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Original Research Article

Levels of Circulating Reticulocytes-C4d: Is There Link to Disease Activity in Patients with SLE?

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ABSTRACT

Objective: The determination of cell-bound complement activation products (CB-CAPs), such as C4d linked to reticulocyte (R-C4d), are potential biomarkers of disease activation state. Within this context, this cross-sectional study aimed to assess whether, in SLE, R-C4d levels can be predictors of the disease activity in a set of patients selected from Brazilian population.
Method: Forty patients with SLE confirmed by the American College of Rheumatology (ACR) classification criteria who were assessed at the time of the visit to the clinic using the SLEDAI-2K index (Systemic Lupus Erythematosus Disease Activity Index 2000) were recruited, besides to 20 adult healthy subjects (controls). Patients with SLEDAI≤ 4 were classified as having low disease activity (SLE-I, n = 20) and those with SLEDAI > 4 as carriers of active disease (SLE-A, n = 20). Peripheral blood samples were collected in EDTA with subsequent separation of reticulocytes population and analyze by flow cytometry to determine the levels of R-C4d.
Results: A positive correlation between R-C4d levels and SLEDAI 2K was observed. Significant differences between controls and LES-A ($p < 0.0001$), and between LES-I and LES-A ($p < 0.0001$) were observed.
Conclusion: Since reticulocytes are present in the peripheral blood for a period of only 24-48 hours and then turning into mature erythrocytes, higher levels of R-C4d may reflect early active disease. Thus, increased R-C4d expression may be a useful tool for screening disease severity at initial diagnosis and for monitoring disease status of patients.

Keywords: Cell bound complement c4d, reticulocytes, systemic lupus erythematosus of disease activation state.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disorder affecting multiple organs whose etiology is not well understood. [(1)] Features of the disease include production of autoantibodies, excessive complement system activation and immune complexes deposition in various tissues. [(2,3)] Among the various manifestations of SLE, lupus nephritis (LN) is the most relevant occurring in up to 50% of the patients at the diagnosis, and up to 60% of patients during the course of the disease. [(4)]

In current clinical practice, diagnosis of SLE is complicated by the diversity of clinical signs and absence of a specific laboratory test. Currently, diagnosis and classification of SLE is made using the criteria proposed by American College of

Rheumatology (ACR) and a clinical index can be used to evaluate disease activity as SLEDAI-2K (Systemic Lupus Erythematosus Disease Activity Index 2000) that records clinical manifestations and laboratory data of affected patients. [(5)]

Given the complexity of LES, many studies have been conducted to better understand its pathophysiology and such studies enabled the development of some biomarkers to be used in clinical practice. Complement system activation is observed in patients with SLE inducing pathological immune responses. Within this context, the discovery and validation of complement activation products associated with blood cells (CB-CAP = Cell -bound complement activation products) can be an important tool for clinical management of SLE as potential biomarkers. [(6-8)] Using flow cytometry methodology different CB-CAPs have been identified in circulating blood cells with high specificity to SLE. [(6-10)] Due to number and location of erythrocytes it was hypothesized that these circulating cells could serve as biological vectors in vivo of inflammatory condition and therefore markers of disease activity in patients with SLE. Another fact is that red cells develop from hematopoietic stem cells in the bone marrow and emerge as reticulocytes, with different phenotypic characteristics for 1 to 2 days before reaching their maturation to erythrocytes. Reticulocytes released into the bloodstream during a state of active disease may have been immediately exposed to C4-derived fragments generated from the complement activation occurring after the connection of these fragments to erythrocyte precursors. Thus, it has been hypothesized that reticulocyte-C4d (R-C4d) levels can be considered a predictive biomarker reflecting early disease activity. [(6,7)] Based on the above described, this study intends to reexamine this hypothesis by investigating whether high levels of R-C4d reflects disease activity, as well as whether its levels correlates with the SLEDAI-2K index, in patients with or without active disease, under treatment. Our main motivation for the developing the present study was the frequent severity of the disease, diagnostic difficulties due to its multiple presentations, together with the lack of accurate and reliable laboratory method.

MATERIALS AND METHODS

Patients

Forty patients with SLE diagnosed according to ACR classification criteria (1997) were sequentially recruited in the Rheumatology Clinic of Santa Casa Hospital, Minas Gerais, Brazil, from February 2013 to April 2016. Patients were assisted by medical staff through which clinical and laboratory data were obtained. Patients with SLE- related diseases, immunosuppressive diseases including HIV / AIDS, autoimmunity, patients who did not authorize and / or did not sign the consent and pregnant women were excluded. All patients were under treatment, alone or in combination. Drugs such as azathioprine, prednisone and hydroxychloroquine were the most used by patients. As control group, twenty women were selected with no autoimmune and/or inflammatory diseases and no family history of SLE. Laboratory evaluation of the control group participants was held using conventional biochemical tests as glucose, liver enzymes, blood count, urine routine and proteinuria, and all results were normal. Clinical status and exclusion of drugs with the potential to affect immune system was checked by self reporting.

For this study women were selected with age ranging from 18 to 69 years old. According to Yee et al, 2011 [(11)] and based on SLEDAI -2K criteria, 20 patients were classified with inactive disease or low activity disease (SLEDAI-2K $\leq$ 4) and 20 with active disease (SLEDAI-2K > 4) while simultaneously were selected 20 age matched women without SLE or other diseases (controls). According to renal biopsy results, patients were also classified as having lupus nephritis (n=14) or not (n=21) at the moment of their enrollment in this study. This study was reviewed and approved by Research Ethics Committee of

the Federal University of Minas Gerais, Brazil (protocol number CAAE - 01928412.8.0000.5149), and informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations.

A sample of 5 ml EDTA.K3 of peripheral blood from each fasting eligible participant was collected. Samples were analyzed for R-C4d immediately after venepuncture.

Flow cytometry technique optimization for R-C4d complement fragments

Determination of R-C4d blood levels was performed according to Liu et al., 2005. Briefly, whole blood samples were washed, diluted with physiological saline and aliquoted for staining using a murine monoclonal antibody specific for C4d (reactive with fragments containing C4d; Quidel, San Diego, CA) or isotope control MOPC-21, which was added to red blood cells (RBC) suspensions at concentration of 10 μg/mL (50 μL - 1: 100 dilution). Goat anti IgG F (ab')^2 conjugated to a secondary murine antibody labeled to phycoerythrin was used at a concentration of 10 μg/ml (2,5 μL). After antibody staining, cell suspensions were incubated with thiazole orange (ReticCount reagent; Becton Dickinson) to identify reticulocytes or with saline (unlabeled control). Stained cells were analyzed using flow cytometry (BD FORTESSA, U.S.A.).

Data acquisition and analysis in flow cytometer

Flow cytometer used in this study is equipped with argon lamp that allows a basic evaluation of at least 10 parameters. Identification of the target cell population and determination of percentage of this population and subpopulation were made using BD FACSDiva Software™ (Becton Dickinson Immunocytometry Systems, San Jose, CA) coupled to a cytometer. Analysis of the cell population was performed using Flow Jo (FlowJo Enterprise). For analysis of R-C4d levels, erythrocytes were identified based on their "forward and side-scatter" (Size x Granularity) properties (FIGURE 1Ai and Bi), while reticulocytes were identified based on their forward-scatter properties and positive staining with thiazole orange (size x color) (FIGURE 1Aii and Bii). From previous chart, histograms were performed to analyze expression intensity of C4d on reticulocytes surface (FIGURE 1Aiii and Biii). Values were calculated as specific R-C4d expression less specific isotope control expression.

Statistical analysis

Data analysis was performed using GraphPad Prism software 6™, using t - Student test and analysis of variance (ANOVA), in case of normal distribution data for comparisons among two and three groups, respectively. In case of non-normally distributed data, Mann-Whitney and Kruskal-Wallis tests were used. Kolmogorov-Smirnov test was performed to evaluate normality of variables. In all cases, significance was considered at $p < 0.05$. There were also carried out correlation tests between of R-C4d expression and SLEDAI 2K index using Spearman.

RESULTS

Characteristics of the study participants

Table 1: Clinical characteristics of the patients with SLE

Characteristics	Results (%)
Age, mean +/- sd (range) years	43 +/- 12.18 (18–69)
Clinical manifestations, % positive since SLE diagnosis	
Antinuclear antibodies (fan)	71,5
Arthritis	36,4
Photosensitivity	34,0
Hematologic manifestations	42,0
leukopenia	7,9
Thrombocytopenia	11,4
Anemia	10,23
Proteinuria	38,6
Low complement C4	36
Low complement C3	29
Malar rash	28,4
Anti-dna/anti-sm	20,5

The study population consisted of 40 women with SLE and 20 healthy controls. At the time of entry into the study, mean ± SD age of the patients with SLE was 43 ± 12.18 years (range 18-69 years), while mean±SD age of the healthy control subjects was 44.7±12.9 years. The study included patients with new-onset as well as longstanding disease, showing a broad range

of disease activity, as reflected in the SLEDAI-2K score, and had a wide spectrum of organ involvement. Additional demographic and clinical features of the patients with SLE are shown in Table 1.

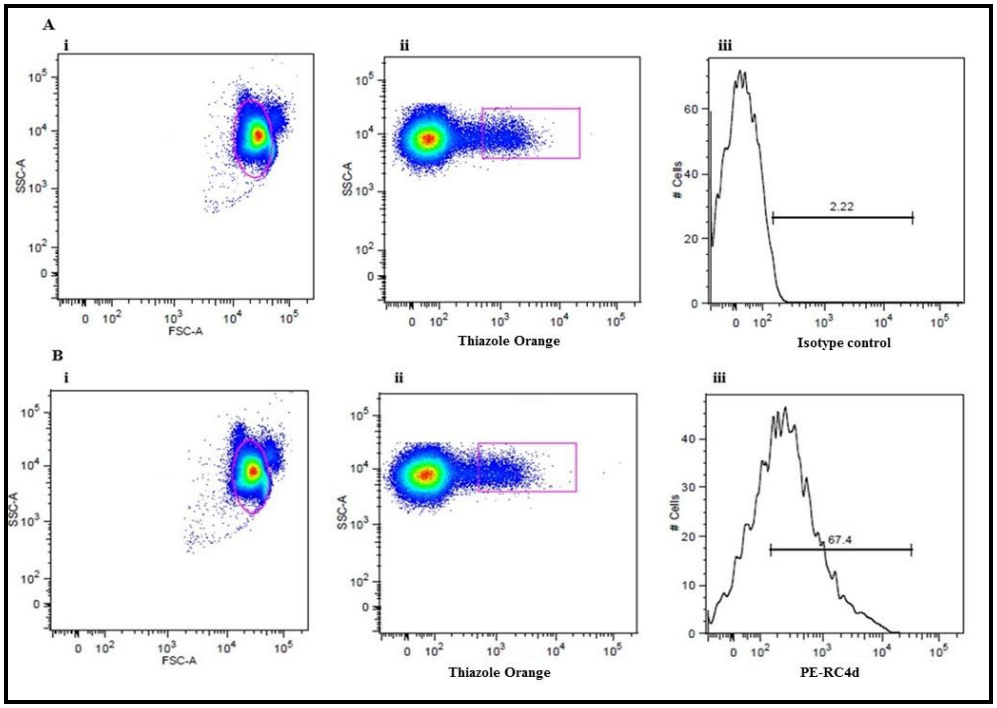

Figure 1: Analysis for R-C4d by flow cytometry. Isotype control R-C4d (Ai, ii and iii). Marking for R-C4d (Bi, ii and iii). Figures Ai and Bi show erythrocytes. Figures Aii and Bii show the population of reticulocytes by Thiazole Orange. Figures Aiii and Biii show the median fluorescence intensity histogram to assess the R-C4d on reticulocytes expression.

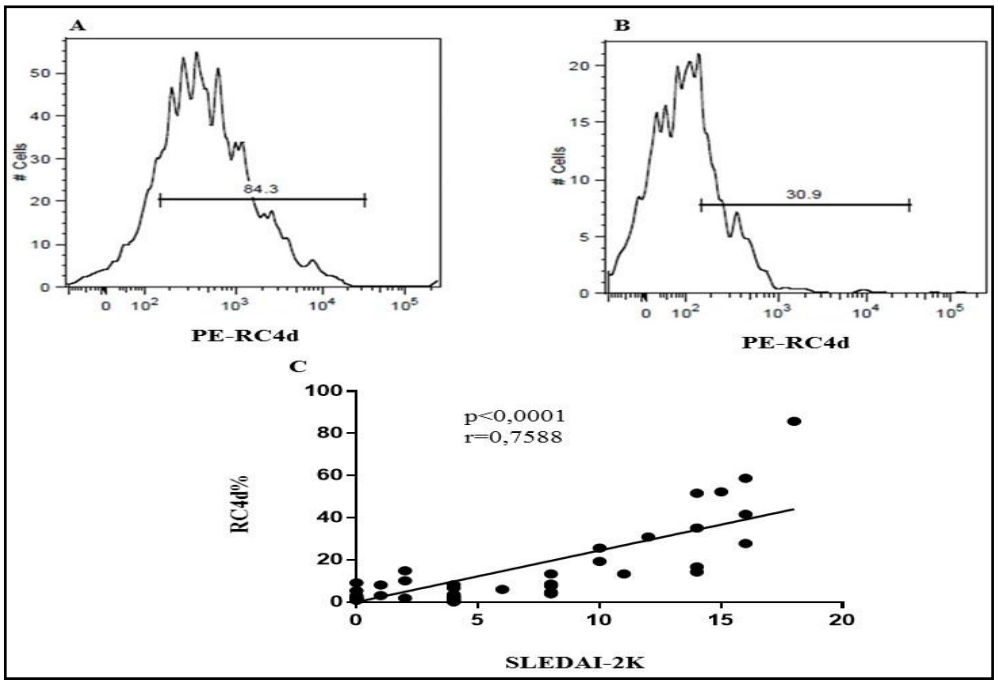

Figure 2: RC4d expression and correlation with SLEDAI-2K. Median fluorescence intensity (SMFI) histograms for R-C4d in patients with SLE and SLEDAI 2K=18 (A), SLEDAI-2K= 12 (B) obtained by flow cytometry as described in the methodology. Spearman correlation of R-C4d expression by flow cytometry and SLEDAI 2K values (C). P values <0.05 were considered statistically significant.

R-C4d expression and correlation with SLEDAI-2K index

For R-C4d technical validation, initially two blood samples obtained from two patients were tested with proven high

activity disease confirmed by high values for the SLEDAI index. A positive correlation between levels of this biomarker and SLEDAI-2K was found for the two patients with SLE, aged eighteen and thirty years old, diagnosed by the Rheumatology team from Santa Casa Hospital, Belo Horizonte, Brazil, according to criteria described in methodology. Clinical activity of SLE was determined and the eighteen years old patient had SLEDAI = 18, while the 30 years patient had SLEDAI = 12. Samples were analysed by flow cytometry and R-C4d levels were correlated to clinical activity of SLE. The newly diagnosed patient with the disease in its more severe form (SLEDAI=18) showed high expression of R-C4d (84.3% reticulocytes) (FIGURE 2A), while the patient with less serious activity (SLEDAI=12) had a lower expression of R-C4d (30.9% of reticulocytes) (FIGURE 2 B).

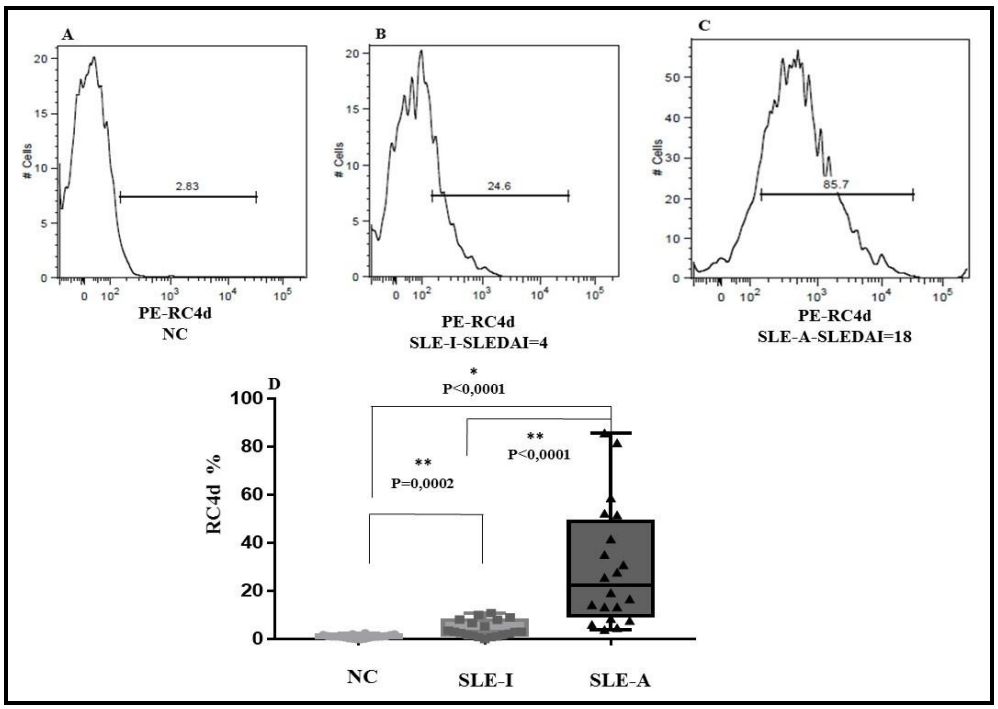

Figure 3: R-C4d expression in individuals with no disease (controls), in patients with inactive or active SLE. Median fluorescence intensity histograms for R-C4d in controls (A), SLE-I (B), and SLE-A (C). Expression of RC4d (in percentage) in controls and in patients with inactive (SLE-I) or active disease (D). Statistical tests: Kruskal-Wallis (*) and Mann-Whitney (**).

R-C4d expression in SLE patients with active, inactive/low activity disease and healthy controls

In a second phase of the study, Thirty-five patients with active and inactive/low active disease, under treatment, were analysed by flow cytometry. After assessment of reticulocytes populations using FlowJo software, R-C4d analysis was performed using technique and antibody concentrations described in standard methodology. Interestingly it was observed highly significant differences in R-C4d percentages among SLE-A (active SLE, SLEDAI-2K > 4), SLE-I (inactive SLE, SLEDAI-2K ≤ 4) and healthy control groups ($p < 0.0001$, Kruskal-Wallis test) (FIGURE 3A, B, C and D). It was observed that R-C4d expression is decreased in both SLE-I and control groups (FIGURE 3, B and D). After applying Mann-Whitney test significant differences between controls and SLE-A ($p < 0.0001$), and between SLE-A and SLE-I groups ($p < 0.0001$) were observed. The highest R-C4d values were observed in SLE-A group (FIGURE 3 C and D). R-C4d expression in women without SLE and in patients with both inactive and active SLE was also evaluated. Significant differences were observed and the highest expression values for R-C4d were observed in SLE patients ($p < 0.0001$ Mann-Whitney

test) (FIGURE 4). The same analysis was applied to patients with and without lupus nephritis (LN) compared to the control group. R-C4d expression was higher in patients with LN ($p < 0.0002$) (FIGURE 5).

In a second phase of the study, correlation analyzes were conducted between R-C4d expression and SLEDAI-2K index in all patients. A positive correlation among of R-C4d expression and higher SLEDAI 2K-index was observed and followed the severity of disease (FIGURE 2 C).

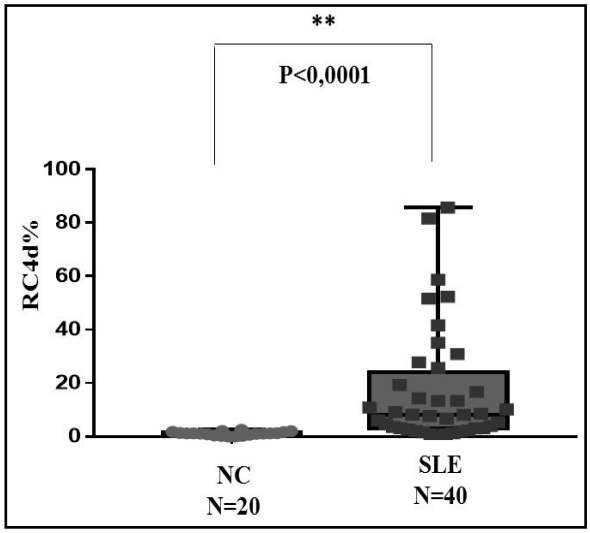

Figure 4: Expression of RC4d (in percentage) in controls and in patients SLE. Statistical tests: Mann-Whitney (**).

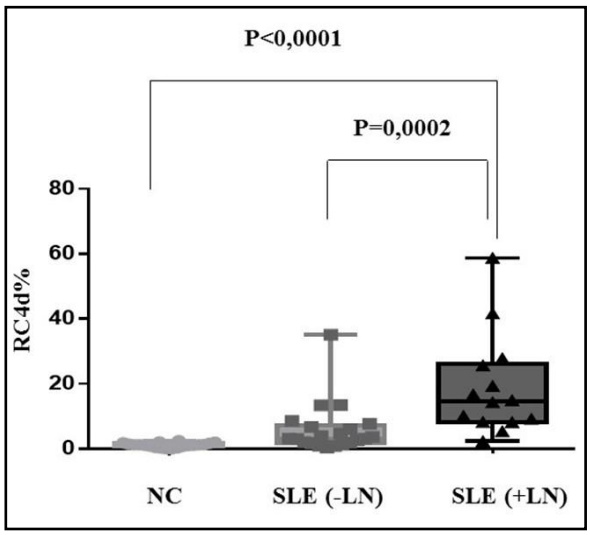

Figure 5: Expression of RC4d (in percentage) in controls and in patients without SLE nephritis (-LN) and SLE nephritis (+LN). Statistical tests used: Kruskal-Wallis (*) and Mann-Whitney (**).

DISCUSSION

Quantification of C3 and C4 fractions of the complement system have been historically viewed as "gold standard" laboratory tests for SLE. Decrease of C3 and C4 in patients with SLE is correlated to increased inflammation and disease activity, however there is a wide range of variation of C3 and C4 in serum among healthy individuals, and this variation is superimposed in SLE patients. Acute phase response during inflammation can lead to an increased synthesis of both C3 and C4. By other side, partial C4 deficiencies are commonly present in the general population. [6-8]

Inactive complement proteins are present in circulation and tissues, and may interact with cells (erythrocytes and lymphocytes) or tissues (endothelial cells). C4 complement system activation products containing thioester bonds can interact covalently with membrane proteins of circulating cells and various hemopoietic cells expressing receptors to proteolytic fragments generated during complement activation. [6-8] Therefore, components of complement system related to cells may be potential biomarkers for SLE, much more reliable than soluble complement proteins.

In this context, considering the rapid exposure of reticulocytes to proteolytic fragments of complement system and its transience in circulation, determination of R-C4d expression may reflect the status of disease at any given time in patients, and may be a very promising tool for monitoring the adopted therapy and signaling of relapses (flares). It is also worth noting that the use of this biomarker to assess severity of disease during diagnosis may better guide physicians in prescribing the most appropriate therapeutic measures when starting treatment.

Results of present study are in line with previous findings and reinforces the potential of this biomarker for use in rheumatology clinic, which still requires a comprehensive method validation and its application in other parts of the world. In short, our results together with those observed in literature give potential value to this biomarker as a screening method for

evaluating the severity of each newly diagnosed patient and to signal treatment failure. This assertion is based on the fact that R-C4d levels were correlated with SLE clinical activity according to the index used to evaluate disease, SLEDAI-2K. In other words, it was observed that higher the SLEDAI score, greater clinical severity, correlated to higher R-C4d expression (FIGURE 2 A, B and C), reflecting a very recent state of active disease. Therefore, R-C4d levels are effectively related to recent SLE activity, according our data.

When comparing SLE-A and SLE-I patients, R-C4d expression levels were significantly higher in population with active SLE. Regard to SLE-I and controls, it was observed very low R-C4d expression for both groups (FIGURE 3A, B, C and D), which indicates that these SLE patients may be under good control.

According to results obtained in this study and in accordance with other studies already published, it can be assumed that complement activation products related to blood cells are potential biomarkers to monitor disease activity in patients with SLE. However, a complete profile of these complement activation products linked to different cell types can provide important information on a specific cellular and molecular mechanism. In particular, from data obtained in this study, the evaluation of complement activation products on reticulocytes (R-C4d) may suggest a strong association between increased expression of R-C4d and recent disease activity in patients under treatment which may indicate treatment failure. In addition greater expression of this R-C4d in patients with lupus nephritis could possibly contribute to renal injury.

It should also emphasize that SLE remains a challenge even for experienced rheumatologists doctors. The heterogeneity of clinical manifestations and the complex and not fully understood mechanisms involved in the disease become difficult to predict activity. In Brazil, approach and monitoring of SLE disease is based mostly on studies and findings conducted in other countries. Studies in our population are still limited and many gaps remain. The mechanisms involved in LES deterioration process and morbidity are still poorly understood. Studies of new biomarkers that predict activity may provide a more immediate and targeted approach to patients with this complex disease, and thus pharmacotherapeutic approaches may be imposed in order to prevent the development and aggravation of SLE.

CONCLUSION

- R-C4d was able to identify early disease activity (flares), reinforcing previous findings.
- A positive correlation between higher levels of C4d and scores obtained by the SLEDAI-2K, whose increased values indicate disease activity may reflects inflammation in vivo.

ACKNOWLEDGMENTS

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