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Correlation between active disease and hypercoagulability state in patients with systemic lupus erythematosus

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ABSTRACT

Objective: This study has investigated whether high levels of Reticulocytes-C4d (R-C4d) and Platelets-C4d (P-C4d) reflecting recent activity in SLE patients are correlated with changes in natural anticoagulation components, coagulation activation and endothelial injury markers.

Methods: This study included three groups: 1) healthy women (control, $n = 30$); 2) women with low activity of the disease (SLEDAI 2 K ≤ 4, $n = 30$); 3) women with active disease (moderate or high activity) (SLEDAI 2 K > 4, n = 30). Median fluorescence intensity (MFI) of R-C4d and P-C4d were determined by flow cytometry using double labeling with specific monoclonal antibodies. Endothelial injury and hypercoagulability were evaluated by measuring Thrombomodulin and D-dimer levels.

Results: Higher MFI index of R-C4d were related to the recent activity of SLE, and higher expression of P-C4d indicated an elevated risk of thrombotic complications. Increased levels of soluble thrombomodulin and D-dimer were observed in patients with active SLE.

Conclusion: R-C4d is helpful to monitor early disease activity and PC4-d may be an important tool to detect a prothrombotic phenotype in SLE. Elevated levels of D-dimer and thrombomodulin add value to P-C4d data and corroborate a hypercoagulable profile in women with SLE, contributing to an increased prothrombotic risk associated with inflammation.

1. Introduction

Among several autoimmune diseases, systemic lupus erythematosus (SLE) is an inflammatory and multisystemic disorder that involves the connective tissue and may be characterized by circulating antigen-antibody complexes deposited in various tissues. Genetic, environmental and hormonal factors might be directly involved in the etiopathogenesis of SLE leading to the loss of immune balance control [1].

For the initial diagnosis of SLE, several clinical criteria are used as suggested by the American College of Rheumatology (ACR). Once the disease is diagnosed, patients should be monitored and the activity status and characterization of SLE should be measured through clinical and laboratory parameters. The SLEDAI-2 K index (Systemic Erythematous Lupus Erythematosus Activity Index 2000) is used for this purpose and is based on the clinical manifestations and laboratory data of patients [2].

Some inflammatory responses associated with SLE may be induced by the pathological activation of the complement system. In this context, the validation of blood cell-bound complement activation products (CB-CAP = Cell–bound complement activation products) as potential biomarkers of SLE activity and patient's stratification may be important tools for a better clinical control of this disease. By means of flow cytometric assays, different CB-CAPs have been identified in circulating blood cells with a high specificity for SLE [3–7].

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According to Liu et al., R-C4d proportions (reticulocyte-bound C4d) may effectively reflect current activity of the disease since reticulocytes released into the bloodstream are immediately exposed to C4-derived fragments produced because complement activation [4].

Also related to CB-CAPs and according to a study conducted by Navratil et al., positive patients to P-C4d (platelet-bound C4d) had a higher frequency of cardiovascular events associated with thrombosis than negative patients to P-C4d [6]. Actually, SLE patients have a significantly increased risk of arterial diseases and venous thrombosis, which is an important cause of morbidity and mortality in this disease. The incidence of myocardial infarction among SLE patients is 50 times higher when compared to people who do not have the disease [8,9].

Patients with SLE may have defects in natural anticoagulant mechanisms and an increase in pro-coagulant factors related to the inflammatory mechanisms and endothelial damage. The natural anticoagulant mechanism is mediated by integrated action of activated protein C (PCa) and its co-factor protein S (PS). The two proteins act together degrading factors Va and VIIIa, thereby limiting thrombin formation [10,11]. Acquired PS deficiencies are known to occur due to the presence of anti-PS antibodies or anti-C4b binding protein (C4BP) leading to decreased PS levels. Nevertheless, neither the prevalence of anti-PS antibodies nor their clinical significance in association with levels of PS in SLE are known [12].

Since thrombotic phenomena are frequent in patients with SLE and hypercoagulability seems to be directly related to inflammation and endothelial damage, measurement of soluble thrombomodulin as a marker of vasculitis and D-dimer of fibrinolysis is interesting [13]. As it is well known, D-dimer evaluation plays an important role in the diagnosis of venous thromboembolism and disseminated intravascular coagulation. However, D-dimer has also been reported as a marker of atherosclerotic diseases [13,14]. Thrombomodulin is a glycoprotein expressed on the luminal surface of vascular endothelial cells, and it is also a key molecule to maintain anticoagulant properties of the vascular endothelium. In diseases linked to endothelial injury, increased soluble Thrombomodulin levels are observed [15].

In this context, the present study had as aim to investigate whether high expression of R-C4d and P-C4d reflecting recent activity in SLE patients are associated with changes in levels of natural anticoagulation components, coagulation activation and endothelial injury markers, and whether their median fluorescence intensity (MFI) are correlated to the SLEDAI-2 K index.

It is believed that use of new biomarkers may help in the diagnosis and monitoring of disease activity, evaluation and prediction of clinical characteristics and stratification of SLE subtypes, which will certainly benefit the patients.

2. Methods

2.1. Patients

For this study women were recruited with age ranging from 18 to 69 years old by a medical team specialized in rheumatology. Based on SLEDAI -2 K criteria assigned to each patient by rheumatologists, 30 patients were classified with inactive disease or a low activity disease (SLEDAI-2 K ≤ 4) and 30 with an active disease (SLEDAI-2 K > 4) while simultaneously 30 age matched women without SLE or other diseases (controls) were also recruited.

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 written informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations.

Sixty SLE patients diagnosed according to ACR classification criteria (1997) were sequentially recruited in the Rheumatology Clinic of Hospital Santa Casa, 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 other autoimmune diseases associated with SLE (such as rheumatoid arthritis, Sjögren's syndrome, scleromyositis, scleroderma, ankylosing spondylitis, among others), as well as other immunosuppressive diseases including HIV/AIDS, 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, thirty 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 such 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 the immune system were checked by self-report.

A sample of EDTA.K3–5 mL of peripheral blood from each fasting eligible participant was collected. Samples were analyzed for R-C4d and P-C4d immediately after venipuncture. A volume of 5 mL of blood was also collected in 3.2% sodium citrate to obtain poor platelet plasma (PPP). Aliquots of PPP were stored at −80 °C until analysis.

2.2. Flow cytometry technique optimization for R-C4d and P-C4d complement fragments

R-C4d blood expression were determined according to Liu et al. [5]. Briefly, whole blood samples were washed, diluted with phosphate buffered saline (PBS) and stained using a mouse monoclonal antibody (mAb) specific for human C4d (Quidel®, San Diego, CA) or isotype control, MOPC-21, at the concentration of 10 μg/mL. Finally, anti IgG secondary antibody $F(ab')_2$ conjugated to phycoerythrin (PE) was added at a concentration of 10 μg/mL. After antibody staining, cell suspensions were incubated with thiazole orange (ReticCount reagent; Becton Dickinson) to identify reticulocytes or with saline (unlabeled control).

P-C4d frequencies was determined according to Navratil et al. [6]. Briefly whole blood samples were diluted in PBS and labeled using monoclonal antibodies. First, fluorescein isothiocyanate (FITC) conjugated anti-CD42a antibody (BD Biosciences, San Jose, CA) and anti-CD4d or the isotype control, MOPC-21, was used at the concentration of 10 μg/mL. PE conjugated goat anti-mouse IgG $F(ab')_2$ was added at the concentration of 10 μg/mL.

2.3. Data acquisition and analysis in flow cytometer

Samples were acquired on FACS LSRFortessa using BD FACSDiva™ Software (BD Bioscience, San Jose, USA) and data were analyzed with FlowJo V10.0.2 (Tree Star). Erythrocytes were identified based on forward scatter area (FSC-A) versus side scatter area (SSC-A) properties, while reticulocytes were identified based on FSC-A versus thiazole orange positive stained. MFI of C4d was defined within reticulocytes population (Fig. 1A). Prior measurements using isotype control (MOPC21) were run to exclude non-specific binding from real events.

Platelets were identified by their characteristic on FSC-A versus SSC-A and by CD42a expression as a specific platelet marker. The expression of C4d within platelets population was analyzed and MFI was determined (Fig.1B). The C4d-specific labeled was confirmed by running isotype control (MOPC21).

2.4. Determination of plasma levels of thrombomodulin (TM), D-dimer (DDI), protein S (PS) and C4b-binding protein (C4BP)

Quantitative determination of Thrombomodulin (TM) was carried out using IMUNOBIND® Thrombomodulin ELISA diagnostic set (American Diagnostica Inc. - Stanford, CT), levels of D dimer (DDi) was carried out by IMUNOCLONE® D-DIMER ELISA diagnostic set (American Diagnostica Inc. - Stanford, CT), Protein S (PS) by

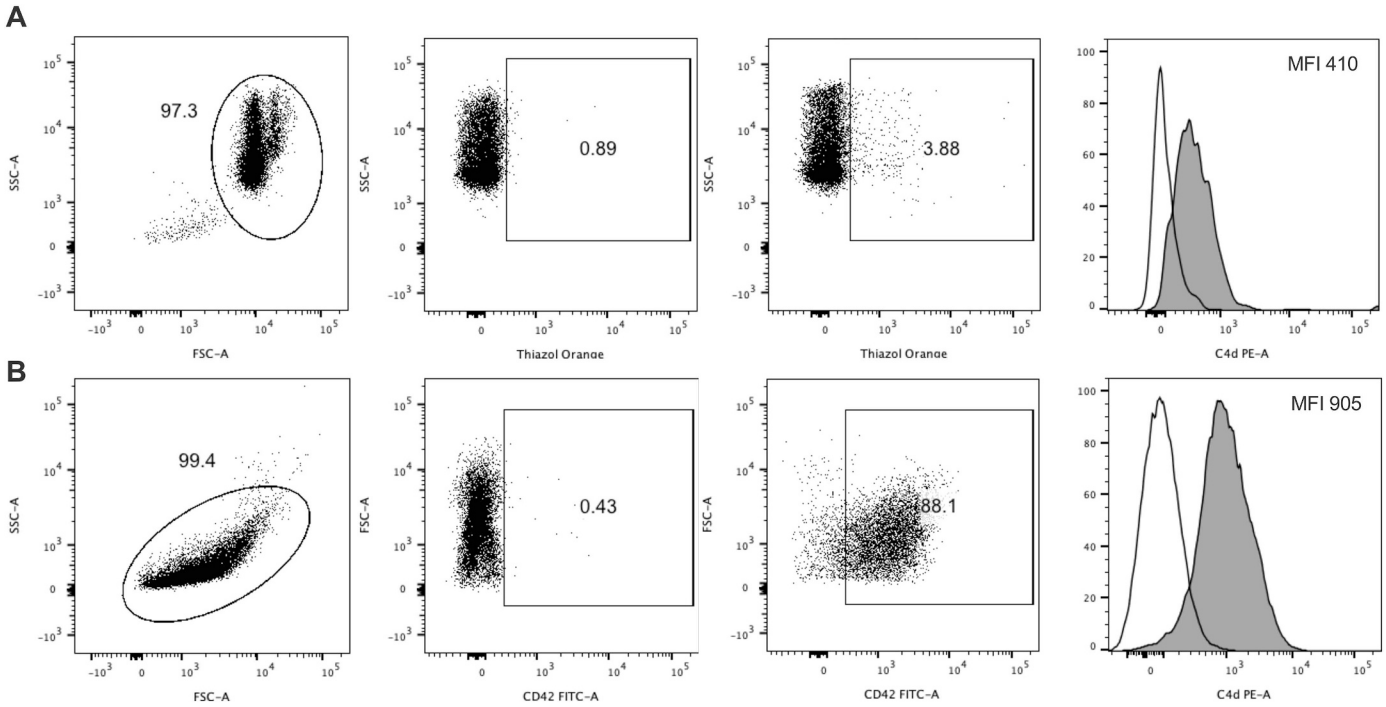

Fig. 1. Analysis for R-C4d and P-C4d by flow cytometry. (A) To analyze R-C4d, erythrocytes was first defined by FSC-A x SSC-A and reticulocytes by Thiazole Orange labeling. The expression of C4d within reticulocytes was calculated using median of fluorescence intensity (MFI) and the label using isotype control was also checked (unfilled). (B) To label P-C4d platelets was defined using FSC-A x SSC-A and by stained with CD42a-FITC. The expression of C4d within platelets was calculated using MFI and and the label using isotype control was also checked (unfilled).

Asserachrom® PS:Ag diagnostic kit (Diagnostica Stago®, Asnière, France), and plasma levels of C4BP by immunological method using latex (LIATEST C4BP; Diagnostica Stago®), following the manufacturer's instructions.

2.5. Statistical analysis

Data analysis was performed using GraphPad Prism® software 7. D'Agostino-Pearson test was performed to assess the normality of variables. In case of non-normally distributed data, Mann-Whitney was used and in case of normality distribution, ANOVA was performed followed by Tukey's multiple comparisons test. Correlation tests were also carried out among R-C4d, P-C4d, DDi and TM expressions versus SLEDAI -2 K index using Spearman test. In all cases, significance was considered at $p < .05$.

3. Results

3.1. Characteristics of the participants

The study population consisted of 60 women with SLE and 30 healthy controls. At the time of entry into the study, mean ± SD age of the SLE patients was 43.2 ± 12.0 years (range 18–69 years), while mean ± SD age of the healthy control subjects was 44.7 ± 12.0 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-2 K score, and a wide spectrum of organ involvement. Additional demographic and clinical features of SLE patients are shown in Table 1.

3.2. R-C4d and P-C4d expressions in SLE patients and their correlation with SLEDAI-2 K index

The expression of R-C4d and P-C4d was assessed among reticulocytes (Fig.1A) and platelets (Fig.1B), respectively, of SLE patients.

Table 1
Clinical characteristics of the patients with SLE.

Characteristics	*N* = 60
Age mean ± SD (range) years	43.2 ± 12.0 (18–69)
Clinical manifestations	**(%)**
Antinuclear antibodies (ANA)	72.3
Arthritis	35.7
Photosensitivity	35.0
Hematologic manifestations	43.0
Leukopenia	8.1
Thrombocytopenia	12.3
Anemia	10.1
Proteinuria	39.3
Low complement C4	37.0
Low complement C3	30.0
Malar rash	29.1
(anti-DNA/anti-Sm)	18.5
Mucosal ulcers	21.8
Psychosis	9.0
Pleurisy/Pericarditis	6.0
Discoid rash	7.6

SLE, Systemic Lupus Erythematosus; SD, standard deviation, anti-DNA, Autoantibodies anti- deoxyribonucleic acid; anti-Sm, autoantibodies anti-Smith.

Thiazole Orange were used to define reticulocytes and anti-CD42a-FITC was labeled to better define platelets, both gates were determined using samples labeled only with isotype control (Fig. 1, center panels). Finally, expression of C4d was evaluated in each sample (Fig. 1 A, B right panels).

Regarding the R-C4d, significant differences were found when compared negative control group (NC), i.e., individuals without the disease and SLE-I (SLEDAI-2 K ≤ 4) versus SLE-A (SLEDAI-2 K > 4) (Fig. 2A). After applying the ANOVA statistical test, the group with SLE-A had higher expression for R-C4d when compared to SLE-I (mean of 255.3 versus 185.3; $p < .05$, respectively) and NC groups (mean of 133.1; $p < .0001$).

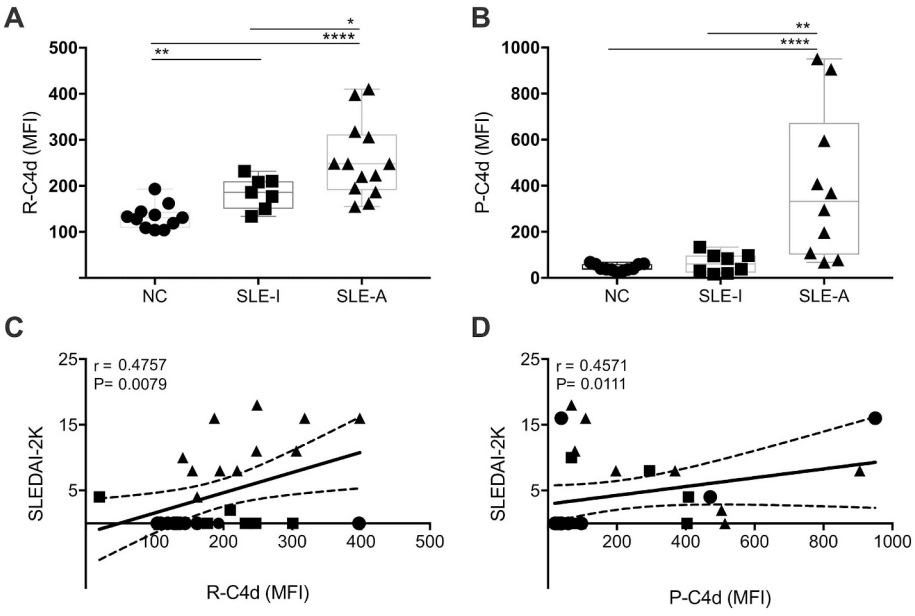

Fig. 2. R-C4d and P-C4d expressions and correlation with SLEDAI-2 K. R-C4d and PC4d expression in controls (NC), in patients with inactive (SLE).—I) or active (SLE-A) systemic lupus erythematosus. Expression in NC and in patients with SLE-I or SLE-A disease of R-C4d (A) and P-C4d (B), both in MFI index. Spearman correlation of R-C4d (C) and P-C4d (D) expressions by flow cytometry versus SLEDAI-2 K values. *P* values < .05 were considered significant. * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, **** $P \leq .0001$.

Patients with SLE-I and NC individuals had very low expression of P-C4d. The highest expressions of P-C4d were observed in the SLE-A group. Significant differences were found when comparing SLE-A versus SLE-I patients (mean of 397.5 versus 64.33; $p < .01$, respectively) and SLE-A versus NC group (mean of 397.5 versus 44.35; $p < .001$, respectively) (Fig. 2B).

In order to evaluate whether increased expression of both R-C4d and P-C4d markers followed increased severity of the disease, correlation analyses were calculated between expression of R-C4d, P-C4d and the SLEDAI-2 K index. A positive correlation was found between expression of R-C4d, P-C4d and increased SLEDAI-2 K index ($r = 0.4757$, $p < .0079$; and $r = 0.4571$, $p < .0111$, respectively), (Fig. 2C–D). As a timely complementation of our data an inverse and significant correlation between SLEDAI-2 K and serum C4 expression ($r = -0.421$; $p = .009$) was observed as expected (data not shown).

3.3. Levels of hypercoagulability (D-dimer) and endothelial lesion (Thrombomodulin) markers in SLE patients and their correlation with the SLEDAI-2 K index

Related to DDi, a significant difference was found for its levels among the three groups ($p < .0001$). Significant differences were also found between NC and SLE-I groups (medians of 0.37 versus 0.93 μg/mL; $p = .01$), and between SLE-I and SLE-A groups (medians of 0.93 versus 1.44 μg/mL; $p = .004$) (Fig. 3A). By other hand, TM levels were significantly different when comparing the three groups ($p < .0001$). In other words, significant differences were found between NC and SLE-I groups (medians of 0.80 versus 1.00 ng/mL; $p = .03$), and between SLE-I and SLE-A groups (medians of 1.00 versus 1.32 ng/mL; $p = .0007$) (Fig. 3B).

Analyses of correlation among levels of DDi ($r = 0.3477$; $p = .0028$), TM ($r = 0.4765$; $p = .0001$) and the SLEDAI-2 K index were conducted. A positive correlation was shown of these markers with the SLEDAI-2 K index (Fig. 3C–D). Therefore, in the present study, it was shown that the greater the severity of the disease, the greater the hypercoagulable potential translated into high plasma levels of DDi and TM, the latter a marker of endothelial lesion, that, ultimately, favors the pro-thrombotic state.

3.4. Levels of PS and C4BP in SLE patients and healthy individuals

In order to evaluate possible deficiencies of the natural anticoagulant mechanisms, plasma levels of protein S and C4BP binding protein were measured in SLE patients and in health individuals. When comparing SLE-A, SLE-I and NC groups no significant difference was observed. After applying the Mann-Whitney test, no difference was verified between NC and SLE-A groups, and between SLE-A and SLE-I (for both markers) (data not shown).

3.5. Determination of a cut off point for defining activity of SLE through expression of R-C4d and P-C4d

Definition of a cut-off point was possible because both markers have presented a strong association with SLE activity evaluated by SLEDAI-2 K. In order to stratify SLE activity, a probable cut-off point was established combining R-C4d and P-C4d MFI index. It was observed that 87.5% of SLE-I patients had MFI index higher than 150 for R-C4d and lower than 100 for P-C4d. It was also verified that all patients with SLE-A (except one of them) had MFI index for R-C4d higher than 150 and MFI index for P-C4d higher than 100. When NC group was analyzed all the samples had R-C4d MFI index lower than 150 and P-C4d MFI index lower than 100 (Fig. 4). Therefore, expression of R-C4d and P-C4d allows to distinguish between the different types of SLE patients and the control group.

4. Discussion

An important aspect to be considered is the fact that inflammatory phenomena occurring in SLE are closely associated with a pro-thrombotic state. Inflammatory mechanisms contribute to an increase in procoagulant factors, reduction of natural anticoagulants and inhibition of the fibrinolytic activity [16]. In this context, the importance of the study focusing on the relationship between SLE activity and exacerbation of hemostasis activation should be highlighted considering that thrombotic events increase morbimortality associated with the disease [17].

R-C4d marker appears to be a quite promising tool for therapeutic monitoring since it is able to signal possible relapses (flares) in SLE patients at a given moment. This statement is based on the fact that R-C4d expression was correlated to the clinical activity of SLE according to the index used to assess the disease, SLEDAI-2 K. It was found that the higher the SLEDAI-2 K index, that is, the greater the clinical severity, the greater the expression of R-C4d, which would be reflecting a recent activity of the disease, given the fugacity of reticulocytes in the

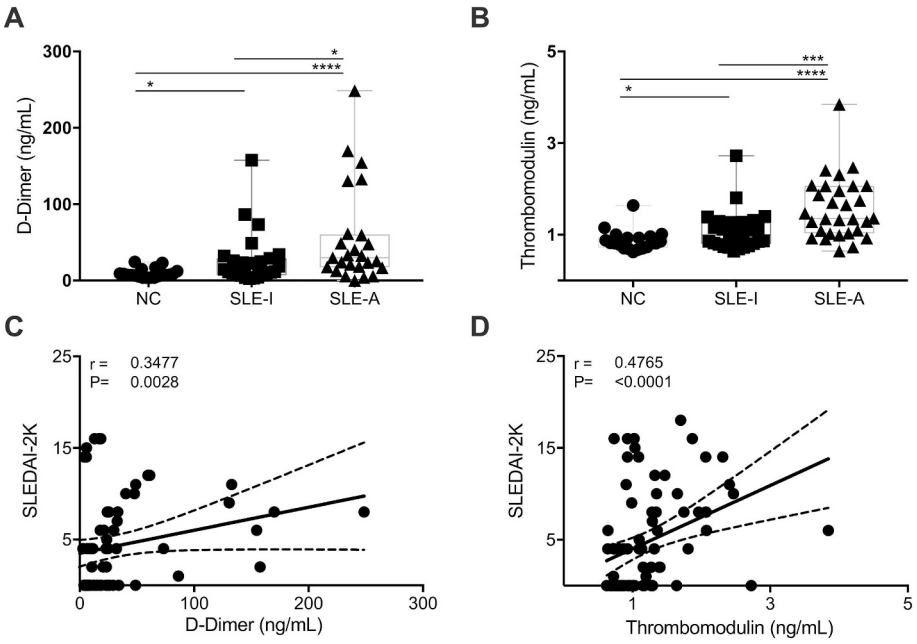

Fig. 3. D-dimer and thrombomodulin levels and correlation with SLEDAI-2 K. D-dimer and thrombomodulin levels in controls (NC), in patients with inactive (SLE−I) or active (SLE-A) systemic lupus erythematosus. Levels in NC and in patients with SLE-I or SLE-A of D-dimer (A) and thrombomodulin (B). Spearman correlation of D-dimer (C) and Thrombomodulin (D) levels versus SLEDAI-2 K values. * P ≤ .05, *** P ≤ .001, **** P ≤ .0001.

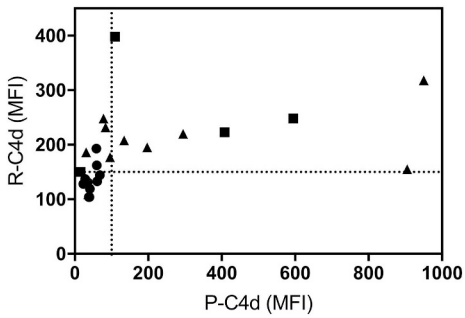

Fig. 4. Association between the expression of R-C4d and P-C4d and determination of a cut-off point for discrimination of SLE activity. In y-axis the cutoff point for R-C4d is shown (MFI index 150). In x-axis the cutoff point for P-C4d is shown (MFI index 100).

peripheral blood. Patients with active disease had a high R-C4d expression, in contrast to the expression observed in patients with the inactive disease and healthy individuals. Therefore, expression of R-C4d are shown to be effectively related to the recent activity of SLE [5]. For the P-C4d and according to data already described in the literature [6], increased of C4d bound to platelets, are related to the hypercoagulated state in SLE patients. In our study, a high expression of P-C4d in patients with active disease has followed the severity of the disease since a positive correlation between its expression and SLEDAI-2 K index was found.

According to the results, it can be suggested that the R-C4d and P-C4d markers together could monitor or even predict SLE activity, as well as guide clinical procedures for the management of a prothrombotic state. Considering SLE as an essentially inflammatory disease and the relationship between inflammation and hemostasis, it may be suggested that the greater the hypercoagulable state the greater the inflammatory process and vice versa. This reciprocity of effects produced by hemostatic and inflammatory changes would favor the risk of thrombotic complications, which, at first, would aggravate the disease. In the present study, patients with active disease had higher levels of markers related to hypercoagulability, such as DDi and TM. It was also observed that the greater the inflammatory nature, the greater the levels of pro-thrombotic markers. This statement may be supported by the direct and significant correlation between plasma levels of both DDi and TM, and SLEDAI-2 K index.

SLE is an essentially inflammatory disease and inflammation is related to endothelial damage mainly in cases of active disease, with consequent hypercoagulability. Vascular integrity can be assessed by determining plasma levels of soluble TM, since this is essentially a membrane protein. Thus, increased plasma levels of TM may indicate laboratory evidence of endothelial lesion and indirectly may indicate reduction of the effectiveness of the natural anticoagulation system via protein C and protein S [18]. Particularly in our study, increased levels of soluble TM in patients with active SLE were found. This fact suggests that the mechanism of hypercoagulability is more related to inflammation and endothelial injury than to the autoimmune component, i.e., presence of anti-PS antibodies, since PS and C4BP levels were very similar between patients with SLE and between those and healthy individuals.

Our data revealed that higher expression of R-C4d is related to the recent activity of SLE. Similarly, higher MFI index of P-C4d is also related to active disease, in addition to indicating an increased risk of thrombotic complications. Thus, the two combined analyzes allowed the establishment of a cutoff point with potential value for stratification of patients with active disease and increased thrombotic risk. In other words, values > 20% for R-C4d were related to recent activity of SLE while values > 40% for P-C4d may show an active disease with thrombotic risk. Together, markers are related to the active disease since only one patient with SLE-A was out of these parameters (95% of SLE-A patients have values > 20% for R-C4d and 40% for P-C4d).

5. Conclusion

From the data obtained in this study, patients with active SLE present a state of hypercoagulability possibly related to endothelial damage and activation of coagulation resulting from the inflammatory process characteristic of the disease. The evaluation of R-C4d and P-C4d together may be useful for early indication of disease activity (flares) while the latter (P-C4d) may be considered an important auxiliary tool for risk stratification of thrombotic complications. The definition of a prothrombotic profile may have an impact on the decision of individualized prophylaxis benefiting patients.

Acknowledgments

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Conflict of interest

The authors of this study declare having no conflicts of interest.

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