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ORIGINAL ARTICLE

Relation of Platelet C4d with All-Cause Mortality and Ischemic Stroke in Patients with Systemic Lupus Erythematosus

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Abstract Systemic lupus erythematosus (SLE) is an autoimmune disease associated with significant morbidity, including premature cardiovascular disease, and mortality. Platelets bearing complement protein C4d (P-C4d) were initially determined to be specific for diagnosis of SLE and were later found to be associated with acute ischemic stroke in non-SLE patients. P-C4d may identify a subset of SLE patients with a worse clinical prognosis. This study investigated the associations of P-C4d with all-cause mortality and vascular events in a lupus cohort. A cohort of 356 consecutive patients with SLE was followed from 2001 to 2009. Primary outcome was all-cause mortality. Secondary outcomes were vascular events (myocardial infarction, coronary artery bypass graft, percutaneous coronary transluminal angioplasty, ischemic stroke, venous thromboembolism, pulmonary embolism, or other thrombosis). P-C4d was measured at study baseline. Seventy SLE patients (19.7 %) had P-C4d. Mean follow-up was 4.7 years. All-cause mortality was 4 %. P-C4d was associated with all-cause mortality (hazard ratio 7.52, 95 % confidence interval (CI) 2.14–26.45, $p=0.002$) after adjusting for age, ethnicity, sex, cancer, and anticoagulant use. Vascular event rate was 21.6 %. Patients with positive P-C4d were more likely to have had vascular events compared to those with negative P-C4d (35.7 vs. 18.2 %, $p=0.001$). Specifically, P-C4d was associated with ischemic stroke (odds ratio 4.54, 95 % CI 1.63–12.69, $p=0.004$) after adjusting for age, ethnicity, and antiphospholipid antibodies. Platelet-C4d is associated with all-cause mortality and stroke in SLE patients. P-C4d may be a prognostic biomarker as well as a pathogenic clue that links platelets, complement activation, and thrombosis.

Keywords Complement C4d · Platelet · Systemic lupus erythematosus · Vascular event · Mortality · Ischemic stroke

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Background

We have identified complement activation product C4d bound to the surface of platelets (platelet C4d; P-C4d) in 18 % of patients with systemic lupus erythematosus (SLE) [1], a systemic autoimmune disease in which autoantibody production and complement activation are key participants in the pathogenesis of organ injury. In our previous studies,

platelet C4d was highly specific (99 %) for SLE compared to more than 30 other autoimmune and inflammatory disorders. Platelet C4d positivity was significantly associated with the presence of antiphospholipid antibodies in these SLE patients. We have also demonstrated that platelets bearing C4d are associated with acute ischemic stroke and stroke severity in non-lupus patients [2]. Abundant evidence suggests that therapeutic strategies focused on platelet and/or complement inhibition hold great promise in the treatment of ischemic stroke [3–11]. Together, these considerations suggest that platelets (as the cellular participant in thrombosis) bearing the complement activation product C4d (as a molecular mediator of inflammation) may be an informative biomarker in characterizing patients with SLE.

Utilizing our well-characterized cohort of lupus patients, we retrospectively evaluated the clinical features associated with platelet C4d and also investigated its association with vascular events and all-cause mortality.

Methods

Study Participants

All study participants were 18 years of age or older, and all provided written informed consent. The University of Pittsburgh Institutional Review Board approved this study.

Consecutive patients with SLE were recruited and followed during routine visits to the University of Pittsburgh Lupus Patient Care and Translational Research Center beginning in July 2001 when we began performing the P-C4d assays. Only patients who met the 1982 or 1997 American College of Rheumatology (ACR) revised classification criteria for definite SLE were included [12, 13]. As part of their routine care, all patients underwent medical history and physical examination by physicians (AK and SM) who were blinded to the results of the P-C4d assays. SLE disease activity was measured at the time of each clinic visit using the Systemic Lupus Erythematosus Activity Measure (SLAM) [14] and Systemic Lupus Disease Activity Index (SLEDAI) [15]. The modified SLEDAI excluded the parameters of complement and anti-dsDNA antibodies. We did not include the Systemic Lupus International Collaborating Clinics (SLICC) damage index [16] in this report since this index was not measured routinely in all of our SLE subjects until 2008. Patients in this study were followed until August 2009.

Outcome Characteristics

Primary outcome was all-cause mortality. Secondary outcomes included vascular event, defined as myocardial infarction (MI), coronary artery bypass graft (CABG), percutaneous transluminal coronary angioplasty (PTCA), ischemic stroke, deep vein thrombosis, pulmonary embolism, or other thrombosis. Vascular event was further categorized as coronary heart event (MI, CABG, and PTCA) or thrombotic event (ischemic stroke, deep vein thrombosis, pulmonary embolism, and other thrombosis). Data on vascular events, cancer, and death were collected prospectively and verified by CM and AK through chart review, death registry, and the Pennsylvania Cancer Registry. Renal disease was defined using ACR criteria as having (1) renal biopsy showing lupus nephritis, (2) persistent proteinuria greater than 0.5 g per day or greater than 3+ protein by urine dipstick if quantification was not performed, or (3) evidence of cellular casts in the urine. Presence of antiphospholipid antibodies was defined as a positive test for anticardiolipin IgG/IgM by ELISA and/or lupus anticoagulant. Anti-double-stranded DNA antibodies were measured by either Crithidia luciliae immunofluorescence test or ELISA.

P-C4d Measurement

C4d deposition on platelets (P-C4d) was assessed by two-color flow cytometry as previously described [1]. Briefly, platelets were identified and electronically gated by expression of the platelet-specific marker CD42b and by forward scatter properties, which reflect cell size. Whole blood was diluted in phosphate-buffered saline and labeled by immunofluorescence for flow cytometry, using phycoerythrin-conjugated anti-CD42b (BD Biosciences, San Jose, CA) and a second monoclonal antibody (mAb) conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) or using Alexa Fluor 488 with a Zenon Mouse IgG labeling kit (Molecular Probes). The mAb were either anti-C4d (reactive with C4d-containing fragments of all major allotypes of C4) (Quidel, San Diego, CA) or the IgG1-isotype control, MOPC21. Samples were analyzed on a FACSCalibur flow cytometer (BD Immunocytometry Systems). To minimize the day-to-day variability of PC4d measurement, the FACSCalibur cytometer was calibrated daily using the CaliBrite 3 beads (BD). To ensure the long-term reliability of PC4d measurement, the same mAb clones and labeling reagents from the same providers were used throughout the study period; the laser of the FACSCalibur was maintained by the manufacturer on a regular basis to ensure the performance of the instrument.

Since P-C4d was not measured at each follow-up visit, we utilized only the baseline P-C4d measurement. P-C4d was considered positive for complement deposition based on the cut point of 2.15 median fluorescence intensity as previously reported [1], [2] which is derived from repeated measures of platelet samples from healthy controls. This cut point took into account the slight variations in fluorescence labeling between the MOPC21 isotype control and the anti-C4d antibodies, as well as the detection limitations of the flow cytometer.

Statistical Analysis

Data are presented as mean (standard deviation) or median (interquartile range/IQR 25th–75th percentile) based on the distribution of the continuous variables. Categorical variables were analyzed using Fisher's Exact test or chi square test. To determine whether platelet C4d was associated with vascular events, we compared the prevalence of vascular events in SLE patients with positive P-C4d with those who had negative P-C4d at study entry. Multivariable logistic regression was utilized to assess the independent association of P-C4d with the overall vascular outcome variable and then each vascular outcome variable, whereas multivariable Cox proportional hazard regression was performed to assess the independent association between P-C4d and all-cause mortality. The test of proportional hazard assumption was also performed. All analyses used two-tailed tests with a significance level of 0.05. Analyses were performed using STATA/SE version 11.0 for Windows (Stata Corporation, College Station, TX).

Results

Patient Characteristics

The demographics and characteristics of the 356 SLE patients are shown in Table 1. The mean age at the initial biomarker collection was 44.5 years (range:18–81 years). The majority were female (92.7 %) and Caucasian (82 %). Mean SLE disease duration was 15.1±8.8 years (range 0.15–45.5 years). The mean follow-up of consecutive patients since 2001 was 4.7±2 years. The total person-years of follow-up were 1, 657.5 years. Median P-C4d level was 0.55 (IQR 0.03–1.68). Positive P-C4d was found in 70 SLE patients (19.7 %) at study entry, which comprised the top quintile of P-C4d levels. Three SLE patients with positive P-C4d and 10 with negative P-C4d did not have any follow-up visits.

Association Between P-C4d and Prevalent Vascular Event

Even though 13 SLE patients (3 with positive P-C4d and 10 with negative P-C4d) did not have follow-up visits, we were able to obtain vascular event history at their initial visit. Therefore, we did not exclude these subjects from the final analysis. At study entry, 56 SLE patients had a history of vascular event and 21 SLE patients developed an incident vascular event during follow-up. SLE patients with positive P-C4d were significantly more likely to have any incident vascular event compared to those with negative P-C4d (12.8 vs. 3.2 %, $p<0.01$). Due to the low incidence of vascular events after study enrollment, we assessed the association between P-C4d and any vascular event as shown in Table 1. The overall rate of vascular events defined as MI, CABG, PTCA, ischemic stroke, pulmonary embolism, deep vein thrombosis, and/or other thrombosis was 21.6 %. Eleven patients had "other thrombosis" that included lower extremity artery occlusion ($n=3$), upper extremity artery occlusion ($n=2$), thrombosed renal allograft ($n=1$), superficial thrombophlebitis ($n=2$), central retinal vein thrombosis ($n=1$), splenic infarcts ($n=1$) and vasculitic ulcer with thrombosis ($n=1$). SLE patients with positive P-C4d had a significantly higher rate of any vascular event compared to those with negative P-C4d (35.7 vs. 18.2 %, $p<0.01$; unadjusted odds ratio (OR) 2.50, 95 % CI 1.41–4.44, $p<0.01$) as shown in Tables 1 and 2. However, the significant association was found in the thrombosis category and not in the coronary heart disease category. P-C4d was univariately associated with ischemic stroke (12.9 vs. 2.8 %; unadjusted OR 5.13, 95 % CI 1.90–13.82, $p<0.01$) and pulmonary embolism (11.4 vs. 3.5 %; unadjusted OR 3.56, 95 % CI 1.35–9.39, $p=0.01$). Rates of MI, CABG, PTCA, deep vein thrombosis, and other thrombosis were similar between the P-C4d-positive and P-C4d-negative groups. Other covariates that were univariately associated with any vascular event were male sex, history of neurological involvement (seizure or psychosis), antiphospholipid antibodies, greater SLE disease activity by SLAM, history of steroid use, and history of anticoagulant use. In multivariable logistic regression, positive P-C4d was associated with any vascular event (OR 2.18, 95 % CI 1.12–4.04, $p=0.01$) after adjusting for age, ethnicity, sex, history of steroid use, and antiphospholipid antibodies. Furthermore, positive P-C4d continued to be associated with ischemic stroke (OR 4.54, 95 % CI 1.3–2.69, $p<0.01$) independent of the presence of antiphospholipid antibodies (Table 3). Nearly 40 % of baseline P-C4d positive patients were consistently P-C4d positive at follow-up. The baseline PC4d level of persistently P-C4d positive patients were significantly higher compared to those who became negative later (Median 6.7 (IQR 4.6–15) vs. 3.8 (IQR 2.7–6.4), $p=0.001$). Sub-analysis of the persistently P-C4d positive patients showed a significant association with ischemic stroke (OR 5.48, 95 % CI 1.7–17.6, $p=0.004$) after adjusting for the same covariates in Table 3.

Baseline SLE disease activity indices (SLEDAI, modified SLEDAI, and SLAM), serum C3, C4, and platelet count did not contribute significantly to the multivariable regression models ($p>0.30$); the inclusion of baseline SLE disease activity indices separately also did not attenuate the significant association between P-C4d and ischemic stroke. Furthermore, even if we controlled for other variables associated with more severe SLE disease/damage (i.e., renal disease, anti-dsDNA antibodies, and SLE disease duration), P-C4d remained significantly associated with ischemic stroke (OR 4.96, 95 % CI 1.75–14.06, $p<0.01$). On the other hand, positive P-C4d was no longer associated with pulmonary embolism (OR 2.46, 95 % CI 0.84–7.20, $p=0.10$) whereas other

Table 1 Baseline patient demographics and clinical characteristics by platelet C4d status at study enrollment (N=356)

	All (n=356)	Platelet C4d		p value
		Positive (n=70)	Negative (n=286)	
Age, mean (SD), y	44.5 (12.7)	43 (13.8)	44.7 (12.5)	0.46
Duration of SLE, mean (SD), y	15.1 (8.8)	17.1 (10)	14.6 (8.5)	0.08
Follow-up, mean (SD), y	4.7 (2)	4.7 (2.4)	4.6 (1.8)	0.32
Sex, No. (%)				
Women	330 (92.7)	64 (90)	266 (93)	0.65
Men	26 (7.3)	6 (8.6)	20 (7)	
Race/ethnicity, No. (%)				
White	292 (82)	54 (77.1)	235 (82.2)	0.34
Black	53 (14.9)	13 (18.6)	40 (14)	
Others	11 (3.1)	2 (2.8)	9 (3.2)	
Smoking history, %	147 (41.3)	30 (42.9)	117 (40.9)	0.77
SLE manifestations, No. (%)				
Malar rash	164 (46.1)	35 (50)	129 (45.1)	0.46
Discoid rash	35 (9.8)	9 (12.9)	26 (9.1)	0.34
Photosensitivity	206 (57.8)	32 (45.7)	174 (60.8)	0.02
Oral ulcers	190 (53.4)	34 (48.6)	156 (54.6)	0.37
Arthritis	329 (92.4)	64 (91.4)	265 (92.7)	0.73
Serositis	164 (46.1)	36 (51.4)	128 (44.8)	0.32
Renal disease	138 (38.8)	34 (48.6)	104 (36.4)	0.06
Neurological	41 (11.5)	15 (21.4)	26 (9.1)	0.004
Seizure	35 (9.8)	13 (18.6)	22 (7.7)	0.006
Psychosis	17 (4.8)	4 (5.7)	13 (4.6)	0.68
Hematological	260 (73)	59 (84.3)	201 (70.3)	0.02
Hemolytic anemia	29 (8.2)	12 (17.1)	17 (5.9)	0.002
Leukopenia	149 (41.9)	31 (44.3)	118 (41.3)	0.65
Lymphopenia	140 (39.3)	34 (48.6)	106 (37.1)	0.08
Thrombocytopenia ($<100\times10^3$)	73 (20.5)	24 (34.3)	49 (17.1)	0.001
Autoantibodies				
Antinuclear antibody (Ab)	353 (99.2)	69 (98.6)	284 (99.3)	0.55
Phospholipid (aPL) Ab	134 (37.6)	39 (55.7)	95 (33.2)	<0.0001
Cardiolipin Ab	106 (29.8)	32 (45.7)	74 (25.9)	0.001
Lupus anticoagulant	55 (15.5)	17 (24.3)	38 (13.3)	0.02
Double-stranded Ab	188 (52.8)	46 (65.7)	142 (49.7)	0.02
Smith Ab	42 (11.8)	7 (10)	35 (12.2)	0.60
SLE activity indices				
Median SLEDAI score (n=339)[a]	2 (IQR 0–4)	3 (IQR 2–6)	2 (IQR 0–4)	0.04
Median modified SLEDAI score (n=339)[b]	0 (IQR 0–2)	0 (IQR 0–4)	0 (IQR 0–2)	0.55
Median SLAM score (n=339)[a]	5 (IQR 3–8)	6 (IQR 3–8)	5 (IQR 3–7)	0.13
Platelet count, ×1,000	253 (IQR 192–303)	236 (IQR 180–288)	256 (IQR 196–305)	0.08
Serum C3, % below normal range	115/328 (35.1 %)	31/60 (51.7 %)	84/268 (31.3 %)	0.003
Serum C4, % below normal range	154/328 (47 %)	36/60 (60 %)	118/268(81.7 %)	0.03
Medication use ever[c]				
Steroid	271 (76.1)	55 (78.6)	216 (75.5)	0.59
Antimalarial	231 (64.9)	49 (70)	182 (63.6)	0.32
Anticoagulant	42 (11.8)	17 (24.3)	25 (8.7)	<0.0001

Table 1 (continued)

	All (n=356)	Platelet C4d		p value
		Positive (n=70)	Negative (n=286)	
Antiplatelet	100 (28.1)	21 (30)	79 (27.6)	0.69
Immunosuppressant	169 (47.5)	43 (61.4)	126 (44.1)	0.009
All-cause mortality (%)	14 (3.9)	8 (11.4)	6 (2.1)	0.002
Any vascular event, ever[d] (%)	77 (21.6)	25 (35.7)	52 (18.2)	0.001
Coronary heart disease				
MI	14 (7.1)	5 (4.3)	9 (3.2)	0.16
CABG	6 (1.7)	1 (1.4)	5 (1.8)	0.66
PTCA	10 (2.8)	2 (2.9)	8 (2.8)	1
Thrombosis				
Ischemic stroke	17 (4.5)	9 (12.9)	8 (2.8)	0.002
Pulmonary embolism	18 (5.1)	8 (11.4)	10 (3.5)	0.007
Deep vein thrombosis	34 (9.6)	8 (11.4)	26 (9.1)	0.55
Other thrombosis	12 (3.6)	4 (5.7)	7 (2.5)	0.16
Others				
Angina	23 (6.5)	6 (8.6)	17 (5.9)	0.42
TIA	11 (3.2)	2 (3.1)	9 (3.2)	1.0
Malignancy, ever	32 (9.0)	6 (8.6)	26 (9.1)	1.0

Continuous variables are presented as mean (standard deviation) or median (Interquartile range/IQR 25th–75th percentile) depending on the data distribution.

MI myocardial infarct, *CABG,* coronary artery bypass graft, *PTCA* percutaneous transluminal coronary angioplasty, *PE* pulmonary embolism, *DVT* deep vein thrombosis, *TIA* transient ischemic attack

[a] Baseline SLEDAI and SLAM were completed in 339/356 SLE subjects (66/70 P-C4d positive and 273/286 P-C4d negative subjects).

[b] Modified SLEDAI score excluded the anti-dsDNA and complement parameters.

[c] Medication use at baseline: antimalarial—hydroxychloroquine, chloroquine, and quinacrine; anticoagulant—warfarin, enoxaparin, and fondaparinux; antiplatelet—aspirin, clopidogrel, enoxaparin; immunosuppressant—azathioprine, methotrexate, mycophenolate mofetil, mycophenolic acid, cyclophosphamide, tacrolimus, leflunomide, cyclosporine, and 6-mercaptopruine.

[d] Vascular event, ever (at baseline and follow-up): MI, CABG, PTCA, ischemic stroke, PE, DVT, or other thrombosis

covariates, specifically antiphospholipid antibodies (OR 5.75, 95 % CI 1.67–19.76, p<0.01) and history of renal disease (OR 3.58, 95 % CI 1.18–10.87, p=0.02), were significantly associated with pulmonary embolism in the multivariable logistic regression model.

Association Between P-C4d and All-Cause Mortality

Fourteen (3.9 %) SLE patients died during this study. The mean age of death was 46.9±15.2 years. The overall mortality rate was 8.4 (95 % confidence interval 5–14.3) per 1,000 person-years. In univariate analysis, more P-C4d-positive patients died compared to those who were P-C4d-negative (11.4 vs. 2.1 %, p=0.002). Both groups had similar duration of follow-up. SLE disease duration appeared to be longer but not statistically significant in the deceased group compared to the living group (median 18.6 vs. 14.9 years, p=0.06). The causes of death were infection (n=4), cardiac arrest (n=2), congestive heart failure (n=1), ovarian cancer (n=1), lung cancer (n=1), hemorrhage (n=1), and unknown (n=4). Of note, 6 of these 14 deceased patients had cancer (ovarian carcinoma, T-cell lymphoma, non-Hodgkin's lymphoma, small-cell lung cancer, epithelial hemangioendothelial lung cancer, anal squamous cell carcinoma). The overall prevalence of cancer in this study sample was 9 % (32/356) and the cancer rates were similar in both groups. Five subjects had a history of cancer prior to the SLE diagnosis. Immunosuppressive therapy (including cyclophosphamide use) or smoking history was not associated with malignancy. Figure 1 illustrates the Kaplan–Meier survival curves for P-C4d positive and P-C4d negative SLE subjects. A log-rank test and Wilcoxon test for equality of survival both showed significant differences between the two groups (p=0.0005 and p=0.002, respectively). As shown in Table 2, positive

Table 2 Univariate association with vascular events and all-cause mortality

	Any vascular event			All-cause mortality		
Variables	OR	95 % CI	*p* value	HR	95 % CI	*p* value
Age	1.02	0.99–1.04	0.06	0.99	0.95–1.03	0.71
Duration of SLE	1.01	0.98–1.04	0.58	1.03	0.98–1.09	0.27
Sex (Male)	2.93	1.29–6.68	0.01	1.04	0.14–7.92	0.97
Race (Caucasian)	0.65	0.35–1.20	0.17	0.27	0.09–0.78	0.02
Smoking history	1.52	0.91–2.52	0.10	1.02	0.35–2.95	0.97
SLE Manifestations						
Arthritis	0.72	0.27–1.91	0.51	1.03	0.13–7.94	0.98
Serositis	1.18	0.71–1.96	0.51	1.07	0.37–3.05	0.90
Renal disease	1.52	0.92–2.54	0.11	2.73	0.91–8.16	0.07
Neurological	2.35	1.18–4.71	0.02	2.87	0.90–9.16	0.08
Hematological	1.69	0.91–3.14	0.097	1.77	0.40–7.92	0.46
aPL antibodies	2.61	1.56–4.37	<0.01	1.09	0.38–3.11	0.88
dsDNA antibodies	1.64	0.98–2.76	0.06	0.79	0.28–2.28	0.67
SLEDAI score (n=339)	1.06	0.99–1.14	0.10	1.04	0.89–1.21	0.64
SLAM score (n=339)	1.08	1.01–1.14	0.02	1.09	0.98–1.22	0.12
Serum C3	1.00	0.99–1.01	0.83	0.98	0.98–1.02	0.85
Serum C4	1.02	0.99–1.05	0.18	1.06	1.00–1.14	0.06
Medication use, ever						
Steroids	2.46	1.20–5.04	0.01	4.03	0.53–30.79	0.18
Antimalarial	0.87	0.51–1.46	0.60	0.52	0.18–1.47	0.22
Anticoagulant	22.5	10.08–50.22	<0.01	5.62	1.95–16.2	<0.01
Antiplatelet	1.31	0.76–2.26	0.34	0.18	0.02–1.37	0.10
Immunosuppressant	1.64	0.99–2.73	0.06	1.46	0.51–4.21	0.48
Vascular event, ever	NA	NA	NA	3.43	1.20–9.78	0.02
Malignancy, ever	2.05	0.94–4.46	0.07	8.22	2.84–23.77	<0.01
Platelet C4d	2.50	1.41–4.44	<0.01	5.36	1.85–15.51	<0.01

P-C4d at baseline was associated with an unadjusted hazard ratio for all-cause mortality of 5.36 (95 % CI 1.85–15.51, p = 0.002). Positive P-C4d at baseline was significantly associated with a 7.5-fold increased risk of all-cause mortality (HR 7.52, 95 % CI 2.14–26.45, p =0.002) after adjusting for age, race, sex, cancer, and anticoagulant use (Table 4). Even though any vascular event was univariately associated with all-cause mortality, any vascular event did not attenuate the association between P-C4d and all-cause mortality in the multivariable regression model (HR 7.58, 95 % CI 2.16–26.54, p =0.002). The addition of baseline modified SLEDAI and SLAM to the separate multivariable regression models did not attenuate the significant association between P-C4d and all-cause mortality. Similarly, the addition of variables for more severe SLE disease/damage (i.e., renal disease, cardiovascular event, and anti-dsDNA antibodies) also did not attenuate the significant association between P-C4d and all-cause mortality. No interactions were found and the proportional hazard assumption was met.

Table 3 Association between baseline positive platelet C4d and ischemic stroke in patients with systemic lupus erythematosus

Independent variables	Odds ratio	95 % Confidence interval	*p* value
Positive P-C4d	4.54	1.63–12.69	<0.01
Age	1.04	0.99–1.08	0.06
Race (Caucasian)	0.32	0.11–0.96	0.04
Antiphospholipid antibodies	2.11	0.67–5.71	0.22

P-C4d platelet C4d

Discussion

In this retrospective cohort study, we demonstrated that C4d deposition on platelets at baseline is significantly associated with all-cause mortality and ischemic stroke in patients with SLE. The mortality rate in our study was 3.9 %, which is lower than in other studies [17–19]. This low mortality rate may be related to the potential survival bias based on the relatively long SLE disease duration (mean 15.1 years) and a short mean follow-up

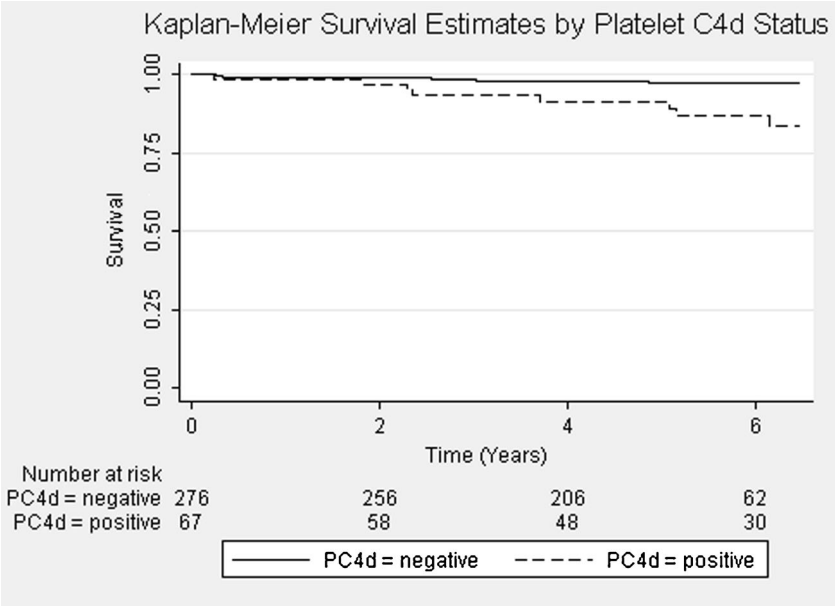

Fig. 1 Kaplan–Meier survival curves of patients with systemic lupus erythematosus (SLE) stratified by platelet C4d (P-C4d) status. P-C4d positive SLE patients (*dash line*) had worse survival or higher all-cause mortality compared to P-C4d-negative SLE patients (*solid line*). Log-rank test and Wilcoxon test for equality of survival both showed significant differences between the two groups ($p=0.0005$ and $p=0.002$, respectively)

since study entry (4.7 years) in our study cohort. Infection, as expected, was a leading cause of mortality [19–21].

We noted a higher frequency of malignancy in our cohort (9 %) compared to the other studies (4.3 %, 4.5 %) [22, 23] even though the SLE duration and age at the time of cancer diagnosis in our study were similar to those of the other published studies. When we excluded patients with pre-existing cancer prior to SLE diagnosis, the incidence of cancer was reduced to 7.6 %. Factors which could potentially contribute to malignancy include immunosuppressive therapy, chronic inflammation, and cigarette smoking, even though these factors were not significant in our study.

Our current findings of the association between P-C4d and a history of stroke corroborate previous work that demonstrated activation of complement in acute ischemic stroke [4–6, 24–27] and the interaction between complement activation and platelets in cerebral ischemic injury. Furthermore, P-C4d was significantly associated with stroke and not venous thromboembolism (i.e., pulmonary embolism) in the multivariable regression analyses. These findings suggest a potential dual role of platelets which involves inflammation mediated by complement activation and arterial thrombosis through platelet activation in cerebrovascular event (i.e., stroke) in SLE. Although TIA was not significantly associated with P-C4d positivity, the small number of TIA events in this study would preclude any meaningful postulation. Our finding is consistent with a previous study by Peerschke et al. which demonstrated a positive association between in vitro complement activation (C1q and C4d) on platelets and arterial thrombosis (predominantly stroke) in patients with SLE and with antiphospholipid antibodies but not in non-lupus patients with primary antiphospholipid syndrome [28]. This study utilized a solid phase assay to measure the capacity of sera from either SLE patients with antiphospholipid antibodies or non-SLE patients with antiphospholipid syndrome to activate the classical complement pathway on immobilized heterologous platelets from healthy donors. A recent study by Lood et al. investigated in vivo deposition of the complement proteins C1q, C4d, and C3d on platelets of patients with SLE. [29] Using a different method from that of the current study, this group reported values of complement deposition as a percentage of platelets being positive compared with a negative isotype antibody and found increased complement C1q, C4d, and C3d depositions on platelets of SLE patients with venous and not arterial thrombosis in their univariate analyses. We initially also found an association between P-C4d and venous thrombosis, specifically pulmonary embolism, in a univariate analysis. However, this association was no longer significant after adjustment for the presence of antiphospholipid antibodies. Even though the assays utilized by Peerschke et al [28]. and Lood et al [29]. were not the validated P-C4d assays, their findings support the link among platelets, complements,

Table 4 Association between baseline positive platelet C4d and all-cause mortality in patients with systemic lupus erythematosus

Independent variables	Hazard ratio	95 % Confidence interval	*p* value
Positive P-C4d	7.52	2.14–26.45	<0.01
Age	0.97	0.93–1.01	0.16
Race (Caucasian)	0.09	0.03–0.35	<0.01
Sex (Male)	0.09	0.01–1.11	0.06
Malignancy, ever	50.96	9.99–259.70	<0.01
Anticoagulant use	12.47	3.03–51.29	<0.01

P-C4d platelet C4d

and thrombosis in SLE. Antiphospholipid antibodies are also known to activate the classical pathway of the complement system. Further studies are needed to examine the mechanisms underlying the interaction of antiphospholipid antibodies with platelets and complement activation in venous and arterial thrombosis. Thus far, we speculate that deposition of C4d on platelet surfaces may influence platelet aggregation and/or platelet interactions with circulating leukocytes and endothelial cells. There is growing literature regarding the proinflammatory capacity of platelets [30–32] and the anti-inflammatory effects of antiplatelet therapy through reduced platelet activation in patients who had thrombotic events [33].

Our study was limited by the small sample size, few patients with positive P-C4d, and few numbers of events; thus, the 95 % confidence interval was wide. Our patients were predominantly Caucasian. We did not have SLICC damage index completed for all subjects in this study. Thus, we cannot exclude the potential mediating effects of greater damage related to SLE disease or its treatment in P-C4d-positive patients. Another limitation is that the majority of vascular events, including ischemic stroke, occurred prior to the initial study visit. Therefore, we did not have the exact etiology or subtype of the stroke events to determine if stroke was related to SLE and/or other unrelated conditions, such as atrial fibrillation or carotid stenosis. There are other factors, such as socioeconomic status and cumulative steroid use, which we have not accounted for in this study. Nevertheless, our retrospective cohort study was a relatively large single center study with longitudinal patient follow-up. Our sub-analysis of the persistently P-C4d-positive patients showed a significant association with ischemic stroke (OR 5.48, 95 % CI 1.7–17.6, $p=0.004$). This observation further identifies a much higher risk group of SLE patients who have persistently positive P-C4d. Previously, P-C4d was found to be highly specific for the diagnosis of SLE [1]. In a recent multicenter cross-sectional study, P-C4d followed the best predictors (i.e., C4d on erythrocytes and B lymphocytes) for SLE diagnosis using the receiver operating characteristic curve analysis comparing SLE and other rheumatic diseases [34]. In our current study, we discovered an important observation regarding the association between platelet C4d and poorer prognosis in patients with SLE, specifically all-cause mortality and ischemic stroke. Taken together, P-C4d has potential value as a lupus biomarker for diagnosis, prognosis, and vascular risk stratification.

In summary, our findings indicate that platelet C4d is associated with all-cause mortality and ischemic stroke in patients with SLE. Furthermore, these observations may have important implications for identifying a subset of SLE patients who may have more severe clinical manifestations related to cellular and molecular mechanisms of complement and platelet activation. Future mechanistic studies of the function of C4d-positive platelets and their cellular interactions may provide insight into the pathogenesis of SLE and the potential therapeutic role of anti-platelet and/or anti-complement interventions in the treatment of SLE.

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Conflict of interest Drs. Joseph M Ahearn and Susan Manzi are consultants for Exagen Diagnostics, Inc.

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