Evaluation of Serum Complement C3 and C4 Levels as biomarkers for Systemic Lupus Erythromatosus

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Abstract

Background: Systemic Lupus Erythmatosis (SLE) is a chronic autoimmune disorder that affects multiple organ systems and also affects the skin and oral mucosa, with the exact cause is unknown. Many hypotheses try to explain the role of the complement C3, C4 in the pathogenesis of SLE. The aim of this study is to determine levels of serum complement C3 and C4 in patient with SLE, so that we may explain its role in diagnosis and pathogenesis of the disease.

Methods: Twenty patients were informed from outcome patients of Dermatology Unit in El-Azhar University suffering from SLE. All the patients included in this study fulfilled 4 or more of the American Rheumatism Association classification Criteria for SLE. Blood samples from These 20 SLE patients (18 females and 2 males) aged from 20 to 45 years old were collected. Complement C3 and C4 were measured using radial immunodiffusion plates system technique. Clinical parameters such as Erythrocyte Sedimentation Rate (ESR), Total Protein (TPR), Serum Creatinine and Antinuclear Antibody (ANA) of those patients were considered in order to compare and explain the data obtained for the levels of C3 and C4. The data were collected and statistically analyzed.

Results: Most of patients were female 90% and only 10% male. Of all patients, 60% have low level of serum C4, 40% have normal level of serum C4, 25% have abnormal level of serum C3, and 75% have normal level of serum C3. Statistical analysis of the data on the correlation between C4, and disease activity revealed significant (P< 0.05) correlation, however no significant correlation was found between C3 and disease activity. Analysis on the correlation between C3 and C4 with TPR, S. creatinine, and ESR, showed no significant correlation. No significant relationship was also found between C3 and C4. All patients have had high TPR, S. creatinine and ESR. All patients have had positive ANA which is an important marker of SLE as an auto immune disease.

Conclusions: Patients showed different degrees of oral and systemic manifestations, which exacerbate and become acute with decreased level of complement C4 and instability of C3 level. Accordingly, the low level of C4 was associated with the development and exacerbation of SLE. Increased C3 levels is solely due to activity through the alternative pathway in SLE patients.

Key words: Complement, C3, C4, SLE,
Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterised by the production of antibodies to components of the cell nucleus in association with a diverse array of clinical manifestations (Bhaviya et al., 2010; Yang et al., 2009 and Bernknopf et al., 2011). Clinically, SLE usually affects adults, women of childbearing age (20 to 40 years), female to male ratio is 9:1 approximately 8% to 15% of SLE cases occur in children (Rahman et al., 2008). Estrogen (mainly 17-oestradiol, estradiol) and their metabolites seem to play an important role in SLE (Lords et al., 2002 Weidler et al., 2004; and Ali Khan et al., 2009). Gonadotrophin releasing hormone (GnRH) has been shown to exacerbate lupus. There is preliminary evidence that there is a defective hypothalamo-pituitary-adrenal (HPA) axis in SLE patients (Juha Kere, 2004). The primary pathological findings in patients with SLE are those of inflammation, vasculitis, immune complex deposition, and vasculopathy (Bernatsky et al., 2008; Habibi et al., 2011 and Löfgren et al., 2012).

The exact aetiology of SLE is unknown. SLE shows a strong familial aggregation, with a much higher frequency among first degree relatives of patients. Moreover, in extended families, SLE may coexist with other organ specific autoimmune diseases such as haemolytic anaemia, immune thrombocytopenic purpura, and thyroiditis. The genetic factors play an important role in the predisposition of the disease (Lee and Bae, 2010; Yuan et al., 2010; Krishnamurthy and Mahadevan, 2011). However, most cases of SLE are sporadic without identifiable genetic predisposing factors, suggesting that multiple environmental factors may also be responsible (Bernknopf et al., 2011). The immune system is a network of molecules, cells, tissues, and organs that work together to defend the body against attacks by “foreign” invaders. Sometimes the immune system’s recognition apparatus breaks down, and the body begins to manufacture antibodies directed against self antigens in its own cells and tissues (Kelly, 2007 and Anaya, 2010). The complement system consists of a tightly regulated network of proteins that play an important role in host defense and inflammation. Some of these proteins are soluble plasma constituents and others are cell membrane proteins. A main task of complement components is to stop invasion by microbes; membrane complement proteins act as receptors for target-bound complement (Janeway et al., 2001). Complement activation results in opsonization of pathogens and their removal by phagocytes, as well as cell lysis (Liu et al., 2011). Complement...
activation is known to occur through three different pathways: classical, alternate and lectin involving proteins that mostly exist as inactive zymogens that are then sequentially cleaved and activated. All the pathways converge at C3 (which is the most abundant complement protein found in the blood), resulting in the formation of the activation products, C3a, C3b, C5a and the membrane attack complex (C5b-9) (Sarma and Ward, 2011). Regulators of complement activation protect self-tissue from damage (Zipfel et al., 2006 and DeFranco et al., 2007). During previous years it has become more and more obvious that the complement system is not only involved in killing of invasive pathogens, but also contains important regulators and activators of several humoral and cellular immune functions (Sturfelt and Truedsson, 2005).

The association between the complement system and SLE is contradictory. The complement system has long been known to be activated in exacerbations of SLE (Chen et al., 2009). There are many hypotheses try to explain the role of the complement in the pathogenesis of SLE (Hussain et al., 2008):

First, as complement is known as a mediator of inflammation, complement deficiency might predispose to the development of SLE. Inherited complement C4 deficiency, whether partial or complete, confer a high risk to developing SLE, whereas C3 deficiency is only rarely associated with SLE-like illness (Cohen, 2004). The deficiency of complement leading to the inappropriate clearance of apoptotic cells which lead to formation of immune complexes. Impaired handling of immune complexes is a major pathogenetic factor in SLE (Horák, 2009).

Second, complement deficiency is strongly associated with SLE, while on the other hand the activation of complement plays a major role in the inflammation. Furthermore, the complement system may become a target of the adaptive immune response as autoantibodies against several complement components (Fliereman and Daha, 2007). In patients with SLE, autoantibodies against components of the classical pathway are often found. SLE patients have elevated serum of Immunocoaglutinins which are autoantibodies against C3 or C4 fragments. Anti-C1q autoantibodies are found in approximately 30–50% of SLE patients C3 and C4 nephritic factors are IgG autoantibodies that stabilize the alternative pathway C3 convertase and the classical pathway C3 convertase, respectively (Peter, 2011).

The third theory postulates the possible positive role of the complement in induction of autotolerance by determination of activation thresholds of B and T. the complement system protects against immune response to autoantigens by enhancing the elimination of self-reactive lymphocytes (Trendelenburg, 2005).
It has been detected that Serial estimation of anti-dsDNA titre, C3 and C4 levels help in diagnose of lupus flare and make appropriate therapeutic decisions in patients with high SLEDAI score (Yang et al., in 2009 and Col et al., 2010).

**Patients and Methods**

**Patients:** twenty patients were randomly selected from the Dermatology Department, El-Azhar University with confirmed diagnosis of SLE were included in the study, and filled an informed consent. The age of the patients were 19-45 years old (Mean age 28.72 ±7.32).

All the patients included in this study fulfilled the inclusion and exclusion criteria. Inclusion criteria are: all the patients included in this prospective study were evaluated and fulfilled 4 or more of the American Rheumatism Association classification Criteria for SLE, all the patients confirmed to had SLE by clinical & laboratory examination, all patients were stopping their immunosuppressive therapy for at least 3 months and Patients' age ranged from 19 to 45Y. Exclusion criteria were to exclude Cases with drug-induced LE from this study when suspected.

**Serum collection:** Consent was taken from all patients before blood sampling. Serum samples were obtained in an empty vacutainer tube for the preparation of serum. The serum was obtained by allowing the blood to clot at room temperature for two hours and the tube was then centrifuged. Then serum was removed and aliquoted and stored at -80 till all samples were collected.

**Laboratory examination:** ESR has been measured by Westergren’s method. ANA titer and profile done by ELISA technique (positive >120 units/ml), using Stanbio reagent supplied by stanbio laboratory, Texas USA for titre and its pattern depending on a method by Cabral and Alarcon (1998). TPR and S. Creatinine are measured by using method of Bowers (1980).


**Calculation of results**

Data are coded, entered and checked to SSPS software version 10. Quantitative data are presented as mean ± standard deviation or presented as mean & range when data are not normally distributed. Qualitative data are expressed in number and percentages.

Comparisons regard the activity is estimated by student t test. Comparison of median of S. creatinine and TPR is estimated by Mann Whitney test.

Correlation coefficient r is calculated for estimating the association between 2 quantitative variables. P is considered significant when it is less than 0.05.
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**Results**

All patients examined and included in the study fulfilled the selection criteria. Clinical and laboratory characteristics of studied SLE patients are shown in table (1).

**The patient sex**

In the present study it was found that most of patients were female 90% and only 10% were male (Figure 1).

**Levels of serum complement C4, C3 in SLE patients**

In the present study, the complement C3 and C4 has been measured. It has been found that 60% of all patients have low level of serum C4, 40% have normal level of serum C4, 25% have abnormal level of serum C3, and 75% have normal level of serum C3 as shown in Table 2, Figure 2.

In the current study, patients were divided into 2 groups, exacerbation and remission (Table 3 and Figure 3). Moreover, a positive correlation (P< 0.05) between low level of C4 and disease activity was noticed in all SLE participating patients. While, that was not the situation in case of C3 (Table 4).

In this category of patients 40%, oral lesions ranged from erythema in the palatal mucosa and buccal mucosa to purpuric macules. Petechiae and Ulcerations were also found in palatal mucosa and buccal mucosa. Bullous LE: labial blisters, intra-oral intact or ruptured blisters, (Figure 5a and 5b).

**Active disease group:**

From this study, it was found that the low level of C4 was significantly correlated (P< 0.05) with disease activity as shown in table (4). Also acute and subacute oral manifestations were revealed in this group of patients meaning that the decrease in the level of C4 is correlated with presence of these acute oral manifestations.

**Acute lesions:**

In this category of patients 40%, oral lesions ranged from erythema in the palatal mucosa and buccal mucosa to purpuric macules. Petechiae and Ulcerations were also found in palatal mucosa and buccal mucosa. Bullous LE: labial blisters, intra-oral intact or ruptured blisters, (Figure 5a and 5b).

Subacute lesions:

20% of SLE patients revealed discrete red patches, which diffusely and
discretely arranged in labial and palate (Figure 6).

**Inactive disease group**

Through the current study, normal or increased level of C4 was associated with decreased disease activity (40%) which means that the increase in the level of C4 was correlated with the remission of the disease. Interestingly, it was noticed that chronic oral manifestations were found in 50% of this group of patients (about 20% of total SLE patients), tables 2 and 4.

**Chronic lesions**

20% of SLE patients revealed atrophic or ulcerated round lesions with peripheral keratotic striae asymmetrically distributed in buccal mucosa. Ulcers ranged from linear with keratotic striae, intensely keratotic lesions were also found on palatal mucosa, (Figure 7a & 7b).

**DISCUSSION**

Systemic lupus erythematosus (SLE) is an autoimmune disease of uncertain etiology that is complex in multiple aspects. At the clinical level, SLE has been described as a wide array of disorders and patients manifestations ranging from immediately life threatening disease of major organs such as heart, lungs or kidney. Deficiency of C4, whose gene is found on chromosome 6 in the MHC III region, imparts a risk of SLE (Bajaj et al., 2010).

Complement system has important protective functions in both the innate and the adaptive immune systems but can also, when inappropriately activated, cause tissue damage. Complement deficiency predisposes to infection and also to development of autoimmune disease, especially SLE, and complement is at the same time involved in the pathogenesis of this disease (Sturfelt and Truedsson, 2005).

There are three major complement activation routes: classical, alternative and lectin pathways. Regardless of how these pathways are initiated, the complement activity leads to proteolytic activation and deposition of the major complement proteins C4 and C3, which induces phagocytosis, and the subsequent assembly of the membrane attack complex which lyse the invading microbes. However, complement is a double-edged sword; adequate complement activation is necessary for killing the bacteria and removing the apoptotic cells, while excessive complement activation can harm the host by generating inflammation and exacerbating tissue injury (Chen et al., 2009). The evidence that products of complement activation really contribute to tissue damage in SLE is somewhat circumstantial (Sturfelt and Truedsson, 2005).

Attention has been paid to other factors beside C3 and C4 as: age, sex, ESR, TPR and S. Creatinine in relation to SLE, in this study. Furthermore, Clinical studies suggested that the gender in SLE is influenced by sex hormones, such as
estrogen and androgen; in agreement with results obtained from numerous studies (Weidler et al., 2004; Horák et al., 2009; Bernknopf et al., 2011).

In the current study C4 and C3 were measured in serum of patients suffering from SLE. C4 was chosen to be measured as a component of the classical or lectin pathway of complement activation; the easiest to measure is C4. Additionally, C3 was measured in the current study because its measurement together with C4 could enhance the understanding of the mechanisms involved and aid in the clinical definition of SLE (Hussain et al., 2008).

In this work 60% of all SLE patients participated in the study had low levels of C4, all those patients presented criteria of disease flare, while 40% had normal C4 levels and those patients presented criteria of disease remission. These results were in agreement with results obtained from previous studies (Hussian et al., 2008; Horák et al., 2009 and Peter et al., 2011).

Hussian et al. (2008) suggested that low C4 levels may falsely be regarded as classical pathway activation and they reported that several other factors may explain low C4 level such as: Partial defects or homozygous defects in either C4A or/and C4B will result in reduced levels of total C4, reduced synthesis or increased catabolism of C4 without corresponding complement activation may also explain low C4 values.

In 2002, Eniav found that the development of severe SLE in the absence of both classical and alternative complement pathways suggests that it is the absence of C4, and not the presence of C3 that is critical in SLE pathogenesis. Thus, this study supports that complement C4 provides an important protective role against the development of SLE.

In the present study 25% of the patients had low C3, while, 75% had normal C3. Cameron et al. (1973) studied 55 samples of SLE and he also found that low plasma C4 concentrations were commonest in lupus group, but a C3 concentration of below 20% of reference normal serum was not seen in their study; in contrast to our data.

It was found that more than 75% of all individuals with deficiency of C4 have SLE, which is commonly severe. Deficiency of Complement may lead to abnormal in vivo processing of dying cells that, in the context of an inflammatory response, could initiate and drive an autoimmune response leading to the development of SLE (Carroll, 2004). Another mechanism in the pathogenesis of SLE associated with Complement deficiency that Complement may be involved in the recognition of self by B cells and thereby defects in Complement might result in failure of B cell negative selection. This failure allows auto-reactive B cells to survive and propagate when they would normally undergo apoptosis (Botto et al., 2009).
Moreover, analysis of serologic factors in SLE has revealed that in many patients, lower C4 levels occur before the depression of other complement components. After the induction of remission, C4 has a tendency to return to normal levels more slowly than C3; decreased C4 levels are mainly present in those with exacerbation of SLE. Prolonged decrease in serum C3 levels is associated with the development of chronic SLE (Ceribell et al., 2009).

Clinically, most patients included in this study presented with multiple oral lesions. Locations more frequently affected were buccal mucosa, hard palate and lower lips. Some patients had lesions affecting more than one oral site. These findings agree with previous studies, in which buccal mucosa, palate and vermilion of lips (more the lower than the upper lip) are referred as the commonest sites for lupus oral lesions (Lourenço et al., 2007 and Simonsen et al., 2008).

Considering morphologic aspects, the oral lesions examined presented varied clinical aspects, ranging from the classic plaques with central erythema surrounded by a white rim with radiating keratotic striae and occasionally telangiectasias. In this study classic lesions were present in nearly half of the patients. This reflects the importance of considering other clinical hypotheses when examining oral lesions suggestive of lupus erythematosus and shows that in many circumstances their diagnosis is challenging (Chen et al., 2009 and Peter et al., 2011).

The data presented in this work has not found significant correlation between S. Creatinine, TPR and C3 or C4. However, a significant correlation between S. Creatinine, TPR and disease activity has been found. The decreased levels of C4 could either be due to the low production or its consumption in a classical activity of the complement (Hussain et al., 2008).

No authors have discussed a correlation between TPR and C4 or C3. In this study, no significant correlation has been found between the increased level of TPR and the high activity of C3. These findings indicated that the decreased levels of C4 are merely because of the low production. These findings also explained that the complement activation is mainly through the alternative pathway in the active SLE. An obvious increase in ESR in all participants was revealed. However, the statistical analysis of the data presented here revealed neither significant correlation between ESR and C3 nor between ESR and C4. ESR is used as a screening test to identify patients with inflammatory conditions. It is a useful marker to monitor disease activity and is commonly elevated in patients with SLE (Luis et al., 2005).

In addition, findings in this study have shown that all patients had positive ANA which is an important marker of SLE. It has been found that all patients with SLE have a positive ANA test, with a
sensitivity of 93% to 95% (Wallace, 2007).

Ultimately, SLE is a complex autoimmune disease with multiple pathogenic mechanisms. In this study, serum level of C3 and C4 were assessed, and a correlation between complement and different clinical aspects including: oral manifestations has been established. This correlation suggests possible use of decreased level of C4 as a biomarker for SLE. This study also suggests that the increased level of C3 is due to alternative activation of complement pathway in SLE patients.

REFERENCES


Allergy and Infectious Diseases Science Education, United States.


Table (1): Clinical and laboratory characteristics of studied SLE patients.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Female NO (%)</th>
<th>Male NO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 (90.0)</td>
<td>2 (10.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>$\bar{X} \pm SD$</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28.7 ± 7.32</td>
<td>19-4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum Creatinine</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.55</td>
<td>(1.10-16.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TPR</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>692.5</td>
<td>(135-6320)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ESR</th>
<th>$\bar{X} \pm SD$</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>61.05 ± 12.51</td>
<td>35.0-80.0</td>
</tr>
</tbody>
</table>

Table (2): Means of serum complements C4, C3 values of SLE patients.

<table>
<thead>
<tr>
<th>Complement</th>
<th>$\bar{X} \pm SD$</th>
<th>Range</th>
<th>Normal value</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4</td>
<td>0.15 ± 0.07</td>
<td>0.10-0.30</td>
<td>8 (40.0)</td>
<td>12 (60.0)</td>
</tr>
<tr>
<td>C3</td>
<td>1.15 ± 0.48</td>
<td>0.10-2.20</td>
<td>15 (75.0)</td>
<td>5 (25.0)</td>
</tr>
</tbody>
</table>

Table (3): Frequency of exacerbation and remission among studied SLE patients.

<table>
<thead>
<tr>
<th>Activity</th>
<th>N = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remission</td>
<td>13 (65.0)</td>
</tr>
<tr>
<td>Exacerbation</td>
<td>7 (35.0)</td>
</tr>
</tbody>
</table>
Fig 1: Sex of 20 SLE cases in the present work.

Fig 2: shows levels of C4, C3 in SLE patients.
Fig 3: Groups of exacerbation and remission.

Table 4 shows relation of C3, C4 and disease activity.

<table>
<thead>
<tr>
<th>Complement</th>
<th>Remission (N=13)</th>
<th>Exacerbation (N=7)</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4</td>
<td>0.164±.067</td>
<td>1.128±.075</td>
<td>1.07</td>
<td>0.04*</td>
</tr>
<tr>
<td>C3</td>
<td>1.16±0.41</td>
<td>1.12±0.62</td>
<td>0.14</td>
<td>0.88</td>
</tr>
</tbody>
</table>

P< 0.05 significant correlation between C4 and exacerbation

Table 5: Relation between median of S. creatinine and TPR of SLE patients, with disease activity using Mann Whatin P (Z value)

<table>
<thead>
<tr>
<th></th>
<th>Remission</th>
<th>Exacerbation</th>
<th>Mann. Whatin P (Z value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Creatinine</td>
<td>1.9 (1.1-4.5)</td>
<td>4.5 (3.6-16.5)</td>
<td>3.00 [0.002* H.S]</td>
</tr>
<tr>
<td>Median (Range)</td>
<td>1.9 (1.1-4.5)</td>
<td>4.5 (3.6-16.5)</td>
<td>3.00 [0.002* H.S]</td>
</tr>
<tr>
<td>TPR Median</td>
<td>600 (135-750)</td>
<td>1523 (1200-6320)</td>
<td>3.61 [0.000** H.S]</td>
</tr>
<tr>
<td>Median (Range)</td>
<td>600 (135-750)</td>
<td>1523 (1200-6320)</td>
<td>3.61 [0.000** H.S]</td>
</tr>
</tbody>
</table>

P <0 .001 Highly significant
Table (6): Means of age and ESR of studied SLE patients by exacerbation and remission

<table>
<thead>
<tr>
<th>Laboratory variables</th>
<th>Remission (N=13)</th>
<th>Exacerbation (N=7)</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>29.0±7.41</td>
<td>28.14±7.71</td>
<td>0.24</td>
<td>0.81</td>
</tr>
<tr>
<td>ESR</td>
<td>60.5±13.7</td>
<td>62.0±10.87</td>
<td>0.24</td>
<td>0.81</td>
</tr>
</tbody>
</table>

(P< 0.05 Significant)

Table (7): Correlation coefficient (r) between C₃, C₄, certain laboratory variables among studied SLE patients.

<table>
<thead>
<tr>
<th>Laboratory variables</th>
<th>C₃</th>
<th>p</th>
<th>C₄</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Creatinine</td>
<td>-0.11</td>
<td>0.63</td>
<td>-0.04</td>
<td>0.85</td>
</tr>
<tr>
<td>ESR</td>
<td>0.26</td>
<td>0.27</td>
<td>0.08</td>
<td>0.72</td>
</tr>
<tr>
<td>TPR</td>
<td>-0.07</td>
<td>0.76</td>
<td>-0.28</td>
<td>0.22</td>
</tr>
</tbody>
</table>

(P< 0.05 Significant)

Fig (4): Butterfly rash (a) and skin ulcer in hand (b), skin ulcer in feet (c) and skin ulcer in arm (d) in SLE
Evaluation of Serum Complement C3 and C4 Levels….

Fig (5): lip ulceration (a) palatal petechiae (b)

Fig (6): palatal plaque.

Fig (7): Atrophic or ulcerated round lesions with peripheral keratotic striae in buccal mucosa (a) atrophic lip ulcer (b)
The study aimed to determine the levels of complement C3, C4 in the blood of patients with red spotted disease, which helped us in interpreting the role of complement in diagnosis and onset of this disease.

Red spotted disease is a chronic inflammatory disease caused by the body's immune system. This means that the immune system's cells recognize some of the body's tissues as foreign and attack them, causing damage to these tissues. A patient with red spotted disease produces antibodies in the blood, with the target of destroying living tissues. From these tissues, skin, heart, lungs, kidneys, joints, and nerves.

Where red spotted disease is an immune disease, it is possible that there is a strong relationship between changes in the percentage of complement in the blood and the development of the disease in the form of indications that can help us guide on the occurrence and emergence of red spotted disease.

This study included twenty patients with red spotted disease, according to the criteria of the American Lupus Patients Association, and the levels of complement C3, C4 were measured using the radioimmunoassay technique.

The results of this study indicate that the complement C3, C4 play a significant role in the emergence and development of this disease and also play a role in the emergence of symptoms. The study also indicated the possibility of using a decrease in the level of C4 in the patient's blood as a sign of red spotted disease.