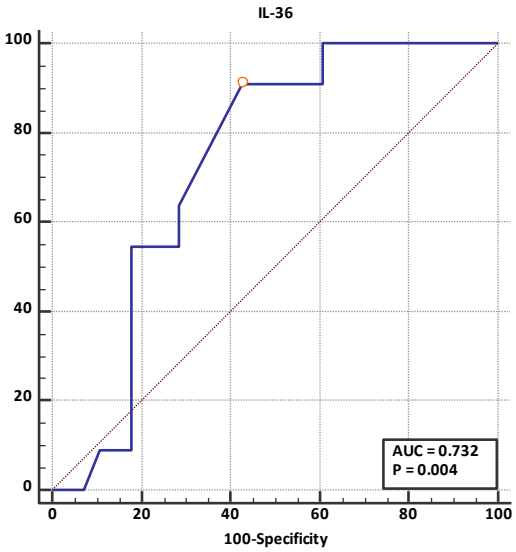

Figure 3. Receiver-operating characteristic (ROC) curve and corresponding area under the curve (AUC) for *IL-36α* expression for detection of SLE patients with high disease activity (SLEDAI > 11). The AUC value was 0.73 (95% CI: 0.57-00.86, *P*=0.004). Using the cut-off > 4.4-fold of *IL-36α* expression, sensitivity and specificity were 90.9 and 57.1%, respectively. The positive and negative predictive values were 45.5% (95% CI 34.3-57.1) and 94.1% (95% CI 70.6-99.1) respectively.

The association of organs involvement and *IL-36α* expression are presented in table 2. A significantly higher level of *IL-36α* expression was found in patients with arthritis to those without arthritis (4.2±1.5 vs 2.8± 1.8, *P*=0.039). Also, there was significant difference between patients with mucocutaneous involvement and those without mucocutaneous involvement (4.1±1.4 vs 2.8±2.1, *P*=0.041). On contrast, there was a non-significant increase in *IL-36α* expression in SLE patients with thrombo-cytopenia, neuropsychiatric involvement, serositis, proteinuria, and low C3 or C4. However, SLE patients with mild decrease in kidney function with (eGFR 60-89 ml/min/1.73m^2) had significantly higher median (IQR) mRNA expression compared to those with (eGFR ≥ 90 ml/min/1.73m^2) [4.29 (4.28-5.50) vs 4.25 (1.76-4.29), *P*=0.035] (Figure 4). Finally, there was a non-significant difference between patients on or off treatment with glucocorticoids, hydroxychloroquine, and azathioprine and *IL-36α* expression (*P*=0.960, *P*=0.949, and *P*=0.386, respectively).

Table 2. *IL-36α* expression in patients with systemic lupus erythematosus and disease manifestations

Characteristics	IL-36α expression Mean ± SD	*P* Value
Arthritis		
Present	4.2 ± 1.5	0.039*
Absent	2.8 ± 1.8	
Mucocutaneous manifestations		
Present	4.1 ± 1.4	0.041*
Absent	2.8 ± 2.1	
Proteinuria ≥ 0.5 gm/day		
Present	4.2 ± 1.4	NS
Absent	3.4 ± 1.8	
Biopsy-proven lupus nephritis		
Positive	2.9 ± 2.1	NS
Negative	3.8 ± 1.6	
eGFR — ml/minute /1.73 m2		
eGFR ≥ 90	3.5 ± 1.8	
60 ≥ eGFR <90	4.7 ± 0.7	
eGFR ≤ 30	1.6 ± 2.2	
Thrombocytopenia, < 100,000 /ul		
Present	3.3 ± 1.4	NS
Absent	3.6 ± 1.9	
Neuropsychiatry affections		
Present	4.2 ± 1.6	NS
Absent	3.5 ± 1.8	
Low C3		
Present	4.3 ± 1.6	NS
Absent	3.3 ± 1.8	
Low C4		
Present	4.3 ±1.4	0.112NS
Absent	3.3 ± 1.9	
SLEDAI		
SLEDAI ≤ 5	2.7 ± 2.0	0.006*
SLEDAI > 5	4.3 ± 1.2	

SLE, Systemic Lupus Erythematosus; eGFR, estimated glomerular filtration rate; C3, complement 3; C4, complement 4; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index. **P* value >0.05 is not significant (NS).

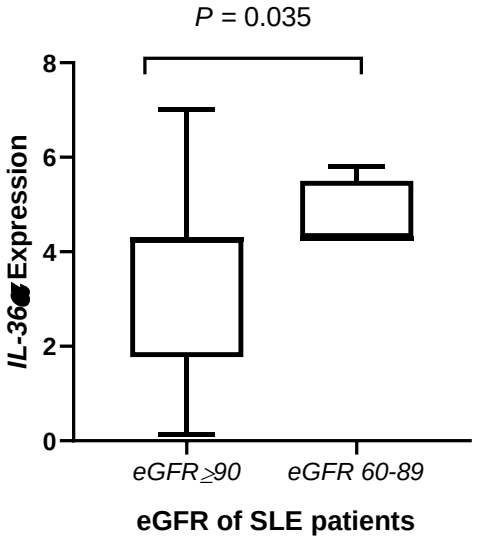

Figure 4. Relative expression of *IL-36α* in SLE patients according to estimated glomerular filtration rate (eGFR). SLE patients with mild decrease in eGFR (60-89 ml/min/1.73m^2) had significantly higher median expression for *IL-36α* compared to SLE patients with normal function (eGFR ≥ 90 ml/min/1.73m^2) (*P value* = 0.035).

Discussion

The dysregulation expression of IL-36 cytokines might prompt or activate autoimmune inflammatory disorders such as RA, psoriasis, and inflammatory bowel disease (IBD).[21,22] Herein, we report on the results of mRNA expression of *IL-36α* in Egyptian SLE patients and its association with disease activity and organ involvement. Our results showed significantly higher expression of mRNA of *IL-36α* in peripheral whole blood of SLE patients compared to healthy control group. In addition, *IL-36α* expression had significantly positive moderate correlation with disease activity in SLE patients. Also, a significant increase in *IL-36α* expression was noted in patients with arthritis, mucocutaneous involvement and those with mild decrease in kidney function (stage 2) compared to those with normal kidney function (stage 1). These findings support the role of IL-36α cytokine and extend the importance of IL-36α as a potential biomarker of disease activity in SLE patients.

Our results of higher mRNA expression of *IL-36α* are in accordance with and extends the previous studies which investigated a potential role of IL-36 family in SLE patients. Chu *et al.*, investigated the plasma level of IL-36α and IL-36γ with ELISA in 43 SLE patients and 16 normal control. Higher levels of IL-36α and IL-36γ were found in SLE patients compared to controls.[12] Also, there were higher levels of IL-36α and IL-36γ in patients with active SLE disease compared to those with inactive disease and normal controls. Also, they reported that increased circulating level of IL-36α can exert a proinflammatory effect in SLE peripheral blood mononuclear cells (PBMCs) through stimulating the production of IL-6 and CXCL8. In addition, they noted that IL-6 and CXCL8 were higher in SLE patients compared with normal controls (all $P<0.05$).[12]

Ismail et al., also using ELISA, reported an elevated serum level of IL-36α level in 40 Egyptian SLE patients compared to 20 normal controls and it was found to correlate with SLEDAI.[23] Mai et al., studied the serum levels of IL-36α, IL-36β, IL-36γ, and IL-Ra with ELISA in 72 SLE patients and 63 normal controls.[13] They reported a markedly increased plasma IL-36α and IL-36γ in SLE patients compared to the normal control group. However, they noted that although serum IL-36α level was significantly elevated in SLE patients, mRNA level was decreased in SLE patients compared to the controls. We can explain this discrepancy between serum level of IL-36α and its mRNA level as they measured *IL-36α* mRNA levels in only 30 of 72 SLE patients and 20 of 63 healthy controls, not all patients and controls assessed previously in ELISA.

Regarding organs involvement and expression of *IL-36α,* our results of higher expression in SLE patients with arthritis is consistent with Mai et al., and Ismail et al.[13,23] However, the significant elevation of *IL-36α* expression in patients with mucocutaneous involvement in our study contrasts with other study in SLE.[13] The possible role of IL-36 family in inflammatory joint and skin diseases such as rheumatoid arthritis and psoriasis had been extensively investigated recently.[24-26] Regarding major organ involvement, there was no association between *IL-36α* expression and proteinuria or biopsy-proven lupus nephritis.

However, we observed a higher expression of *IL-36α* among (10/49) patients with a mild decrease in kidney function compared to 34/49 patients with normal kidney function. Previous studies in murine models and humans demonstrated a role of IL-36 in tubulointerstitial diseases. For example, Ichii O *et al*, studied a mouse model of renal disease and showed that IL-1F6 (now called IL-36) overexpression was associated with tubulointerstitial lesions (TILs).[24] Similarly, Chi *et al.,* showed increased *IL-36α* expression in renal biopsy and urine samples from patients with renal TILs and confirmed the increased expression of *IL-36α* in the renal tubular epithelial cells of a mouse model of TILs.[25] It should be pointed out that our findings must be interpreted with cautions due to the small sample size. This may explain why we did not notice any difference in *IL-36α* expression and/or drug response especially, glucocorticoids, hydroxychloroquine, and azathioprine. However, our findings are consistent with a previous study.[13]

A follow up study in SLE patients is recommended to examine changes , if any, in *IL-36α* expression over time and response to treatment to validate its use as a disease activity biomarker, especially for clinical trials.

In conclusion, *IL-36α* gene expression is associated with disease activity in Egyptian SLE patients and may be a useful biomarker for disease progression. Future studies in larger sample size of patients with lupus nephritis might provide an insight into the pathogenesis of this complex disease.

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Author Contributions

Idea generation, NHA & AEM. Idea formulation, MMH, HMZ & MMF. Methodology, AEM, HM., MMH, MMF & NHA. Statistical analysis, AEM, HMZ, MMH. Data analysis, writing, editing and reviewing, AEM, HMZ, MMH, MMF & NHA. All the authors have read and agreed to the published version of the manuscript. They shared all in drafting and revision of the article critically for important intellectual content.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study was approved by the institutional research ethics committee of faculty of medicine, Suez Canal university, Ismailia, Egypt (approval number: 4482).

Informed consent

A signed consent form was obtained from each study participant.

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