Evaluation of Complement (C3d) as Urinary Immune-Related Biomarker in the Diagnosis of Lupus Nephritis

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Abstract
Background: Kidney involvement is a key problem in systemic lupus erythematosus (SLE), affecting about 50% of patients and responsible for a large amount of morbidity and mortality. Early identification and treatment can significantly alter the course of renal illness and enhance long-term survival. This study aimed to evaluate the diagnostic value of Complement 3d as urinary immune-related biomarker for the diagnosis of lupus nephritis in patients of SLE. Methods: A cross sectional study on 78 patients with SLE (72 females and 6 males) was conducted from September 2021 to March 2022. Renal involvement was determined using the renal SLEDAI, which incorporates the SLEDAI-2k kidney-related parameters: "hematuria, pyuria, proteinuria, and urinary casts." Results: Urinary complement 3d levels were statistically higher in the LN group than those without LN (p value <0.000). Levels of urinary C3d positively correlated with renal SLEDAI. Conclusions: This study concluded that urinary C3d had a high accuracy and can be considered a good predictor for the diagnosis of active LN in SLE patients, and C3d can be used to evaluate nephritis activity in SLE patients.
Keywords: u Complement 3d, urinary biomarker, lupus nephritis biomarker.

1. Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune connective tissue disease associates with the production of a variety of autoantibodies directed against native DNA and other cellular constituents. It is a prototypic disease with heterogeneous clinical manifestations that may involve many different organs and systems [1].
Most patients with SLE develop kidney disease (nephritis). Lupus nephritis (LN) is an important cause of morbidity and even mortality in patients with SLE. Lupus nephritis has diverse morphologic manifestations with varying clinical presentations and consequences. The pathogeneses involve immune complexes, which can deposit anywhere in the kidney [2].
Current laboratory markers for LN such as "proteinuria, urinary protein-to-creatinine ratio, creatinine clearance, anti-dsDNA, and complement" levels are unsatisfactory. They lack sensitivity and specificity for differentiating renal activity and damage in LN. Significant kidney damage can occur before renal function is impaired and first detected by laboratory parameters. Renal biopsy is the gold standard for providing information on the histological classes of LN and the relative degree of activity and chronicity in the glomeruli. However, it is invasive and serial biopsies that are impractical in the monitoring of LN. Thus, novel biomarkers that are able to discriminate lupus renal activity and its severity, predict renal flares, and monitor treatment response and disease progress are clearly necessary [3] [4].
In patients with SLE, the levels of C3 and C4 in the blood are often decreased. However, serum levels of low complement are unreliable as disease activity markers in lupus since they remain at low or normal levels regardless of the disease activity and their inability to accurately forecast flares. Components of the complement are detectable in the urine of LN patients, particularly those with active kidney disease. The presence of these components in the urine may suggest activation of the complement in the kidney and represent inflammation in the renal tissue [5]. Levels of complement 3d, have been identified as "potential biomarkers of complement-mediated injury in renal diseases. Increased urine levels of C3d are found in tubulointerstitial nephritis, membranous nephropathy and non-membraneous glomerular diseases" [6].
This study aimed to evaluate the diagnostic value of C3d as urinary immune-related biomarker for diagnosis of nephritis in SLE patients.

2. Methods

A cross sectional study on 78 patients with SLE (72 females and 6 males) was conducted from September 2021 to March 2022. All the patients were recruited from AL-Sader teaching medical city in the province of Najaf, Iraq. The age range of patients was between 12 to 53 years.

Inclusion Criteria

Each patient should have at least 4 scores of

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"American college of rheumatology" of SLE to be enrolled in this study as a SLE patient.

Exclusion Criteria

Any patient with other connective tissue diseases such as rheumatoid arthritis or scleroderma, diabetes mellitus, any disease that can affect urinary biomarker such as immunodeficiency disease, autoimmune disease, malignancy, and chronic infection, urinary tract infections, LN but undergoing hemodialysis or has a history of renal transplantation and LN but with renal insufficiency from non–lupus-related causes.

Patients Groups

Renal involvement in SLE patient was assessed with the renal SLEDAI, which consists of the 4 "kidney-related parameters of the SLEDAI-2K: hematuria, pyuria, proteinuria, and urinary casts. Each item in the renal SLEDAI is assigned 4 points. Thus, scores for the renal SLEDAI can range from 0 (inactive renal disease) to a maximum of 16."

Therefore, patients at the time of their clinic visit were randomly selected and classified into:

1. Group of patients without LN.
2. Group of patients with LN.

For the purposes of the present study, the group of patients without LN was prospectively defined as those having a renal SLEDAI score of 0 or 4 (one abnormal result for renal parameters, when hematuria, pyuria, or urinary casts but not proteinuria was the renal-related criterion. While "the LN group was prospectively defined as those having a renal SLEDAI score of ≥8 (i.e, at least 2 abnormal results for renal parameters) or, when proteinuria was the renal-related criterion, a renal SLEDAI score of 4." The latter limit was used in order to select those patients who had already displayed changes in glomerular permeability [7].

The samples (blood and urine) were collected from the SLE patients. Afterwards, aliquots of fresh urine samples from each patient were frozen at -40°C for later analysis. The Human Complement Fragment 3d was measured using ELISA Kit (E5115Hu, bioassay technology laboratory®, China; Sensitivity: 0.023µg/ml).

3. Statistical Analysis

Data of both study groups were entered and analyzed using the statistical package for social sciences (SPSS) (version.25 Inc, Chicago, USA) and GraphPad Prism software, version 9.3.1 (La Jolla, CA, USA). Descriptive statistics presented as "median with interquartile range (IQR)" frequencies and proportions. Comparison between groups was calculated using "Mann-Whitney U test. Multiple comparisons were done using the Kruskal-Walli's test". Pearson's Chi-square and Fisher's exact (when proportions were too small) tests used alternatively to compare frequencies. Correlations between urinary immune biomarker, "laboratory parameters, and SLEDAI scores were performed using Spearman's rank correlation coefficient. "Level of significance of ≤ 0.05 was considered as significant difference or correlation. "The receiver operating characteristic curve (ROC)" analytical curve has been used to estimate the diagnostic efficiency of C3d as clinically viable by assay the ratio of area under the curve (AUC).

4. Results

According to renal SLEDAI score, 46 (59%) SLE patients were with LN and 32 (41%) SLE patients were without LN as shown in table (1).

There was a significant difference (P = 0.003), between the mean age of SLE patients with LN [28 year (± 9.03)] and SLE patients without LN [35.1 ± 10.7], and there was an insignificant difference regarding the distribution of both groups according to gender (P = 0.187). The group of SLE patients with LN included 2 males and 44 females, accounting for 4.3 % and 95.7%, respectively, whereas SLE patients without LN included 4 males and 28 females, accounting for 12.5 % and 87.5 %, respectively, as shown in table (1).

Table 1: Demographic Characteristics and Medications of SLE Patients with and without LN

Characteristic	Patients with LN n = 46	Patients without LN n = 32	P- value
Age, mean (± SD) years	28 (± 9.03)	35.1 (± 10.7)	0.003*
Adult patients; no. (%)	37 (80.4 %)	30 (93.8 %)	0.097
Adolescent patients; no. (%)	9 (19.6 %)	2 (6.2 %)	
Gender: Male no. (%) Female: no. (%)	2 (4.3%)	4 (12.5%)	0.187
	44 (95.7%)	28 (87.5%)	

Blood and Urinary Parameters of SLE Patient Groups

The outcomes of blood and urinary parameters results in the two patient groups are shown in tables (2) and (3). Patients with LN had significantly higher levels of blood urea and serum creatinine [35 mg/dl (27.0-58.2) and 0.8 (0.66-1.35), respectively] compared to that of patients without LN [22 mg/dl (17.5-28.0) and 0.7 mg/dl (0.6-0.7), respectively] (P < 0.01) and there was a significant difference in glomerular filtration rate (P- <0.05) between patients with LN [99.5 mL/min (53.5-122.5)] and patients without LN [114.5 mL/min (104-124)], while the hematological parameters (hemoglobin, WBC and platelets) showed an insignificant difference between patients with LN [11.2 mg/dl (10.3-11.7), 6.3 x 109/liter (4.1-9.5) and 232 x 109/liter (196-301), respectively] and patients without LN [11.5 mg/dl (10.4-12.9), 5.5 x 109/liter (4.5-7.5) and 243 x 109/liter (200-296), respectively] as revealed in table

(2).

Table 2: Blood Biochemical and Hematological Parameters of SLE Patient Groups			
Characteristic	Patients with LN	Patients without LN	P- value
Blood urea (mg/dl)	35 (27.0-58.2)	22 (17.5-28.0)	<0.0001**
Serum creatinine (mg/dl)	0.8 (0.66-1.35)	0.7 (0.6-0.7)	0.003*
Glomerular Filtration Rate (EPI) (mL/min/1.73m2)	99.5 (53.5-122.5)	114.5 (104-124)	0.050*
Hemoglobin (mg/dl)	11.2 (10.3-11.7)	11.5 (10.4-12.9)	0.374
WBC (x 109/liter)	6.3 (4.1-9.5)	5.5 (4.5-7.5)	0.384
Platelets (× 109/liter)	232 (196-301)	243 (200-296)	0.872

There were a highly significant differences (P-<0.0001) in spot urinary protein, and spot protein to creatinine ratio between patients with LN [105.4 mg/dl (24.1-225.0) and 917.9 mg/g (344.6-3159.7), respectively] and patients without LN [14.0 mg/dl (6.5-20.0) and 109.3 mg/g (52-222.5), respectively], while there was no significant difference in urinary creatinine between patients with LN [70.7 mg/dl (33.8-143.2)] and patients without LN [107.3 mg/dl (66.0-175.6)].

Table 3: Urinary Biochemical Parameters in SLE Patient Groups			
Characteristic	Patients with LN	Patients without LN	P- value
Urinary protein (mg/dl)	105.4 (24.1-225.0)	14.0 (6.5-20.0)	<0.0001**
Urinary creatinine (mg/dl)	70.7 (33.8-143.2)	107.3 (66.0-175.6)	0.076
Protein to creatinine ratio (mg/g)	917.9 (344.6-3159.7)	109.3 (52-222.5)	<0.0001**

Urinary Complement 3d in SLE Patients with and without LN

Urinary C3d was normalized to urinary creatinine levels and comparison between lupus patients with and without LN was done to evaluate its association with lupus renal disease. Urinary C3d levels were significantly higher (P = 0.010) in the LN group (median 5.0 ug/mg creatinine, IQR 1.5-15.9; n = 46) compared to those without LN (median 1.73 ug/mg creatinine, IQR 0.78-5.8; n = 32) as shown in figure (1).

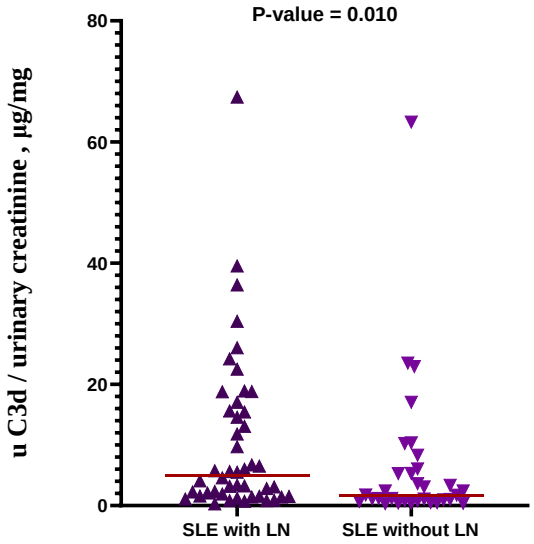

Figure 1: Urinary Levels of C3d in SLE Patients with and without Nephritis.

Renal Histopathology Characteristics of LN Patients

A Twenty-six of the 46 LN patients who were selected for the study had a kidney biopsy. According to the "World Health Organization's system" for classifying diseases [8], 5 of these patients had type II glomerulonephritis (GN), 14 had type III GN, 6 had type IV GN, and 1 had type V glomerulopathy as shown in figure (2).

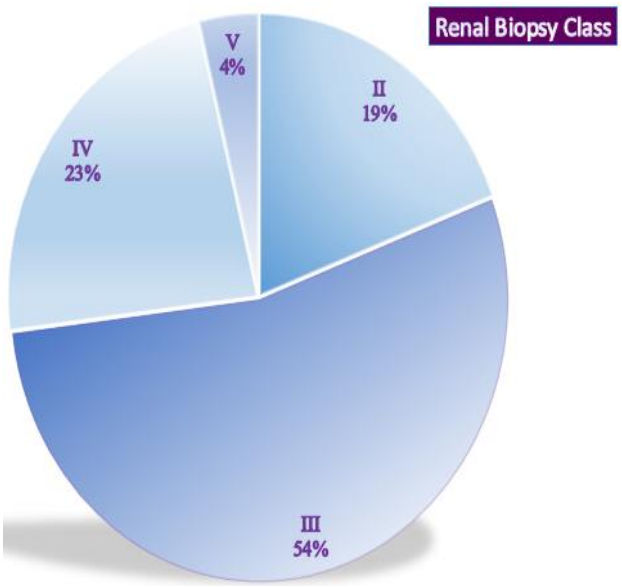

Figure 2: Renal Histopathology Characteristics of Lupus Nephritis Patients

Diagnostic Performance of Urinary Complement 3d

A receiver operating characteristic (ROC) curve was used to quantify the diagnostic utility of urinary complement 3d by ELISA between LN patients proven by biopsy (the current gold standard) and SLE patients without renal involvement. The results were shown in figure (3) and table (4). Indeed, the cutoff values obtained for urinary complement 3d, were good predictors because of an area under the curve of more than 0.8 and significant P values (P <0.05), as shown in table (4). The level of accuracy was more than 70%.

At a cutoff value ≥ 1.19 ug/mg creatinine, the sensitivity of urinary C3d levels for the diagnosis of LN was 84 %, with a specificity of 57%.

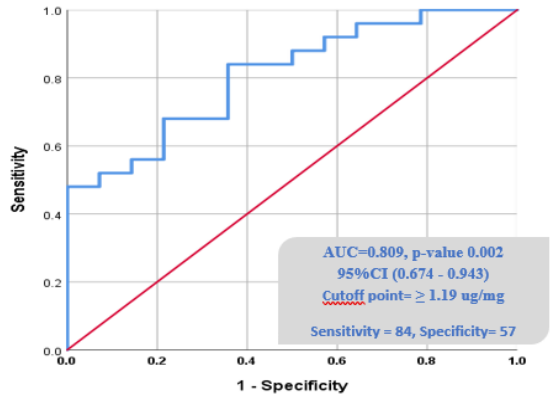

Figure 3: Receiver Operator Characteristic (ROC) Curve Analysis to Find the Best Urinary Complement 3d / Urinary Creatinine Cutoff Value that Can Predict Lupus Nephritis in Systemic Lupus Patients.

Table 4: Characteristics of Receiver Operator Characteristic (ROC) Curve in LN Patients	
Characteristic	u C3d /u creatinine
AUC	0.809
SE	0.069
Sig.	0.002
95% Confidence Interval	0.674 – 0.943
Cut off point	≥ 1.19 ug/mg
Sensitivity (%)	84
Specificity (%)	57
PPV (%)	77.7
NPV (%)	66.6
Diagnostic effectiveness (accuracy)	74 %
Youden's index	0.41

AUC, Area under the Curve; SE, stander error, Optimal Cut-Point Value, PPV (Positive Predictive Value), NPV, (Negative Predictive Value); Youden's index is a measure for evaluating the biomarker effectiveness.

Urinary Complement 3d and Renal Activity

When comparing the renal SLEDAI score in LN patients to the urinary C3d/urinary creatinine, a significant correlation was also found (r=0.25, P=0.029) as shown in figure (4).

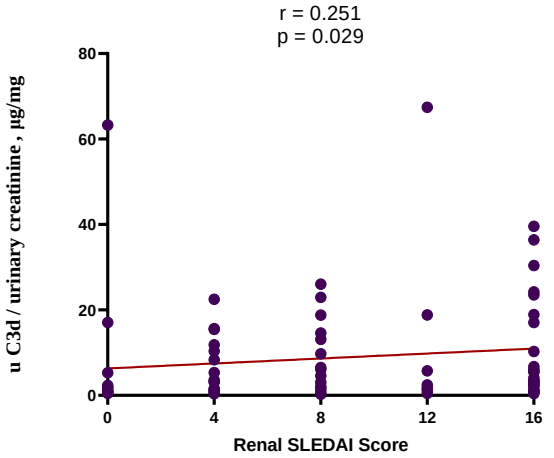

Figure 4: Correlation Between Urinary C3d Levels in SLE Patients and Indices of Renal Activity.

In the group of patients with LN, although urinary protein was not significantly associated with urinary C3d, those patients showed a significant correlation between urinary protein (quantitative) and renal SLEDAI. Renal SLEDAI was recalculated without proteinuria to indicate that the increase of this biomarker was due to increased local production rather than nonspecific protein loss.

There was a significant association between urinary C3d/urinary creatinine and renal SLEDAI in LN patients as shown in Figure (5)

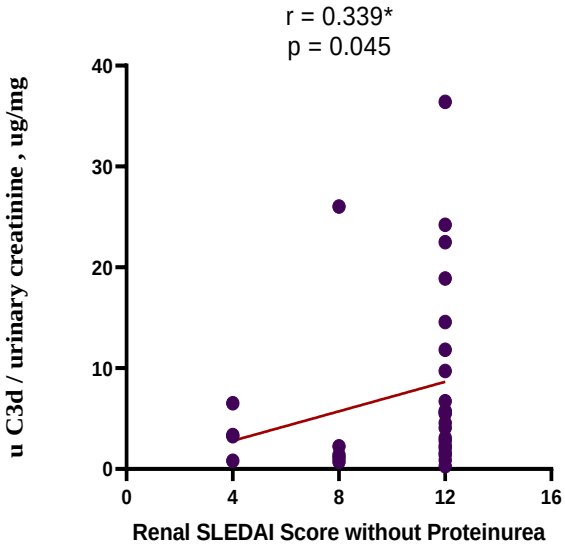

Figure 5: Correlation Between Urinary C3d Levels in LN Patients and Indices of Renal Activity.

5. Discussion

Complement 3d is a stable C3b degradation product produced by the constitutively active serine protease Factor I in the presence of complement receptor 1 (CR1) and Membrane Cofactor of Proteolysis (MCP) [9].

The present study revealed a significant difference in urinary C3d/ urinary creatinine level between the LN and non-LN groups. Several previous studies supported this result as the studies of Negi et al [10] and Ganguly et al [9], which showed a significant difference between active LN patients and SLE nonrenal disease patients. Furthermore, Ganguly et al [9] reported the patients' response to treatment, in which urinary C3d levels are high at the onset and significantly fall at the end of 3 months of treatment in the entire cohort.

The level of urinary C3d/urinary creatinine showed a correlation with the disease activity (renal SLEDAI) (r = 0.251, P = 0.029). This finding agrees with Negi et al [10] and Ganguly et al [9]Thus, it is a possible biomarker to predict the presence of active lupus nephritis. In addition, these results indicate the correlation of urinary C3d level with the degree of renal inflammation in LN.

To further support of this correlation between urinary C3d level and the degree of renal inflammation in LN, the proteinuria parameter was removed from the renal SLEDAI, and reanalysis of the data was done. The correlation remained statistically significant even after exclusion of proteinuria from the score (r 0.339, P 0.045). Thus, the elevation of urinary C3d observed

in patients with LN may be due to increased local production rather than nonspecific loss of filtered protein (this nonspecific loss of filtered protein that led to persistent proteinuria may not necessarily indicate ongoing inflammation in the kidneys and may be contributed by pre-existing chronic lesions or recent damage in the kidneys during the course of the disease). Flares of nephritis can occur without any observable and recent increase in the degree of proteinuria, and the correlation of the remaining parameters in the renal SLEDAI, (urine sediment: hematuria, pyuria, and the presence of casts) with the levels of urinary C3d can reflect the inflammation rather than an increased protein filtration rate.

Theoretically there are several ways that could lead to an increased excretion of C3d in the urine. Complement 3d is a relatively small molecule of 35 kDa, which will be filtered to a certain degree. Like other low-molecular weight proteins, filtered C3d may be reabsorbed by the proximal tubular cells. In such a case, urinary C3d excretion should be elevated in patients with tubular interstitial diseases. Urinary C3d can also be derived from filtered C3. Complement 3 has a high molecular weight (195 kDa) and will only pass in the urine of patients with glomerular diseases whose glomerular permeability is increased. Alternatively, C3 may be locally produced. C3 gene expression has been observed in both glomerular epithelial cells and tubular cells [11].

The receiver operating characteristic (ROC) curve analysis was constructed to quantify the utility of urinary C3d in the diagnosis of LN in SLE patients with nephritis proven by biopsy (the current gold standard) and SLE patients without renal involvement. At a cutoff value of ≥ 1.19 ug /mg creatinine, urinary C3d was considered as a good predictor for LN because the area under the curve was more than 0.7 and a significant P value. The level of accuracy was 74%, with a high sensitivity (84%) but relatively low specificity (57%). Thus, urinary C3d level can be used as a cheap, readily available biomarker to diagnose LN because urinary complement fragments may better reflect kidney disease as they are less likely to be influenced by systemic activity and can be used to predict the presence of active lupus nephritis.

In the current study, there is no significant correlation between urinary C3d and the age or the gender of the LN patients. These findings demonstrate ideal features of complement 3d as biomarker for LN when this biomarker is not affected by age or gender [12].

In the present study, there is no significant correlation of the blood biochemical parameters (blood urea, serum creatinine and GFR) with urinary C3d in LN patients because this marker reflect the renal inflammatory state rather than the renal insufficiency, where abnormal eGFR decline or increase in serum creatinine occurs in stage 4 chronic kidney disease (CKD) [12] which indicate severe renal insufficiency.

Patients with LN did not exhibit a significant correlation between hemoglobin and urinary C3d. This finding may indicate the lack of an important effect of this biomarker on the renal production of erythropoietin in LN patients of this study.

In addition, there was no apparent correlation between WBC and C3d in the urine of patients with LN. Although thrombocytopenia is known as one of the hematological criteria of SLE, according to the American College of Rheumatology (ACR) classification criteria. Patients diagnosed with LN did not demonstrate a significant correlation between platelets and C3d in urine, which may indicate that this biomarker doesn't have an important role in the development of thrombocytopenia in LN patients.

The present finding revealed that the urinary protein had no apparent correlation with C3d in the urine of patients with LN. Manzi et al [13] likewise found no correlation between urinary C3d and proteinuria. Lack of such correlation between of C3d and spot urinary protein in LN patient may be due to low sensitivity of spot urinary protein increase with the increase of the immune biomarker, since the spot measurement of protein in urine may vary depending upon the hydration status of the patient and on renal function. In addition, this immune related biomarkers may reflect the inflammatory status of the kidney rather than the loss of protein in urine because persistent proteinuria may not necessarily indicate ongoing inflammation in the kidneys and may be contributed by pre-existing chronic lesions or recent damage in the kidneys during the course of the disease [3].

This limitation of spot urinary protein can be overcome by the use of spot urinary protein to creatinine ratio by normalization of spot urinary protein with urinary creatinine.

Spot urinary protein/creatinine ratio has been increasingly widely adopted as a simpler method than 24-hour timed urine collections to estimate the degree of proteinuria. The numeric ratio of protein and creatinine concentrations approximates the number of grams per day of proteinuria [14]. In addition, American College of Rheumatology (ACR) renal disease subcommittee recommends measuring protein-to-creatinine ratio in a morning void urine sample or even a spot urine sample [12].

The protein to creatinine ratio has proven to be more beneficial for glomerular disorders in general and lupus nephritis in particular due to its simplicity and convenience [15].

The current finding showed that the urinary creatinine had a strong negative correlation with C3d in the urine of LN patients. In spite of this strong correlation, most of the results of urinary creatinine of LN patients were within the normal range. These findings may imply that the level of biomarker rise with a relative decrease in creatinine clearance but without an abnormal decline in renal efficiency because this biomarker reflect the renal inflammatory state rather than the renal insufficiency.

There was a significant correlation of urinary C3d/urinary creatinine level with protein to creatinine ratio in the group of patients with LN ($r = 0.279$, $P = 0.025$). This result was supported by Ganguly et al [9]. As mentioned above, the

limitations of spot urinary protein were overcome by the use of spot PCR. So, the spot PCR rather than the spot urinary protein correlates with the severity of the renal inflammation, which was indicated by the increase in the level of urinary C3d.

6. Conclusions

Based on this study, the following conclusions could be made

1. Urinary complement 3d levels were statistically higher in the LN group than those without LN, and had a high accuracy, and can be considered as good predictors for diagnosis of active LN in SLE patients.
2. Levels of urinary C3d positively correlate with rSLEDAI and can be used to evaluate disease activity.
3. From the above two points, urinary C3d measurements may be an ideal non-invasive measure, which could reduce the need for renal biopsy.

7. Recommendations

Measure the urinary C3d in 24-hour urine collections instead of spot urine collection, which is more sensitive.

1. Measure the serum C3d in LN patients to identify if the production of this biomarker is local (renal) or systemic.

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