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Usefulness of erythrocyte-bound C4d as a biomarker to predict disease activity in patients with systemic lupus erythematosus

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Objective. SLE is an autoimmune disorder characterized by abnormal complement activation. Numerous new biomarkers have recently been used to diagnose or monitor disease activity in patients with SLE. We checked the levels of erythrocyte-bound C4d (E-C4d), an activation-derived fragment of C4 that is deposited on the erythrocytes, under different conditions of SLE in order to correlate these levels with disease activity.

Methods. We conducted a cross-sectional investigation of three groups of patients: (i) 63 patients with SLE; (ii) 43 patients with other diseases; and (iii) 26 healthy controls. Erythrocytes were analysed by flow cytometry to determine the levels of E-C4d.

Results. We found a significant elevation in the mean levels of E-C4d in SLE patients compared with patients with other diseases or healthy controls. In SLE patients, the levels of E-C4d were correlated with the SLEDAI and inversely correlated with serum C3/C4 levels. In the subgroup of SLE patients with haemolytic anaemia (HA), a significantly higher level of E-C4d was observed than that in SLE patients without HA. However, in SLE patients with HA, there was no correlation between the levels of E-C4d and other markers of disease activity, including SLEDAI and levels of anti-dsDNA, C3 and C4.

Conclusion. E-C4d levels are useful diagnostic markers for SLE and can serve as biomarkers of disease activity in patients with SLE. However, E-C4d is of limited value in monitoring disease activity in SLE patients with HA.

Key words: Systemic lupus erythematosus, E-C4d, Complement, Autoimmune haemolytic anaemia.

Introduction

SLE is a chronic systemic inflammatory disease that can be either benign or fatal; the benign form predominantly targets the skin and joints, whereas the fatal form induces multiple organ failure. Before a patient develops clinical symptoms, prompt diagnosis of SLE represents a major challenge for rheumatologists. A current diagnosis of SLE is defined by four of the 11 revised ACR criteria published in 1982 [1]. In SLE patients who do not fulfil standard ACR criteria, delays in treatment and diagnosis may increase the probability of multiple organ damage. Multi-organ inflammatory injury in SLE appears to be caused by tissue deposition of ICs consisting of autoantigens, autoantibodies and activated complements, which can elicit a subsequent 'cytokine storm' [2–4]. The formation of ICs is an important mechanism of the adaptive immune response that promotes removal of foreign antigens. In this process, complements maintain solubility of the ICs via complement receptors, and clearance by phagocytosis occurs through mechanisms involving Fcγ receptors [2, 4]. Abnormal autoimmunity with activation of the classical complement pathway is a part of the pathogenesis of SLE. Components of the classical complement pathway, including C1q, C1r, C1s, C4 and C2, can be activated for clearance of ICs in the circulation [4]. Traditional biomarkers for the diagnosis of SLE include ANAs, anti-dsDNA, anti-Sm and aPL antibodies. In our clinical experience, these indicators are not always optimal for effective diagnosis of SLE. Thus, more reliable biomarkers are needed to facilitate early and accurate diagnosis. Several novel biomarkers for SLE have recently been reported, which, in addition to improving diagnosis, may be useful in future assessment of susceptibility and disease activity. Potential biomarkers include Fc receptor genes (for assessing disease susceptibility), erythrocyte-bound C4d (E-C4d) and erythrocyte-bound complement receptor type 1 (for diagnosis and assessment of disease activity) as well as $CD27^{high}$ plasma cells, IFN signatures and anti-C1q antibodies (all three for assessing disease activity) [5, 6]. Traditionally, the anti-dsDNA level along with serum C3 and C4 levels have been used to monitor lupus disease activity. However, these traditional biomarkers are not always appropriate for clinical monitoring because high levels of anti-dsDNA or low levels of C3/C4 are persistent in some lupus patients.

Increased expression of E-C4d is found in SLE patients and can be used as a tool to diagnose lupus and evaluate disease activity, as discussed in previous studies [7, 8]. C4d is one of the products degraded from C4. C4d can be bound on various cells including erythrocytes, reticulocytes and platelets in the peripheral circulation; however, C4d is bound mostly on erythrocytes [9]. In our clinical experience with the evaluation of E-C4d levels in lupus patients, various degrees of increased expression of E-C4d can be found under different conditions of SLE. E-C4d levels are not always high in some patients with lupus, but they may be very high in other lupus patients. In this study, we focus on the evaluation of E-C4d levels under different conditions of lupus and correlate these levels with disease activity.

Patients and methods

Study participants

All study participants were $\geqslant$18 years of age, and each provided a written informed consent. None of the patients was excluded from participation on the basis of sex or ethnicity. This study was approved by the Tri-Service General Hospital Institutional Review Board.

SLE patients. Blood samples were collected from 63 SLE patients who met the 1982 ACR revised criteria for the classification of definite SLE. This group of patients consisted of 53 women and 10 men with ages ranging from 18 to 77 (mean age 38) years. Disease activity was evaluated in each patient according to the SLEDAI. E-C4d levels were measured by flow cytometry. Serum level of anti-dsDNA was checked by enzyme-linked

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fluorescent immunoassay (Phadia immunoCAP system, Phadia GmbH, Freiburg, Germany). Quantitative determination of C3 and C4 was measured by means of immunonephelometry on the BN* System. Through the machine of Sysmex XE-2100L, white blood count (by flow cytometry method using semiconductor laser), haemoglobin (by SLS haemoglobin detection method) and platelets (by direct current detection method) were also assessed. The concentration of blood urea nitrogen (BUN) and creatinine (Cr) was detected by Kinetic UV method (for BUN) and Jaffe method (for Cr) in the machine of Roche Modular P Analytics (Roche Diagnostics, Indianapolis, IN, USA).

Patients with other diseases. Forty-three randomly selected patients with various other rheumatic or autoimmune diseases were recruited. The other diseases included RA, SS, APS, MCTD, UCTD, Raynaud's disease (RD), AS, PsA and ANCAs-associated vasculitis (AAV).

Healthy controls. Twenty-six healthy individuals were recruited as controls. These participants were required to complete a brief questionnaire regarding previous or current medical conditions.

Flow cytometric characterization of erythrocytes

For each assay, 3 ml of blood was collected from every participant. Blood samples were placed in a Vacutainer tube (BD Pharmingen^{TM}, Franklin Lakes, NJ, USA) containing ethylenediaminetetraacetic acid (EDTA). Five microlitres of whole blood was removed and incubated with 50 μl of mouse anti-human C4d monoclonal antibody (Quidel, San Diego, CA, USA; 1 mg/ml) at a 1:200 dilution. After incubation, cells were washed twice with 1 ml of diluent buffer and centrifuged at 1500 *g* for 3 min at 4°C. One microlitre of FITC-conjugated goat anti-mouse immunoglobulin-specific polyclonal antibody (BD Pharmingen^{TM}; 500 μg/ml) was added to the supernatant for 30 min at 4°C. The cells were then washed again, as previously described, and resuspended in 1 ml of phosphate buffered saline (PBS). The samples were analysed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA). Erythrocytes were electronically gated for 30 000 cells on the basis of their forward and side scatter properties. Surface expression of C4d on gated cells was detected by specific mean fluorescence intensity (sMFI), which is the C4d-specific mean fluorescence minus the isotype control mean fluorescence.

Statistical analysis

SPSS version 15.0 software (SPSS, Chicago, IL, USA) was used to perform all statistical analyses. Differences between the median values of defined patient groups were compared using the non-parametric Mann–Whitney U test. A Spearman's rank correlation was used to detect correlations among different study parameters. A *P*-value of <0.05 was considered statistically significant.

Results

Characteristics of the three study groups

The study population consisted of 63 patients with SLE, 43 patients with other diseases and 26 healthy controls. The SLE patients had a mean age of 37.6 ± 2.0 years (53 women and 10 men) and were divided into three subgroups as follows: SLE ($n=49$) without haemolytic anaemia (HA) or chronic renal failure (CRF), SLE with HA ($n=11$) and SLE with CRF ($n=3$). The laboratory data and SLEDAI are shown in Table 1. The laboratory data following haemolysis of 11 SLE patients with HA are shown in Table 2. Forty-three patients identified as having various types of other diseases were distributed as follows: RA ($n=8$), SS ($n=8$), APS ($n=3$), MCTD ($n=3$), UCTD ($n=5$), RD ($n=4$), AS ($n=9$), PsA ($n=2$) and AAV ($n=1$). The patients

Table 1. Laboratory manifestations of SLE patients

Laboratory manifestations	Group 1 ($n=49$)	Group 2 ($n=11$)	Group 3 ($n=3$)
Female/male	41/8	10/1	2/1
Age, years	38.49 ± 2.37	31.36 ± 3.43	46.67 ± 8.57
Anti-dsDNA, IU/ml	112 ± 20	115 ± 54	15 ± 7
C3, mg/dl	68.61 ± 4.64	55.63 ± 5.21‡	79.30 ± 4.94‡
C4, mg/dl	13.82 ± 1.13†	8.05 ± 1.17†,‡	43.00 ± 23.35‡
White blood cells, per μl	5126 ± 425	6163 ± 565	7033 ± 2871
Haemoglobin, g/dl	10.80 ± 0.32†,§	8.36 ± 0.73†	8.53 ± 0.46§
Platelets, per μl	199 596 ± 14 296	168 636 ± 27 200	143 666 ± 50 669
BUN, mg/dl	17.57 ± 1.72§	15.45 ± 1.28‡	89 ± 5.85‡,§
Cr, mg/dl	0.80 ± 0.06§	0.60 ± 0.05‡	10.56 ± 1.84‡,§
Urine DPL, mg	1548 ± 399	1065 ± 1065	3010 ± 1290
SLEDAI	6 ± 0.52	7.81 ± 2.62	10.66 ± 3.71

The values are presented as mean ± S.E.M. Group 1: SLE without HA or CRF; Group 2: SLE with HA; Group 3: SLE with CRF; DPL: daily protein loss. Normal range of anti-dsDNA: 0–10 IU/ml, C3: 90–180 mg/dl, C4: 10–40 mg/dl, white blood cells: 4500–11 000 μl, haemoglobin: 12–16 g/dl, platelets: 150 000–400 000 μl, BUN: 7–20 mg/dl and Cr: 0.5–1 mg/dl. †Significant difference between Groups 1 and 2, $P<0.05$. ‡Significant difference between Groups 2 and 3, $P<0.05$. §Significant difference between Groups 3 and 1, $P<0.05$.

Table 2. The laboratory manifestations of 11 SLE patients with HA[a]

Patient	Sex/age, years	Haemoglobin, g/dl	LDH, U/l	Haptoglobin, mg/dl	Direct Coombs test	Indirect Coombs test	sMFI of C4d
1	F/22	12.5	425	<5.8	+	+	23.34
2	F/32	12	560	<5.8	+	+	20.04
3	F/37	7.9	672	<5.8	+	+	10.24
4	M/25	6.9	346	<5.8	+	+	21.64
5	F/24	5.3	754	<5.8	+	+	32.84
6	F/24	11	452	–	+	+	9.03
7	F/48	8.7	217	<5.8	+	+	26.77
8	F/24	6.2	567	–	+	+	40.11
9	F/38	6.2	466	<5.8	+	+	21.17
10	F/53	8		<5.8	+	+	14.44
11	F/18	7.3	306	–	+	+	9.39

[a]Normal range of haemoglobin: 12–16 g/dl, LDH: 135–225 U/l and haptoglobin: 36–195 mg/dl.

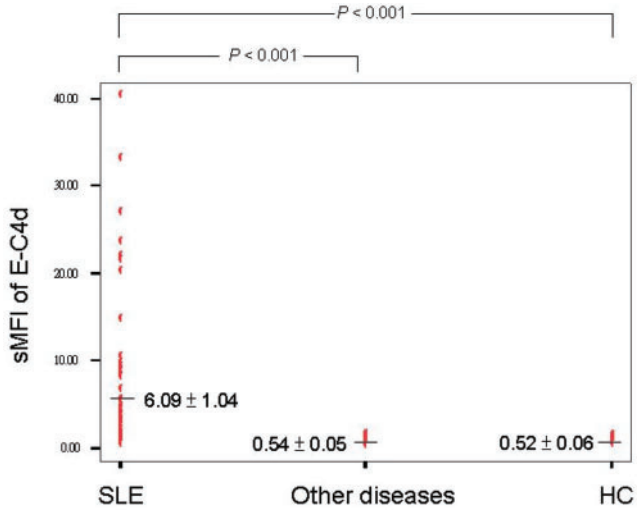

FIG. 1. Levels of E-C4d in specific sMFIs in SLE patients, patients with other diseases and healthy controls (HC).

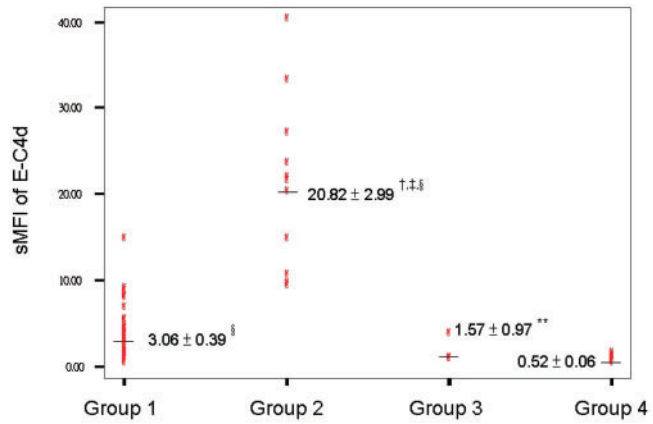

FIG. 2. sMFI values of E-C4d in different subgroups of SLE patients and healthy controls. Group 1: SLE without HA or CRF; Group 2: SLE with HA; Group 3: SLE with CRF; Group 4: healthy controls; †Significant difference ($P < 0.05$) compared with SLE without HA or CRF. ‡Significant difference ($P < 0.05$) compared with SLE with CRF. §Significant difference ($P < 0.05$) compared with healthy controls. **No significant difference ($P = 0.123$) compared with healthy controls. $P = 0.231$ between SLE without HA or CRF and SLE with CRF.

TABLE 3. Correlation of levels of E-C4d with measures of disease activity and serological tests in the SLE patients without HA or CRF, and SLE patients with HA[a]

	Level of E-C4d			
	Group 1		Group 2	
	R	*P*	*R*	*P*
SLEDAI	0.772	<0.0001	0.455	0.16
Anti-dsDNA	0.307	0.032	−0.064	0.853
Serum C3	−0.461	0.001	−0.427	0.19
Serum C4	−0.493	<0.0001	−0.532	0.092

[a]Group 1: SLE patients without HA or CRF; Group 2: SLE patients with HA.

with other disease had a mean age of 40.7 ± 2.7 years (33 women and 10 men). The healthy controls had a mean age of 31.1 ± 2.1 years (18 women and 8 men).

E-C4d levels in controls, SLE patients and patients with other diseases

The SLE patients showed significantly higher levels of E-C4d relative to healthy controls and patients with other diseases (6.09 sMFI units ± 1.04 *vs* 0.52 ± 0.06 and 0.54 ± 0.05, respectively, $P < 0.001$, Fig. 1). In patients with other diseases, E-C4d levels were undistinguishable from those of the healthy controls (0.54 sMFI units ± 0.05 *vs* 0.52 sMFI units ± 0.06, $P = 0.89$). In the total 63 patients including SLE without HA or CRF, SLE with HA and SLE with CRF, there was a statistically significant correlation between increased levels of E-C4d and SLEDAI ($R = 0.472$, $P < 0.001$). A significant inverse correlation was detected in all the above patients between the levels of E-C4d and C3 ($R = -0.442$, $P < 0.001$) and between the levels of E-C4d and C4 ($R = -0.584$, $P < 0.001$). However, no correlation was found between the levels of E-C4d and anti-dsDNA or urine daily protein loss in the total SLE patients. Table 3 shows correlations between E-C4d and parameters for SLE activity in lupus patients without HA or CRF and lupus patients with HA separately.

E-C4d levels in different subgroups of SLE patients

The sMFI values for E-C4d in the three subgroups of SLE patients and healthy controls are shown in Figs 2 and 3. Significantly higher mean levels of E-C4d were found in SLE patients with HA than in SLE patients without HA or CRF or in SLE patients with CRF (20.82 ± 2.99 *vs* 3.06 ± 0.39, 1.57 ± 0.97, respectively, $P < 0.05$). There was no statistically significant difference between the SLE subgroup without HA and the SLE subgroup with CRF (3.06 sMFI units ± 0.39 *vs* 1.57 ± 0.97, $P = 0.23$). No significant difference was found in SLE patients with CRF when compared with healthy controls ($P = 0.123$). In the subgroup of SLE with HA, no significant correlation was found between the levels of E-C4d and the values of lupus disease activity, including SLEDAI and the levels of anti-dsDNA, C3 and C4 (Table 3). However, in the subgroup of SLE without HA or CRF, there was a significant correlation between the levels of E-C4d and the values of lupus disease activity, including SLEDAI and the levels of anti-dsDNA, C3 and C4. The correlation data are shown in Table 3.

Discussion

There are numerous studies evaluating the various cells that bind C4d. Platelet-bound C4d has been shown to be highly specific for SLE and is associated with disease activity [10]. In another study, platelet-bound C4d was associated with severe acute ischaemic stroke and, thus, may be a biomarker for cerebrovascular inflammation and thrombosis [11]. Reticulocyte-bound C4d also serves as a biomarker of disease activity in patients with SLE [12].

E-C4d is also a diagnostic marker for lupus, and the levels of E-C4d can be correlated with lupus disease activity; however, the physiologic function of E-C4d is entirely unclear [3, 7, 8]. The levels of E-C4d are low in normal individuals [13]. In our study, sMFI levels of E-C4d were significantly higher in patients with SLE than in patients with other diseases or healthy controls (Fig. 1). This finding is compatible with previous studies that document a significant correlation between E-C4d levels and disease activity of lupus [7, 8]. However, high levels of E-C4d can also be found in APS, and E-C4d is not specific to differentiate SLE from primary APS [8]. Hypocomplementaemia can be noted, and it can occur before the diagnosis of SLE in some APS patients [14, 15]. Expression of E-C4d may occur in the APS patient without clinical symptoms to fulfil revised ACR criteria of SLE, but these patients still have potential to progress to lupus and need to be followed closely. Our study shows that in most situations, E-C4d may be used as a marker to diagnose SLE. Our data show that these correlated values of lupus disease activity include elevation of SLEDAI and decreased levels of C3/C4 in

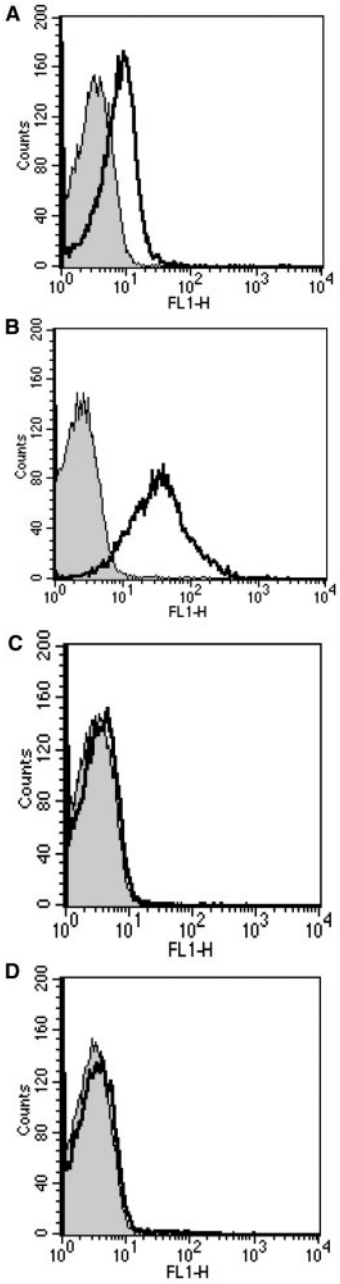

FIG. 3. Histograms in different conditions of SLE (E-C4d: black lines; isotype control: solid grey histogram). (**A**) SLE without HA or CRF (aMFI of E-C4d = 5.02); (**B**) SLE with HA (sMFI of E-C4d = 32.84); (**C**) SLE with CRF (sMFI of E-C4d = 0.71); (**D**) HC (sMFI of E-C4d = 0.43).

our total population of SLE patients. In the SLE patient subgroup without HA or CRF, a highly significant correlation is observed between E-C4d levels and lupus activity (Table 3); E-C4d levels are negatively correlated to a significant degree with serum levels of C3 and C4. In brief, E-C4d may play a role in the mechanism of inflammation *in vivo*, reflecting disease activity in patients with SLE. Increased levels of E-C4d following activation of the classical complement pathway may be a possible explanation for this observation. However, significantly elevated E-C4d levels were not found in SLE patients with CRF compared with healthy controls in our study. Abnormal complement activation in SLE with CRF appeared to be mild. Therefore, E-C4d levels do not serve as an appropriate biomarker when SLE patients are at the uraemia stage. In our study, we observed significantly higher levels of E-C4d in lupus patients with HA than in lupus patients without HA (Figs 2 and 3). This finding is compatible with the recent study in another group. Singh *et al*. [8] found that higher levels of E-C4d could be observed in lupus patients with positive direct Coombs test or HA. Among our lupus patients with HA, all patients have positive direct and indirect Coombs test. When lupus patients present with HA, E-C4d levels are not associated with disease activity because the levels of E-C4d are persistently high, although the levels of E-C4d decrease when HA is controlled. Abnormal activation of the complement system can occur in patients with autoimmune HA (AHA) [16]. In patients with paroxysmal nocturnal haemoglobinuria, erythrocytes are vulnerable to complement activation through the classical, lectin and alternative pathways [17]. This complement-mediated haemolysis is due to a reduction or absence of complement regulatory membrane proteins (CD55 and CD59). However, in patients with SLE, the mechanism of AHA is associated with warm autoantibody-mediated red blood cell (RBC) destruction [18, 19]. These autoantibodies are predominantly IgG globulins, which exhibit relatively high affinity for human RBCs at body temperature (37°C) [18]. The autoantibody-coated RBCs trigger macrophage phagocytosis. During the progression of phagocytosis, classical complement pathway-associated complement proteins C3 and C4 are activated to opsonize. In addition, abnormally diminished expression of CD55 and CD59 on RBCs can be found in patients with SLE [20]. Overall, a hyperactive complement system was observed in SLE patients with secondary HA. In lupus patients with HA, significantly lower levels of C3 and C4 were observed than those in lupus patients without HA in our study (Table 1). This observation suggests that this overexpression of complement cleavage product by the classical pathway plays a major role in the pathogenesis of lupus with AHA when compared with general lupus without AHA. In SLE patients with HA, autoantibodies induced destruction of RBCs to enhance the activation of the complement system. More consumption of complements and more breakdown products of C4d were developed to clear circulating ICs. Therefore, E-C4d levels can be used to evaluate the development of AHA in lupus patients. When SLE patients have HA, E-C4d levels cannot be used as a biomarker to monitor disease activation because there is no correlation between E-C4d levels and disease activity. In the SLE patients with positive anti-cardiolipin IgG or IgM and lupus anticoagulant, higher levels of E-C4d can be found compared with SLE patients without these autoantibodies [8]. The mechanism of APS-induced high level of E-C4d can be explained by the activation of classical pathway of complement system.

More than 75% of individuals with hereditary complement deficiency have SLE with increasing the risk of infections [4, 21]. Early SLE disease onset with higher titres of autoantibiodies or renal involvement can be found in the patients with complete C4 deficiency [22]. Complete deficiencies of C4A and C4B probably impair the clearance of ICs and apoptotic materials, and are the genetic risks for SLE [23–25]. Different mutations in the *C4A* or *C4B* gene have been detected in people with complete C4 deficiencies [24]. In these lupus patients with hereditary complement deficiency, persistent low levels of serum C3 or C4 would be found. Thus, we cannot monitor the disease activity accurately by declined complement levels. The mechanisms of SLE-associated decreased complement include over-activation of complement system, complement proteins deficiency and production of autoantibodies to complement proteins [21, 26, 27]. In hereditary complement deficiency-associated SLE, the classical pathway of complement is still activated for immunoclearance [21, 28]. There are rare reports discussing whether the production of C4d is decreased under the condition of C4 deficiency. In our study, we lack the assay for the SLE patients with complement deficiency and its relationship with C4d production. Further study is needed to evaluate the complement system in lupus patients with hereditary complement deficiency. However, we indeed

observed the persistent low levels of C3 or C4 and detectable C4d in lupus patients without active disease.

As demonstrated in our study and other studies, disease activity in SLE without HA can be effectively evaluated by E-C4d levels. There is a significant correlation between levels of E-C4d and various parameters of disease activity, including SLEDAI; anti-dsDNA, C3 and C4 levels; ESR; anti-cardiolipin IgG; and IgM [7]. However, in another study, the correlation between levels of E-C4d and SLEDAI is mild [8]. Their study showed that E-C4d was associated with serum C3, C4 and anti-dsDNA. Our study also has the same finding, but we observed a higher correlation with SLEDAI because we excluded SLE patients with HA or CRF. Our study shows that in the groups of SLE patients with HA, there is no correlation between E-C4d levels and disease activity, including SLEDAI and anti-dsDNA, and C3 and C4 levels (Table 3). When we excluded the subgroups of HA or CRF among SLE patients, a more significant correlation between E-C4d levels and disease activity of lupus was observed (Table 3). In the recent report of Singh *et al*., they did not exclude the patients with HA or positive direct Coombs test. Therefore, mild correlation between E-C4d and SLEDAI was found in their study. Our findings show that E-C4d levels can serve as a parameter to monitor disease activity in SLE patients without HA or CRF. However, E-C4d levels are of limited use in the evaluation of disease activity of lupus patients with HA (because of persistently high levels of E-C4d) and in lupus patients with CRF (because there is no elevation of E-C4d levels).

In summary, there is a need for lupus biomarkers to monitor disease activity. Towards this goal, we evaluated abnormally elevated levels of E-C4d in SLE patients. We found that a simple test for E-C4d level could provide a specific biomarker for monitoring disease activity in SLE patients. However, the test has limited use with SLE patients with HA or CRF. The mechanism which leads to the increase of active complement fragment in SLE patients with HA is still unclear. Although significantly higher levels of E-C4d can be observed in other disorders, including APS and AHA, which trigger classical complement pathway, it is likely that this novel biomarker may lead to prompt lupus activity evaluation and more timely therapy.

Rheumatology key messages

- In lupus patients with AHA, persistent high levels of E-C4d are found.
- This novel biomarker may be limited to monitor disease activity in lupus patients with AHA.

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