IL 9 as a Prognostic biomarker for Systemic Lupus Erythematosus

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Abstract

Background: Interleukin 9 **(**IL9) controls the activity of different hematopoietic cells. It was initially thought to be a cytokine made by Th2 cells. Recent findings in animal models and patient samples suggest that IL-9 plays a role in several inflammatory disorders such as inflammatory arthritis and systemic lupus erythematosus (SLE).
Objectives: To assess if IL9 can be used as a Prognostic biomarker for SLE.
Materials and Methods: In a cross-sectional study and following informed consent, 59 patients of both sexes with SLE and 26 healthy controls were included in the study. Serum from study population was used for measurement of IL9 and CRP by using Sandwich ELISA and turbidimetric immunoassay respectively. PBMCs were used for the expression of IL9 mRNA. Data were analyzed using SPSS and OpenEpi statistical programs.
Results: The mean age of patients and controls was 27.89 ± 5.31 and 27.1 ± 5.95 years respectively. Female: males' ratio among patients was 4.4:1. Anaemia was the most frequent finding (n=34; 57%) whereas, arthritis is the least (n=12; 20.3%) in patients. The mean levels of CRP in SLE patients and controls were (31.1±27.5 mg/l) and (5.8±6.7 mg/l) respectively, (p=0.01). Higher levels of serum IL9 were indicated in patients (162.7±290.9 pg/ml) compared to controls (55.2±51.3 pg/ml), (p=0.01). The expression of IL 9 mRNA increased by more than fourfold change (4.9) due to SLE.
Conclusion: The high levels of serum IL-9 and its expression as mRNA indicate that the outcome of the IL9 level is upregulated at different levels in patients with SLE.
Keywords: Autoimmune Diseases, SLE, Cytokines, Serum IL9, Expression IL9 mRNA

Introduction

SLE is a B-cell-mediated autoimmune disease characterized by the production of large quantities of antibodies directed against ubiquitous self-antigens, particularly double-stranded DNA (dsDNA) and small nuclear RNA-binding proteins such as Ro, La, Sm, and nRNP (Goulielmos *et al*., 2018), Also, it is characterized by a type III hypersensitivity reaction leading to chronic systemic inflammation (Crow *et al*., 2015). SLE is an unknown aetiology (Shobha *et al*., 2021), and a polyetiological complex autoimmune disease with a chronic relapsing-remitting course and variable manifestations leading a spectrum from mild mucocutaneous to devastating, life-threatening illness, affecting the heart, lungs, and blood vessels, skin, joints, blood, and kidneys, characterized by differences in autoantibody profile, serum cytokines, and multi-system involvement. It is characterized by the dysfunction of both the innate and adaptive immune systems, which increase the production of cytokines and other inflammatory mediators (Frieri & Stampfl, 2016). These molecules produce a strengthening of inflammatory responses, an increase in the apoptosis of circulating cells, a defect in clearing apoptotic bodies, and overproduction of autoantibodies, which are associated with diverse clinical sub phenotypes (Boumpas *et al*., 2018).
The laboratory of Jacques van Snick made the initial discovery and publication of interleukin-9 (IL-9) in 1988. IL-9 belongs to a member of the four-helix bundle cytokine family and is a member of the

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gamma-chain family of cytokines. IL-9, cloned more than 20 years ago, was initially thought to be a Th2-specific cytokine. Its gene has been shown to reside within the Th2 cytokine cluster in the region q31–35 on chromosome 5 (Li & Rostami, 2010). First described as a member of a growing number of cytokines that have crucial roles in the development, proliferation, survival, and differentiation of multiple cell lineages of both the innate and adaptive immune systems (Rochman *et al*., 2009). Although IL-9 was, at one time, described as a Th2 cytokine. Indeed, it is now emerging that other effector T cell subsets such as Th1, Th17, Treg, and especially Th9 cells have the potential for IL-9 production, causing pleiotropic responses in autoimmune disorders (Shimbara *et al*., 2000; Li & Rostami, 2010). IL-9 exerts its diverse biologic functions on target cells via IL-9R, a γc-family receptor. The IL-9 receptor consists of the cytokine-specific IL-9 receptor α-chain and the commonly shared γ-chain, which is present in IL-2, IL-4, IL-7, IL-13, IL-15, and IL-21 receptor complexes. This is concordant with the fact that these cytokine molecules share several overlapping activities in autoimmune regulation (Deng *et al*., 2017).

The molecular characterization of the mouse IL9 gene revealed that the gene is located on chromosome 13, whereas its human homologue is located on chromosome 5 within the TH2 cytokine cluster (IL-2, IL-4, GM-CSF, and IL-13) in the region q31–35 (Mock *et al*., 1990). Human and mouse genes share a genomic structure with five exons and four introns that are quite like each other. 63% similarity is also observed in the three untranslated regions of human and mouse IL9. Even though human and mouse IL-9 are generally comparable, only mouse IL-9 is active in human cells while human IL-9 has no effect on murine cells (Chakraborty et *al*., 2019).

IL-9 is initially thought to be a cytokine made by Th2 cells which mainly express IL-4, IL-5 and IL-13 (Hultner *et al*., 1990). Indeed, the levels of IL-9 expression were high in the Th2-prone BALB/c mouse strain and low in the Th1-prone C57BL/6 mouse strain during infection with Leishmania major (Gessner *et al*., 1993). In addition to Th2, Th17 cells may also secrete IL-9 ex vivo (Nowak *et al*., 2009) (Yan & Richmond, 2020). Beriou et al., 2010 reported human Th17 cells can secrete IL-9, and the long-term culture of human Th17 resulted in the marked co-expression of IL-9 and IL-17A.

Regulatory T cells (Tregs) may also produce IL-9. A study by Lu et al. linking mast cells to peripheral tolerance demonstrated that natural Tregs and inducible Tregs, both Foxp3 populations, secrete I. However, there are contradictions regarding the production of IL-9 from human Tregs (Pavón *et al*., 2013).

Mast cells play a vital role in the production of IL-9 in asthmatic airways. Similarly, IL-9 has been demonstrated to be secreted by human neutrophils and eosinophils (Sun *et al*., 2018). IL-9 has also been discovered to be secreted by innate lymphoid cells, which are crucial parts of the innate immune system (Turner *et al*., 2013). Furthermore, Mouse Natural Killer T (NKT) cells are stimulated by IL-2 to generate IL-9 (Lauwerys *et al*., 2000). In the histological sections of lymphomas from patients with nasal NKT cell lymphomas, there are a lot of IL-9-secreting NKT cells (Nagato *et al*., 2005). Additionally, osteoblasts produce IL-9, which supports osteoblast-mediated megakaryopoiesis (Wang *et al*., 2017). Therefore, it is clear from these data that IL-9 has a variety of biological origins which might influence its pleiotropic functions.

IL-9 is a cytokine that was initially thought to be a T cell-derived factor preferentially expressed by Th2 cells (Chakraborty *et al*., 2019). The newly identified Th9 cells that predominantly produce IL-9 and IL-10 changed this conception. However, unlike Th2 cells, the Th9 cells do not exhibit any regulatory properties (Veldhoen *et al*., 2008), indicating that IL-9/IL-10-producing T cells are not regulatory T cells but effector T cells that induce tissue inflammation (Chakraborty *et al*., 2019).

An early study reported that Th9 cells induced severe colitis and peripheral neuritis upon adoptive transfer into immune-deficient hosts (Elyaman *et al*., 2009). Jäger et al., 2009 observed that Th17 cells secreted large amounts of IL-9 and showed that Th17 can induce EAE upon adoptive transfer.

Meanwhile, Dantas et al., 2015 suggested that IL-9 might normally contribute to Th17 differentiation although Th17 cells were differentiated by other cytokine cocktails (TGF-beta plus IL-6 or TGF-beta

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plus IL-21). These findings suggested that IL-9 might play an important role in the pathogenesis of SLE via regulating Th17 cells.

More recently, in the murine model of rheumatoid arthritis (RA), serum levels of IL-9 accompanied by sustained activation of STAT3 and pathologic cytokines were elevated after the second immunization (Khan, 2016). Multiplex kits were used to test serial plasma samples from six RA patients at baseline as well as several time points (3, 6, and 9 months) following the treatment with rituximab. While down-regulation of inflammatory cytokines such as TNF alpha, IL-1beta, IFN-γ, IL-10, IL-6, and IL-13 was evident by rituximab, the profile of significantly elevated immunomodulators included IL-9 (Khan, 2016), indicating a pro-inflammatory role for this Th9 cytokine (Dantas *et al*., 2015).

IL-9 was believed to have an immunosuppressive function that indirectly inhibits the production of pro-inflammatory cytokines (OUYANG *et al*., 2013). Along these lines, Elyamana et al., 2009 demonstrated that IL-9 (here as a Th17 cell-associated cytokine) acted on Tregs and enhanced their suppressive function both in vitro and in vivo. Furthermore, IL-9 was shown to promote the survival of Tregs and induce the activation of STAT3 and STAT5 signalling in Tregs (Elyaman *et al*., 2009).

Serum IL-23 was known to be upregulated in lupus (Fischer *et al*., 2017) and correlated with the SLE disease activity index (SLEDAI) (Sonmez *et al*., 2018). Recently, it was reported that Th17 cells exposed to IL-23 during a secondary stimulation resulted in a significantly reduced production of IL-9, and the Th17 cells from IL-23R-/- mice produced significantly higher levels of IL-9 (Elyaman *et al*., 2009). These findings suggested that IL-23 is a negative regulator of IL-9 secretion by Th17 cells, and the reverse association of IL-23 with IL-9 also implied a potential protective role of IL-9 in lupus. Furthermore, IL-9 production was found to be important in the recruitment of mast cells (Elieh & Grauwet, 2018), which has the potential to exert both pro-inflammatory and anti-inflammatory effects, depending on different environmental factors it encounters (Deng *et al*., 2017).

However, it is yet unknown if SLE patients exhibit aberrant IL-9 expression and release, and it is also unclear whether IL-9 primarily has pro- or anti-inflammatory effects in this condition. Therefore, in the present study, the serum levels of IL9, IL10, IFN-γ, and expression of IL9 mRNA in SLE patients as well were investigated, in order to provide a better understanding of the interrelationship and immunopathological roles of IL-9 in SLE.

Materials and Methods

Peripheral blood samples from 59 SLE patients from Al-Rayan Specialized Laboratory, including (48 females and 11 males; mean age, 27.89 ± 5.31 years) and 26 healthy controls (19 females and 7 males; mean age, 27.1 ± 5.95 years) were collected. Healthy control volunteers have none of which suffered from any rheumatologic diseases were included. All patients met the 1997 American College of Rheumatology (ACR) revised SLE classification criteria (Hartman *et al*., 2018). Baseline of Clinical features and Laboratory parameters are presented in Table I and Table 2 respectively. The Central Institutional Review Board at Al Neelain University gave its approval to the procedures used in the current study as well as the informed consent forms.

Five ml of venous blood was collected from the participants, and 2 ml of it was put in Ethylene Diamine tetra acetic acid (EDTA) for tubes for gene expression analysis. The remaining 3 ml were put into pyogen-free blood collection tubes and allowed at room temperature for 2 h to separate the serum. The supernatant was centrifuged at 500 r/min for 10 min, after which the upper serum was collected and then stored in an Eppendorf tube at − 80 °C. A human C-reactive protein kit (Adaptec biotechnology/catalogue No. 117Coo-CAA063) was used to measure CRP immediately after serums were prepared in the private lab. The turbidimetric immunoassay (Otsuji *et al*., 1982) determining serum levels was performed on A15 automated chemistry analyzer using goat anti-human polyclonal antibodies, protein standards and reagents from Adaptec Corporation according to users' manuals. The result was expressed as mg/dL and the detection range of this assay was 0 - 22 mg/dL.

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For measuring the serum levels of Interleukin-9 (IL-9), the Enzyme-Linked Immunosorbent Assay technique which was first described by (Engvall & Perlmann, 1971) was used for capturing this cytokine by ELISA kits (SinoGeneclon Co., Ltd. CHINA; KOMABIOTECH Biotechnology Co., Ltd. KOREA). Each sample was tested in duplicate. The result was expressed as pg/ml and the detection range of assay was 20 pg/ml - 800 pg/ml.
Easy-RED™ Total RNA Extraction Kit (iNtRON biotechnology/catalogue No. 17063) was used for extraction of RNA from the whole blood in Eppendorf tubes, followed by reverse transcription using Maxime RT PreMix Kit (iNtRON biotechnology / Oligo dT primer catalogue No. 25081). Approximately 2 µg RNA was converted to cDNA. For measuring the mRNA levels of IL-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control, 3 µl cDNA in triplicate was used for amplification by the HotStarTaq QuantiTect SYBR Green PCR kit (Applied Biosystems, cat. nos. 204141; QIAGEN, German). Under ideal circumstances, qPCR amplification developed by Higuchi et al., 1993 was carried out, and fold induction calculations were made. IL9 mRNA expression was normalized to GAPDH, and the values were expressed relative to control using the Livak method (Livak & Schmittgen, 2001).
The primers used included: GAPDH sense, 5'- ATCCCATCACCATCTTCCAG-3' and anti-sense, 5'-GAGTCCTTCCACGATACCAA-3'; IL9 sense, 5'-GTGCCACTGCAGTGCTAATGT-3' and anti-sense, 5'- CTCTCACTGAAGCATGGTCTG -3'.

Statistical methods:
Student t-test was employed to test whether there were significant differences between the study group regarding IL9 mRNA expression, serum levels of IL9, IL10, and IFN-gamma. All results were presented as Mean ± SD, with the level of significance set at P-value < 0.05. Statistical analysis of the data was performed using the SPSS and OpenEpi (Sullivan *et l*., 2009) statistical programs.

Results

Table 1 and 2 show clinical and laboratory finding among participating patients with SLE.

Table1: Clinical features baseline among patients with SLE.

Clinical features	
Condition	**Frequency**
Anaemia	34(57.6%)
Oral ulcer	18(30.5%)
Butterfly erythema	16(27.11%)
Photosensitivity	15(25.4%)
Arthritis	12(20.3%)

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Table 2: Laboratory parameters baseline among patients with SLE.

Laboratory parameters	
Parameters	**Frequency**
Anti-Nucleosomes	23(38.9%)
Leukopenia	21(35.6%)
Thrombocytopenia	24(40.7%)
Anti-Ribosomal P-protein	8(13.5%)
Anti-Ro-52	12(20.3%)
Anti-SS-A	15(25.4%)
Anti-dsDNA (+)	33(55.9%)
Anti-Sm(+)	18(30.5%)
Elevated creatinine	42(71.1%)
Elevated ESR	33(55.9%)

The mean levels of CRP measured in the serum of patients with SLE, and controls were (31.1±27.5 mg/l) than that of the controls (5.8±6.7 mg/l) respectively. There is statistically significant difference between the patients and the controls, (p=0.01); Fig 1.

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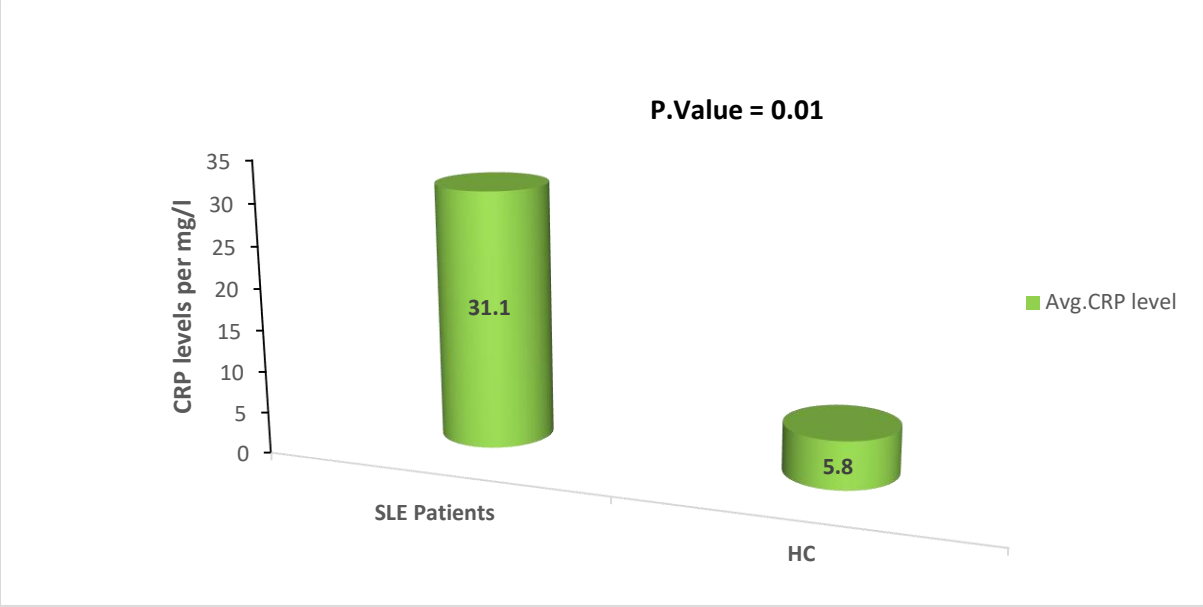

Figure 1: Serum (CRP) levels in each group.

The expression of IL 9 mRNA in patients with SLE and the control group are (21.7 ± 53.7) and (4.4 ± 8.0) respectively. This is interpreted as the expression of IL-9 mRNA was increased by more than fourfold (4.9) due to SLE; (Fig 2), There is a statistically significant difference between the patients and the controls, p=0.01, which is consistent with the ratio of the serum levels of IL 9 between patients and controls 162.7: 55.2 (2.9); (Fig 3) This indicates that the outcome of the IL9 levels is upregulated at different levels in patients with SLE. Fig 4 and Fig 5; show the amplification plot of the IL-9 and GAPDH respectively.

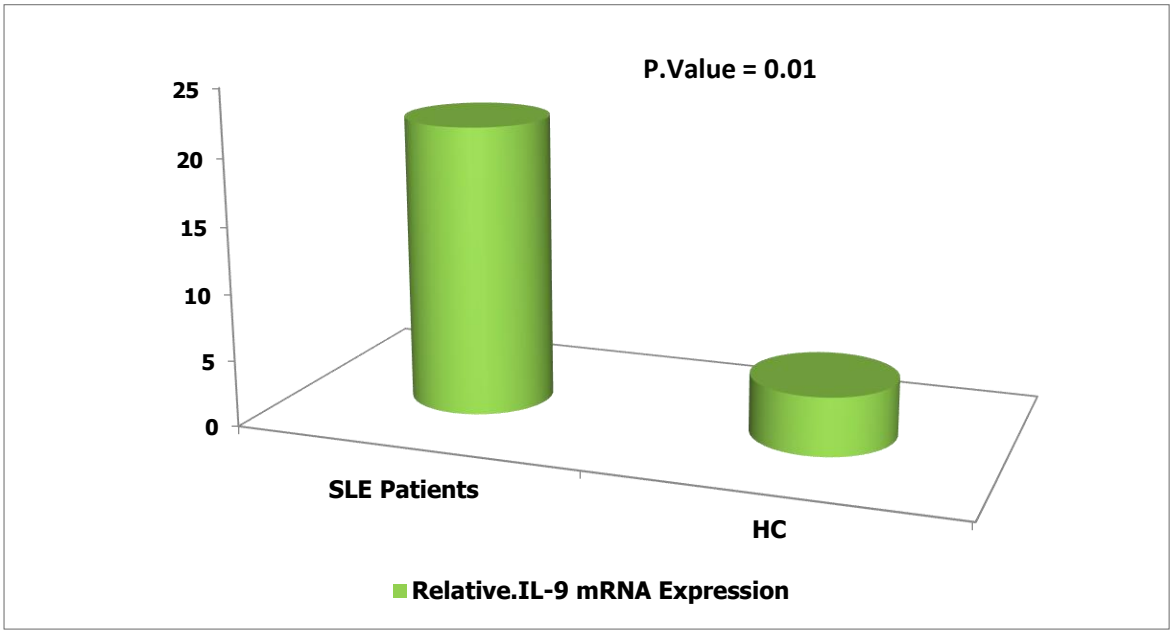

Figure 2: Relative expression of IL-9 mRNA.

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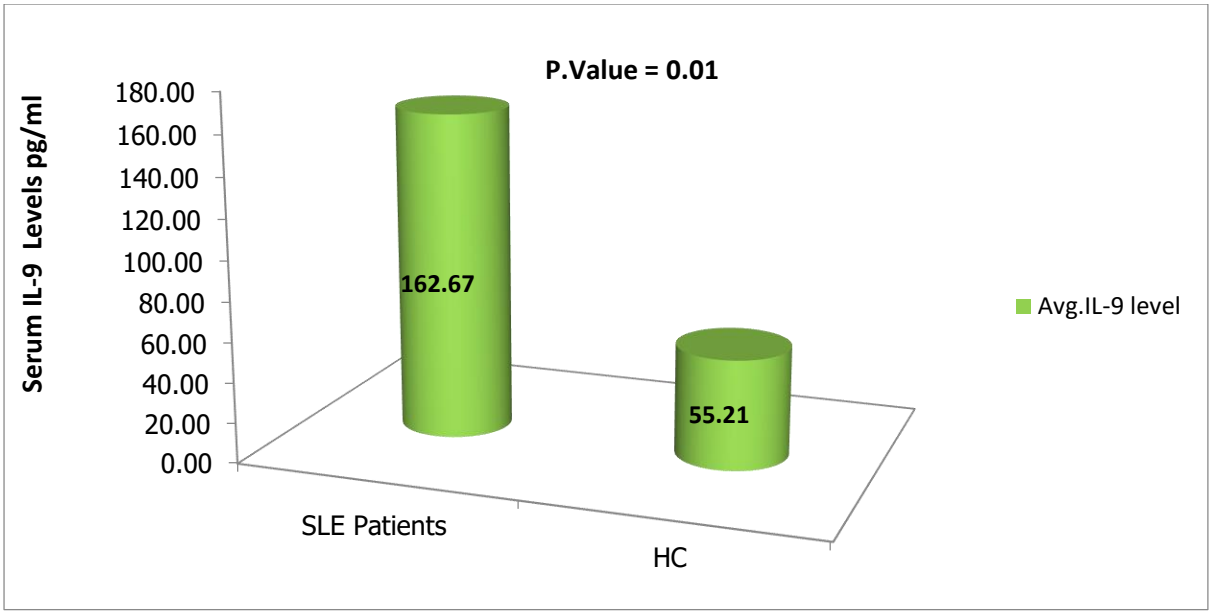

Figure 3: Serum IL-9 protein levels in each group.

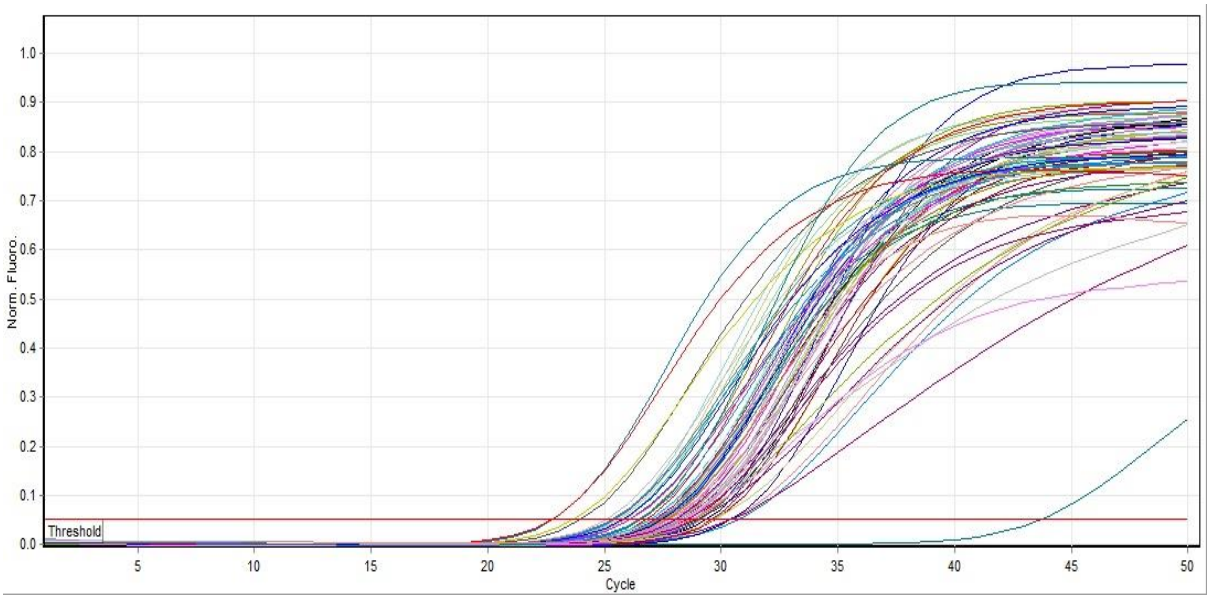

Figure 4: Amplification plot of IL-9mRNA (log scale) using Rotor-Gene Q Series Software.

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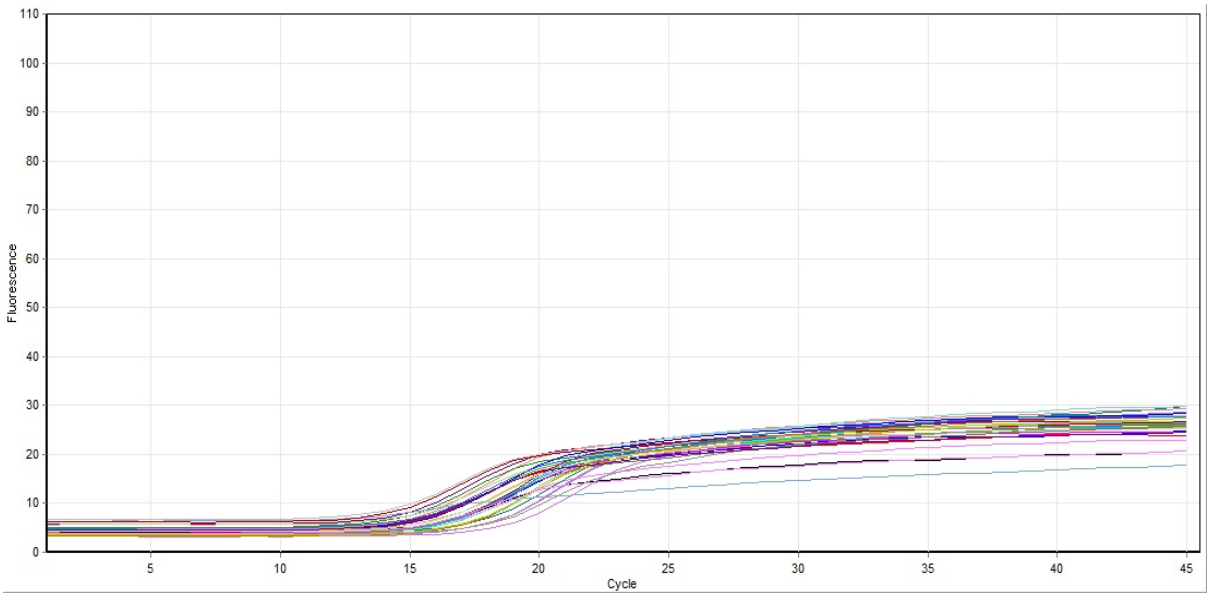

Figure 5: Amplification plot of GAPDH (linear scale) using Rotor-Gene Q Series Software.

Discussion

The immunopathology of SLE is characterized by the production of large quantities of antibodies directed against specific self-antigens, particularly double-stranded DNA (dsDNA) and small nuclear RNA-binding proteins such as Ro, La, Sm, and nRNP (Harley *et al*., 2006; Goulielmos *et al*., 2018). Loss of tolerance to self-antigens is the driving stimulant to the immune response in SLE. Therefore, T and B cells will be activated which results in increased production of autoantibodies and abnormal biological activities of multiple cytokines including B cell activating factor, interferon (IFN-gamma) and cytokines that are involved in the T-helper 17 axis (Oon *et al*., 2016).

Despite the extensive research on SLE immunopathology, there are still aspects that need to be explored. IL-9 is a cytokine produced by T cells that are first classified as a Th2 cytokine (Houssiau *et al*., 1995; Blom *et al*., 2011) Recently, it was discovered that Tregs, Th1, Th17, and Th9 subsets of T cells also release IL-9 (Yazdani *et al*., 2019). Within those subsets, IL-9 has been shown to have pleiotropic effects, and it regulates immune responses in a variety of circumstances by acting in diverse ways (Walker & McKenzie, 2018). Asthma, arthritis, multiple sclerosis, and experimental autoimmune encephalomyelitis are among the autoimmune disorders that may be exacerbated by IL-9 (Hiroshi *et al*., 2021).

Pathogenesis of SLE is brought about by abnormal T helper cell immune response. The package of this response includes proinflammatory and anti-inflammatory cytokines. Therefore, IL-9 as one could play a major role in the pathogenesis of SLE.

It is still unknown whether SLE patients have aberrant expression or secretion of IL-9, and it is also unknown whether IL-9 predominantly has proinflammatory or anti-inflammatory effects in SLE. To better understand the relationships between IL-9 and SLE as well as its immunopathological actions, the expression of IL-9 levels in SLE patients was examined in the present study.

In the current study, High levels of CRP concentration were observed in patients (31.1± 27.5) with SLE compared with healthy controls (5.8± 6.7), (p=0.01). This is in line with the activity of the disease and confirm the previous reports about SLE activity provided by Oeser et al., 2005, Chung et al., 2009, Sahebkar et al., 2016.

The present study revealed that serum IL-9 levels are significantly increased in SLE patients compared to healthy controls (p=0.01); This is in line with OUYANG et al., 2013, and Dantas et al., 2015, who

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indicated that patients with SLE had elevated IL-9 compared with levels in healthy individuals. The fact that the serum levels of IL9 are high in patients may be because of the disease or due to the stimulation of Th2 response by other cytokines. Furthermore, the production of IL9 might stimulate as a response to Th2 stimulation may have positive feedback to produce more IL9 which in turn makes the disease worse.

However, some investigations have shown that IL-9 has anti-inflammatory properties in human autoimmune disorders (Deng et al., 2017; Chen et al., 2019). This may be because IL-9 boosts the suppressive activity of regulatory T cells (Elyaman et al., 2009).
IL-9 mRNA and GAPDH mRNA expression levels were examined in SLE patients and healthy controls to determine whether IL-9 expression is aberrant in the peripheral blood of SLE patients. The results indicated that the fold change of (IL-9 mRNA) to the Internal control gene (GAPDH mRNA) within the control group and SLE patients were 4.4 and 21.7 respectively. Subsequently, the expression of IL-9 mRNA was increased more than fourfold due to disease. This outcome is consistent with OUYANG et al., 2013, Selvaraja et al., 2019, and Zein et al., 2021, who reported increasing in IL-9 mRNA levels in SLE patients so it could have a role in the pathogenesis of SLE. Consistently, Yang et al., 2015, confirmed that high expression of IL-9 was also observed in the kidneys and spleens of lupus-prone mice MRL/lpr.

Conclusion

IL9 as a protein is high in patients with SLE. Likewise, the expression of IL9 mRNA is upregulated in patients with SLE explaining the higher level in the serum. This indicates that IL9 is central in the immune response in SLE both at the gene and the protein levels.

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