Elevated levels of soluble CD40 ligand are associated with antiphospholipid antibodies in patients with systemic lupus erythematosus

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Abstract

Objective

The CD40L/CD40 pathway is involved in the pathophysiology of atherothrombotic disease, and elevated levels of soluble CD40L (sCD40L) were reported in SLE patients. However, the clinical implication of sCD40L in SLE remains elusive.

Methods

We measured levels of plasma sCD40L in 241 SLE patients and 37 healthy controls and investigated its association with clinical manifestation and laboratory parameters.

Results

Levels of plasma sCD40L in SLE patients were significantly elevated compared with healthy controls ($p=0.013$) and positively correlated with levels of soluble P-selectin ($\gamma=0.336$, $p<0.001$). SLE patients who experienced arterial thrombosis had a higher level of sCD40L than those who did not ($p=0.029$). Plasma sCD40L levels were positively correlated with the titers of anti-cardiolipin and anti-β_2 glycoprotein I antibodies ($\gamma=0.338$, $p<0.001$ and $\gamma=0.364$, $p<0.001$, respectively). Its levels were also significantly higher in patients with clinical antiphospholipid syndrome (APS) than in non-APS patients, irrespective of antiphospholipid antibody (aPL) positivity. Of those with arterial thrombosis, sCD40L levels were significantly elevated in patients with positive aPL, compared to those with negative aPL ($p=0.011$). Multiple regression analysis revealed that the presence of hypertension and positive aPL were independently associated with the occurrence of arterial thrombosis in SLE patients. A parallel analysis showed that sCD40L was also an independent variable for arterial thrombosis; however, this association disappeared when aPL, a strong variable, was included in the model because of collinearity between aPL and sCD40L.

Conclusion

Plasma sCD40L levels were elevated in SLE patients who had positive aPL and experienced arterial thrombosis, suggesting that enhanced release of sCD40L through platelet activation presumably by aPL could contribute to the development of atherothrombotic disease.

Key words

systemic lupus erythematosus, CD40 ligand, antiphospholipid

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Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease characterised by production of an array of autoantibodies and abnormal activation of immune response that cause damage and dysfunction to multiple organ systems. Accelerated atherosclerosis is recognised as a major cause of late mortality in patients with SLE (1). The pathogenesis of atherosclerosis is not completely understood, but it is generally accepted that the immune-mediated and inflammatory mechanisms inherent in SLE play an important role in the atherosclerotic process. These mechanisms involve autoantibodies (antibodies against phospholipids and oxidised low-density lipoprotein) (1, 2), cytokines (3) and proinflammatory high-density lipoproteins (4). In addition, we recently demonstrated that elevated osteoprotegerin in SLE patients is actively involved in the pathogenesis of atherosclerosis by inducing apoptosis of endothelial progenitor cells (5). Moreover, elevated levels of soluble CD40L (sCD40L) are considered to mediate the progression of atherosclerosis in several clinical conditions (6-11).

CD40L, also known as CD154, is a 39-kD type II transmembrane protein belonging to tumour necrosis factor superfamily and is expressed mainly on activated T lymphocytes and platelets (12). Engagement of CD40, a cognate receptor on B cells by CD40L induces a variety of B cell responses; B cells undergo proliferation, germinal centre formation, isotype switching, somatic hypermutation of immunoglobulin, and finally differentiate into long-lived plasma cells or memory B cells (13). CD40 can also be found in non-immune cells, such as fibroblasts, endothelial cells, and macrophages. The activation of CD40 leads to the production of both proinflammatory chemokines and VEGF, as well as upregulation of both ICAM-1 and VCAM-1 expression (14, 15). Aside from its proinflammatory properties, the CD40L/CD40 pathway is widely recognised to play a role in the pathophysiology of atherothrombotic disease (12); CD40L induces tissue factor upregulation and thrombin generation in monocytes (16) and endothelial cells (17), and stabilises arterial thrombi by promoting platelet aggregation via a β3 integrin dependent mechanism (18).

Upon activation of platelets and T cells, CD40L is cleaved from the cell surface and its soluble products are released into the extracellular environment (19). Elevated levels of sCD40L were not only detected in patients with clinically overt atherosclerotic diseases, *e.g.* ischaemic stroke (11), stable and unstable coronary artery disease (9, 10), but also in several inflammatory diseases, including systemic sclerosis (20), rheumatoid arthritis (21), SLE (22, 23) and inflammatory bowel disease, *i.e.* Crohn's disease and ulcerative colitis (24). However, there have been inconsistent or contradictory data regarding the association between sCD40L and clinical manifestations/parameters of SLE. Increased sCD40L levels in SLE patients were shown to be associated with higher disease activity (as determined by high titer of anti-double-stranded DNA antibody and SLE disease activity index) (23) and impending disease flare (25), but these associations were not observed in other studies (26-28). On the other hand, increased levels of sCD40L in SLE patients was reported to be significantly associated with the presence of aPL, irrespective of disease activity (22). These discrepancies may be due to the heterogeneity of patient population, sample size, and type of specimen samples obtained (plasma *vs.* serum). In the present study, we thus measured plasma sCD40L levels in a relatively large number of SLE patients and explored the association of sCD40L with various clinical features and laboratory parameters.

Patients and methods

Subjects

Two hundred and forty-one patients with SLE were included. All patients fulfilled the revised American College of Rheumatology (ACR) classification criteria for SLE (29). Patients who had polycythaemia, hyperbilirubinaemia, and hypertriglyceridaemia were excluded because high concentration of haemoglobin, bilirubin, and lipids can interfere with the sCD40L assay (30).

All patients were recruited from the Department of Rheumatology, Yeouido St. Mary's Hospital, The Catholic University of Korea (Seoul, Republic of Korea). Thirty-seven age- and sex-matched healthy individuals were recruited for normal controls. This study was carried out in accordance with the Declaration of Helsinki and approved by the Yeouido St. Mary's Hospital Institutional Review Board (IRB) (no. SC13SISI0119). Written informed consent was obtained from all subjects.

Clinical and laboratory profiles

The clinical and laboratory data of SLE patients were obtained at the time of blood sampling. Disease duration, the presence of organ/system involvement, and clinical features were recorded for these patients. Dyslipidaemia was defined as the presence of one or more of the following: total cholesterol >200 mg/dL, low density lipoprotein cholesterol (LDL-C) (≥130 mg/dL), high density lipoprotein cholesterol (HDL-C) (<40 mg/dL for males and <50 mg/dL for females) or the use of cholesterol lowering agents. Diabetes mellitus was defined as a fasting plasma glucose concentration ≥126 mg/dL, a haemoglobin A_{1C} level ≥6.5%, or the use of glucose-lowering medications. Hypertension was defined as an average systolic blood >140mmHg and/or diastolic blood pressure >90 mmHg, or the use of antihypertensive medications.

The SLE disease activity index (SLEDAI) was used to estimate general disease activity (31). Laboratory parameters included urinalysis, complete blood count, serum creatinine, lipid profiles, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). Complement (C3, C4, CH50) and antibodies against double-stranded DNA (anti-dsDNA Ab), Smith, ribonuclear protein, ribosomal P, SS-A/Ro, and SS-B/La, were measured. Current usage of medications including glucocorticoids, hydroxychloroquine, immunosuppressants (azathioprine, mycophenolate mofetil, methotrexate, and cyclosporine), statin, aspirin, and angiotensin-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) was retrieved.

The record of thrombotic events was retrospectively retrieved from the time of enrolment. The presence of arterial thrombotic events, such as ischaemic heart disease, stroke, and peripheral arterial thrombosis, was confirmed by arteriography, computed tomography scan, or magnetic resonance imaging. Venous thrombosis (deep vein thrombosis, pulmonary embolism, and retinal vein thrombosis) were confirmed by Doppler ultrasonography, ventilation-perfusion lung scintigraphy, fluorescein angiogram, and/or computed tomography. A diagnosis of SLE-associated APS was made only in patients who had experienced thrombotic events concurrently with or after both the confirmation of aPL positivity and diagnosis of SLE.

Assay for antiphospholipid antibodies

For all of SLE patients, antiphospholipid antibodies (aPL) were assayed in duplicate at least 12 weeks apart according to the guidelines of the International Society on Thrombosis and Haemostasis (32). Lupus anticoagulant (LAC) was determined by diluted Russel viper venom time (dRVVT) assay for screening and confirmatory tests using Siemens LA1 screening/LA2 confirmation Reagents Kit (#K013114) (Siemens, Erlangen, Germany) and a normalised ratio >1.3 was considered positive. IgG anticardiolipin (aCL) and IgG anti-β_2GPI (aβ_2GPI) were measured by the enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (#ORG-215G and #ORG-221G respectively; Alegria®, ORGENTECT Diagnostika, Mainz, Germany). IgG aCL was considered positive when value exceeds the medium or high titer (40 GPL)), and IgG aβ_2GPI was considered positive when value exceeds the cut-off value calculated using the 99th percentile of controls (>20 GPL).

Enzyme-linked immunosorbent assay for plasma sCD40L and P-selectin

Plasma samples were collected from all of the subjects enrolled in our study and each plasma sample was from a single person. The measurement of plasma sCD40L and P-selectin were performed for all stored samples at once. According to previous recommendations (33, 34), we used citrated plasma samples. Plasma samples were obtained and stored at -70°C until use. Plasma levels of sCD40L were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, MN, US), according to the manufacture's instruction. Briefly, 100 μL of plasma and diluted standards were first added to wells pre-coated with monoclonal antibody against CD40L. Next, 100 μL of biotinylated anti-CD40L secondary antibody was added to the wells. Following incubation for 2 hours at room temperature and washing the plate three times, 100 μL of streptavidin-horseradish peroxidase was added to each well and left to incubate for 20 minutes at room temperature. After washing again for three times, 100 μL of tetramethylbenzidine substrate was added to the wells and left to incubate for 20 minutes longer. The colour reaction was arrested by adding H_2SO_4, and absorbance was measured at 450 nm. The standard curve ranged from 0 to 4000 pg/mL. Plasma levels of P-selectin were measured using a commercially available ELISA kit (R&D systems, Minneapolis, MN, US), according to the manufacture's instruction. Diluted plasma samples (ratio 1:4) were used for P-selectin assays and assay procedures are similar to that of sCD40L. The standard curve ranged from 0 to 50 ng/mL.

Statistical analysis

For continuous abnormally distributed data, the results are shown as medians with interquartile ranges (IQR); comparisons between groups were performed using the Mann-Whitney U-test. Categorical or dichotomous variables are expressed as number (percentage) and were compared using the chi-square test or Fisher's exact test. Correlation analysis between two variables was carried out using Spearman's correlation coefficient. Multiple regression analysis was performed to assess the relationship between sCD40L and clinical variables. A *p*-value of less than 0.05 was considered statistically significant.

Table I. Demographic and clinical data of SLE patients (n=241).

Variable	SLE patients
Age, years	35 [26–43]
Female, n (%)	222 (92.1)
BMI, kg/m^2	21.2 [19.5–23.3]
Disease duration, years	6 [3–10]
Diabetes mellitus, n (%)	4 (1.7)
Hypertension, n (%)	47 (19.5)
Smoking, n (%)	10 (4.1)
Dyslipidaemia, n (%)	55 (22.8)
Clinical manifestations	
Mucocutaneous, n (%)	148 (61.4)
Musculoskeletal, n (%)	121 (50.2)
Pleuropericardial, n (%)	57 (23.7)
Neuropsychiatric, n (%)	34 (14.1)
Haematologic, n (%)	209 (86.7)
Renal, n (%)	99 (41.1)
Thrombosis, n (%)	36 (14.9)
Arterial, n (%)	26 (10.8)
Venous, n (%)	10 (4.1)
Autoantibody profile	
Anti-Sm Ab, n(%)	51 (21.2)
Anti-RNP Ab, n(%)	98 (40.7)
Anti-Ro/SS-A Ab, n(%)	154 (63.9)
Anti-La/SS-B Ab, n(%)	29 (12.0)
Anti-ribosomal P Ab, n(%)	55 (22.8)
IgG aCL Ab, n(%)	26 (10.8)
IgG aβ_2GPI Ab, n(%)	20 (8.3)
LAC, n(%)	40 (16.6)
Medication	
Glucocorticoid, n (%)	220 (91.3)
Prednisolone*, mg	5 [5–10]
Mycophenolate mofetil, n (%)	17 (7.1)
Azathiprine, n (%)	33 (13.7)
Methotrexate, n (%)	34 (14.1)
Cyclosporine, n (%)	14 (5.8)
Hydroxychloroquine, n (%)	215 (89.2)
ACEi or ARB, n (%)	75 (31.1)
Statin, n (%)	24 (10.0)
Aspirin, n (%)	39 (16.2)

Values are presented as median [interquartile range (IQR)] or number (%).*prednisolone or its equivalent, BMI: body mass index; aCL Ab: anticardiolipin Antibody; aβ_2GPI Ab: anti-β_2 GPI antibody; LAC: lupus anticoagulant; ACEi: angiotensin converting enzyme inhibitor; ARB: angiotensin converting enzyme blocker.

Results

Characteristics of the study subjects

The demographic and baseline clinical characteristics of 241 patients with SLE are summarised in Table I. The median age of SLE patients was 35 [26–43] years and the median duration of disease was 6 [3–10] years. The cumulative frequencies of clinical manifestations were as follows: haematologic (n=209, 86.7%), mucocutaneous (n=148, 61.4%), musculoskeletal (n=121, 50.2%), renal (n=99, 41.1%), pleuropericardial (n=57, 23.7%), and neuropsychiatric manifestations (n=34, 14.1%). In the serum autoantibody profiles (listed in Table I), 58 patients (24.1%) were aPL positive for more than one of the three tests; LAC in 40 (16.6%), IgG aCL in 26 (10.8%), IgG aβ_2GPI in 20 (8.3%) and triple positive in 9 (3.7%) (Fig. 1). Thirty-two (13.3%) patients experienced thromboembolic events (26 arterial thrombosis, 9 venous thrombosis) (Fig. 1). Arterial thrombotic event included ischaemic strokes (n=24), ischaemic heart diseases (n=2), and peripheral arterial diseases (n=2, one of them had both ischaemic stroke and ischaemic heart disease), while venous thromboses consisted of deep vein thromboses (n=6), pulmonary embolisms (n=2) and retinal vein thrombosis (n=1). Two patients were afflicted by ischaemic stroke plus venous thrombosis (n=2). The intersection of the former (aPL-positive SLE patients) and latter (patients suffering from thrombosis) groups was 20 (8.3%). These 20 patients were considered APS. Therefore, there were 38 patients of asymptomatic APS (15.8%).

Increased levels of plasma sCD40L in SLE patients

The levels (median [IQR]) of plasma sCD40L in SLE patients were significantly higher than those of healthy controls (54 [38–68] *vs*. 26 [10–87] pg/mL, p=0.013) (Fig. 2A). We also measured the levels of soluble P-selectin, a marker for platelet activation (35, 36), in the plasma of SLE patients and examined its association with levels of sCD40L. Levels of plasma soluble P-selectin were positively correlated with those of plasma sCD40L (γ=0.336, p<0.001) (Fig. 2B). This indicated that activated platelets were the primary source of sCD40L, which has been shown to constitute more than 95% of circulating sCD40L levels (7, 37). However, there was no correlation between sCD40 levels and platelet count (γ=0.041, p=0.524). This remained unadjusted after controlling for confounding factors (diabetes mellitus, hypertension, smoking, use of statin, ACEi/ARB and aspirin, ESR, and SLEDAI) (γ=0.046, p=0.483). Next, we examined the association of plasma sCD40L levels with

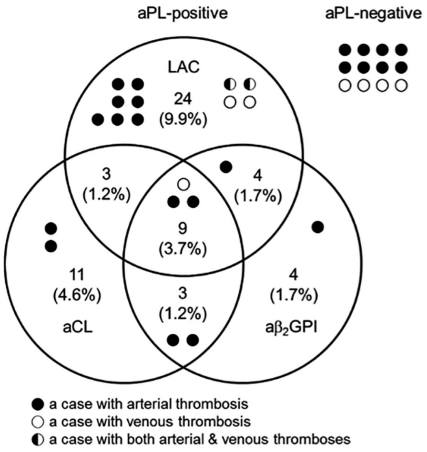

Fig. 1. Venn diagram showing the distribution of antiphospholipid antibodies and thromboses in SLE patients. LAC: lupus anticoagulant; aCL: anticardiolipin antibodies; aβ_2GPI: anti-β_2 glycoprotein I antibodies. A single circle represents a single case.

parameters of disease activity in SLE patients. In correlation analyses (Table II), there was no association between plasma sCD40L levels and complement levels (C3, C4, and CH50), anti-dsDNA titer, or SLEDAI score. After adjusting for confounding factors including diabetes mellitus, hypertension, smoking, ESR, platelet count, use of statin, ACEi/ARB, and aspirin, sCD40L levels tended to correlate negatively with complement C3 although it did not reach statistical significance (p=–0.059).

Association between plasma sCD40L levels and arterial thrombosis

In an effort to investigate the association of plasma sCD40L with clinical manifestations of SLE, sCD40L levels did not differ between patients with and without mucocutaneous involvement, arthritis, cardiopulmonary involvement, neuropsychological disorder, thrombocytopenia, or renal involvement (data not shown). Previous studies have demonstrated that sCD40L levels are significantly higher in patients with stable and unstable coronary syndrome than in healthy controls (38, 39). Thus, we compared sCD40L levels in patients with and without a history of thromboembolic events. In a simple comparison, there was no difference in the sCD40L levels between patients with history of thromboembolic event and without (30 [11–105] *vs*. 25 [9–79] pg/mL, p=0.275). However, after ad-

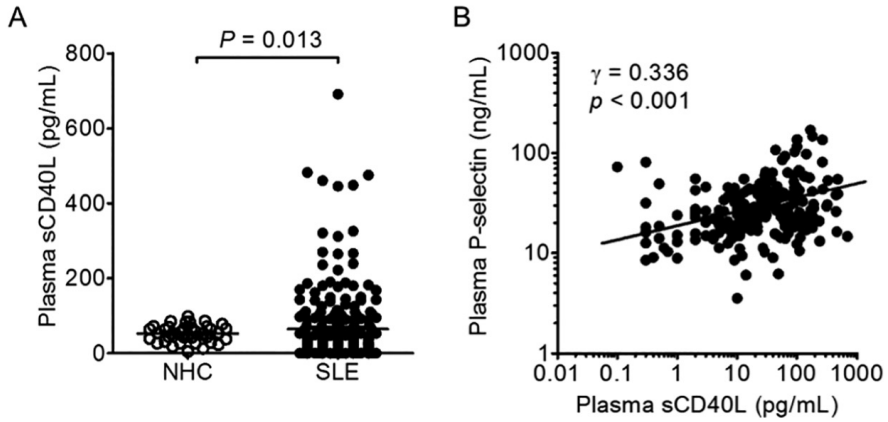

Fig. 2. Plasma sCD40L levels in SLE patients. **A**. Plasma sCD40L levels in SLE patients were significantly elevated compared with healthy controls (54[38–68] vs. 26[10–87] pg/mL, p=0.013). **B**. Plasma sCD40L levels were positively correlated with plasma soluble P-selectin levels (γ=0.336, p<0.001). Logarithmic scale was applied for better visualisation.

Table II. Correlation between plasma sCD40L level and SLE disease activity indices*.

	Crude		Adjusted*	
	γ	*p*-value	γ	*p*-value
SLEDAI	-0.003	0.960	0.053	0.422
C3 (mg/dL)	-0.097	0.134	-0.124	0.059
C4 (mg/dL)	-0.056	0.389	-0.064	0.329
CH50 (U/mL)	-0.042	0.520	-0.067	0.310
Anti-dsDNA titer	-0.044	0.492	-0.034	0.610

*Adjusted for diabetes, hypertension, smoking, use of statin, ACEi/ARB and aspirin, ESR, and platelet count. SLEDAI: Systemic lupus erythematosus disease activity index; ACEi: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker; ESR: erythrocyte sedimentation rate.

justing for factors known to affect the sCD40L levels in previous studies (23, 30, 40-44), including diabetes mellitus, hypertension, smoking, use of statin, ACEi/ARB and aspirin, ESR, platelet count and SLEDAI, increased levels of sCD40L was significantly associated with arterial thrombosis (p=0.029), but not venous thrombosis (p=0.164). In a comparison of sCD40L in patients who had arterial thrombosis regarding to aPL positivity, SLE patients with arterial thrombosis and positive aPL (n=17) had significantly higher sCD40L levels compared to those with arterial thrombosis but negative aPL (n=9) (36 [25–312] *vs*. 7 [6–16] pg/mL, p=0.011). Next, in order to identify factors influencing the occurrence of arterial thrombosis, we performed multivariate logistic regression analysis including traditional and disease-specific risk factors (age, sex, obesity, diabetes mellitus, hypertension, dyslipidaemia, smoking, disease duration, SLEDAI, and aPL positivity). As a result, the presence of hypertension and aPL positivity were associated independently with arterial thrombosis (Table III, models 1 and 2). In logistic regression analysis, sCD40L was independently associated with the occurrence of arterial thrombosis (model 3) (p=0.009), in which aPL was not entered as a variable. However, the significance of this association disappeared when aPL positivity was included in the model 1 due to the collinearity between aPL and sCD40L (Fig. 3A-B). On sub-analyses of each subtype of aPL, LAC and aβ_2GPI were strongly associated with arterial thrombosis (OR 5.677, 95% CI 2.038–15.809, p=0.001 and OR 6.962, 95% CI 1.876–25.835, p=0.001, respectively). However, aCL showed a weak association (OR 2.963, 95% CI 0.872–10.063, p=0.082).

Increased plasma sCD40L in APS patients with arterial thrombosis

Among the two independent variables listed in Table III, SLE patients with positive aPL had significantly higher sCD40L levels than those without aPL (27 [10–138] *vs*. 20 [9–72] pg/mL, p=0.015), while the presence of hypertension was not associated with increased sCD40L levels (data not shown). We examined if there was any difference in the levels of sCD40L in SLE patients according to the presence of each subtype of aPL (see the figure below). When adjusted for potential confounding factors, LAC-positive patients had higher sCD40L levels than LAC-negative ones (20 [9–152] *vs*. 27 [10–75] pg/mL, p=0.040). A significant difference was not found as for aCL and aβ_2GPI (p>0.05). However, when confined to patients with positive aPL (n=58), plasma sCD40L levels were positively correlated with titers of aCL and a_$_2$GPI in (γ=0.321, p=0.014 and γ=0.356, p=0.006, respectively) (Fig. 3A-B). We further investigated the association of sCD40L with respect to APS status. As shown in Fig. 3C, SLE patients with APS exhibited significantly higher sCD40L levels (53 [20–316] pg/mL) than SLE/non-APS patients with positive aPL (14 [8–110] pg/mL) and SLE/non-APS with negative aPL (20 [9–72] pg/mL) (all p<0.05). We did not observe significance between SLE/APS patients with arterial thrombosis and those with only venous thrombosis, due to the small sample size of the latter (n=3), albeit there was no difference in levels of sCD40L between SLE/APS patients with only venous thrombosis and SLE/non-APS patients (p=0.254).

Discussion

Several studies have measured levels of sCD40L in the blood of SLE patients and attempted to determine its clinical significance. However, these yielded conflicting results (26-28), possibly due to different type of specimen used in measurement of sCD40L. Its concentration in serum specimens can be influenced by pre-analytic factors, such as platelet count and storage time at room temperature (9, 30, 33, 34). In fact, platelets are activated during the process of clot retraction, and shedding of sCD40L from the surface of activated platelet during storage results in a progressive rise in serum levels

Table III. Multivariate logistic regression analysis for variables associated with arterial thrombosis in SLE patients.

Variables	Model 1		Model 2		Model 3	
	OR (95% CI)	*p*-value	OR (95% CI)	*p* value	OR (95% CI)	*p*-value
Sex	1.356 (0.278 – 6.613)	0.706	1.294 (0.288 – 5.814)	0.651	1.215 (0.278 – 5.322)	0.796
Age	0.976 (0.924 – 1.032)	0.400	0.965 (0.914 – 1.018)	0.188	0.962 (0.913 – 1.013)	0.143
Disease duration	1.069 (0.972 – 1.175)	0.168	1.068 (0.972 – 1.173)	0.172	1.067 (0.976 – 1.167)	0.152
BMI	1.006 (0.863 – 1.172)	0.940	0.992 (0.853 – 1.153)	0.916	1.039 (0.897 – 1.204)	0.607
Diabetes Mellitus	1.327 (0.122 – 16.845)	0.832	1.348 (0.110 – 17.214)	0.889	1.301 (0.967 – 16.284)	0.803
Hypertension	4.627 (1.443 – 14.831)	0.010	4.889 (1.537 – 15.552)	0.007	4.018 (1.380 – 11.696)	0.011
Dyslipidaemia	2.037 (0.655 – 6.343)	0.219	1.818 (0.600 – 5.507)	0.291	1.258 (0.441 – 3.588)	1.258
Smoking	2.634 (0.354 – 19.614)	0.345	2.468 (0.327 – 18.615)	0.381	2.711 (0.428 – 17.161)	0.290
SLEDAI	0.911 (0.799 – 1.038)	0.160	0.902 (0.792 – 1.027)	0.119	0.937 (0.836 – 1.049)	0.260
aPL	6.892 (2.332 – 20.372)	<0.001	8.006 (2.804 – 22.857)	<0.001	-	-
sCD40L	1.003 (0.999 – 1.007)	0.107	-	-	1.005 (1.001 – 1.008)	0.009

aPL: antiphospholipid antibodies; BMI: body mass index; BMI: body mass index; OR: Odds ratio; SLEDAI: systemic lupus erythematosus disease activity index.

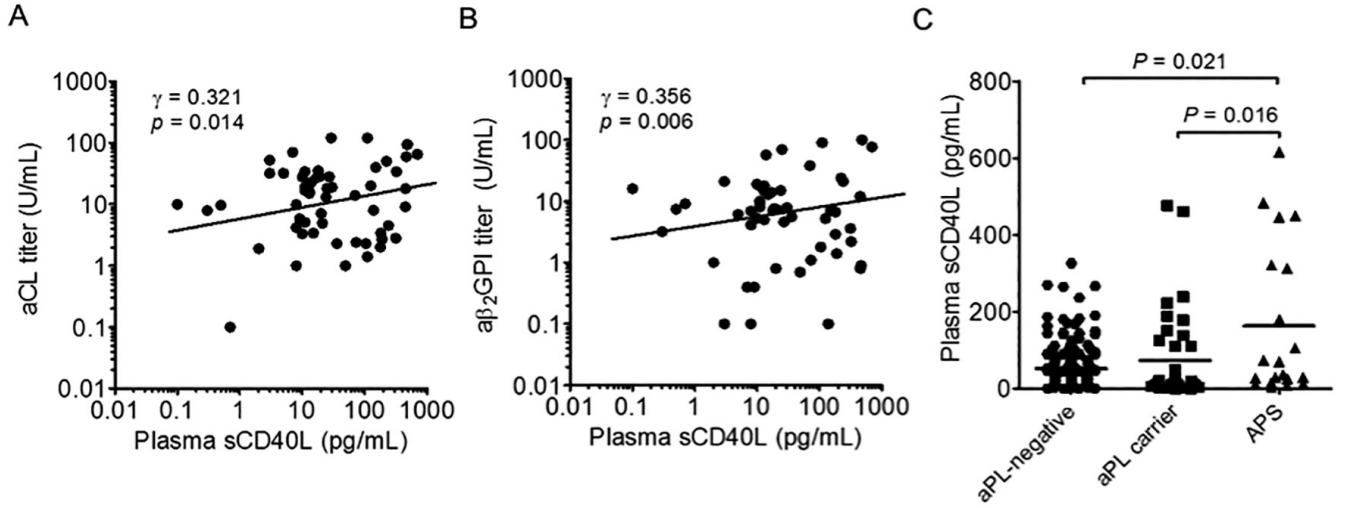

Fig. 3. Plasma sCD40L levels are correlated with antiphospholipid antibodies and increased in SLE patients with antiphospholipid syndrome. **A-B**. Plasma sCD40L levels were positively correlated with titers of aCL and $a\beta_2$GPI in SLE patients with positive aPL (γ=0.321, p=0.014 and γ=0.356, p=0.006, respectively). Logarithmic scale was applied for better visualisation. C. SLE/APS patients had a higher level of plasma sCD40L (53[20–316] pg/mL) than did SLE/non-APS patients with and without aPL (14 [8–110] pg/mL and (20 [9–72] pg/mL).

(33). Therefore, it is generally recommended to use citrated plasma samples for measurement of sCD40L levels (33, 34). Previous studies reported that increased platelet counts were associated with the elevation of plasma sCD40L levels (9, 24, 45). However, elevated plasma sCD40L levels in our SLE patients are not explained by increased platelet count since there was no relationship between plasma sCD40L levels and platelet count.

Estimation of parameters associated with clinical features is often hindered by small number of SLE patients included in the study because clinical features of SLE are fairly diverse and heterogeneous. Thus, we compared plasma sCD40L levels in a relatively large number of SLE patients (n=241) with those in healthy controls, and found that plasma sCD40L levels in SLE patients were significantly higher than in healthy controls, which is consistent with previous data (22, 23). In a report by Kato *et al*. (23), they demonstrated that plasma CD40L levels were correlated with the titers of anti-dsDNA Ab and clinical disease activity (SLEDAI). More recently, Munroe *et al*. showed that levels of plasma sCD40L were significantly higher in preflare SLE patients compared with SLE patients with stable disease (22, 25). These associations were not confirmed in our study (Table II) and, even further, there was no associations found between sCD40L levels and clinical features of SLE such as renal, musculoskeletal, mucocutaneous, cardiopulmonary, neuropsychiatric, and haematologic involvement (data not shown).

There is ample evidence demonstrating that elevated sCD40L levels are associated with increased risk of atherothrombotic events (6-8). In line with these, we found that elevated plasma sCD40L in SLE patients were significantly associated with the occurrence of arterial thrombosis after adjustment for conventional CVD risk factors. However, this association missed out significance when aPL, a strong variable associated with arterial thrombosis (Table II), was entered into the multivariate analysis because of the collinearity between aPL and sCD40L, as shown in Fig. 3A-B. These findings suggest that atherothrombotic events in SLE patients with APS are partly

attributed to the action of sCD40L released from activated platelets by aPL, as supported by a positive correlation of plasma sCD40L with soluble P-selectin, a platelet activation marker, and titers of aCL or aβ_2GPI. Nonetheless, considering that sCD40L levels were significantly lower in SLE/non-APS patients with positive aPL than in SLE patients with APS, it seems likely that aPL are necessary but not sufficient to fully activate platelet as envisaged in the "two-hit hypothesis" (46), in which vascular thrombosis does not occur unless an additional trigger is present. It appears that hypertension, found to be an independent risk factor for arterial thrombosis (Table III), acts as a trigger factor through the induction of oxidative stress (47), and/or other risk factors may stimulate platelets to release sCD40L. The latter notion was supported by a close relationship between increased sCD40L levels and platelet activation in patients with hypercholesterolaemia (44) or smoking (43).

High levels of sCD40L in plasma are not merely a marker but also shown to be a mediator of atherosclerosis. Indeed, *in vitro* treatment of monocyte with sCD40L induces the expression of tissue factor and thrombin generation (16). In porcine coronary arteries, sCD40L is shown to significantly decrease endothelium-dependent vasorelaxation and eNOS mRNA levels, while it increases superoxide anion level (48). In addition to the activation of small GTPase Rac1 and p38 MAP kinase by binding to platelet CD40 (49), sCD40L also functions as a ligand for glycoprotein IIb/IIIa receptor and induces the activation of receptor function (*e.g.* fibrinogen binding, platelet aggregation) (50). Along with above the data, *in vivo* thrombogenic effect of sCD40L has been further demonstrated in animal models. Infusion of sCD40L into wild type mice significantly exacerbated thrombus formation compared with either CD40-/- mice or vehicle-treated wild type mice (49). Moreover, treatment of angiogenic early outgrowth cells (EOCs) with sCD40L attenuated luminal incorporation of EOCs and accelerated neointimal progression (51).

There are some limitations to be addressed in this study. First, retrospective data collection is inherently susceptible to bias, including both misclassification and information bias. Second, aCL IgM and aβ_2GPI IgM tests were not included because those tests were not available in our institution at the time of study subject enrolment. However, the significance of IgM antibody test in the diagnosis of APS has remained controversial (52).

In conclusion, we found that levels of plasma sCD40L in SLE patients were increased and were considered to reflect *in vivo* activation of platelets, as evidenced by a positive correlation with soluble P-selectin. As reported previously (6-11), elevated sCD40L levels were significantly associated with the occurrence of atherothrombotic events in SLE patients as well. Furthermore, sCD40L levels were correlated with aPL and significantly increased in SLE/APS patients with arterial thrombosis compared with in SLE/APS with only venous thrombosis and in non-APS/SLE patients irrespective of aPL positivity. Future research is required to elucidate the effect of aPL and its underlying mechanism(s) on platelet activation and sCD40L release. Also, large-scale prospective cohort studies are required to better clarify the tripartite association among aPL, sCD40L, and accelerated cardiovascular disease in SLE patients.

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References

1. ADINOLFI A, VALENTINI E, CALABRESI E *et al*.: One year in review 2016: systemic lupus erythematosus. *Clin Exp Rheumatol* 2016; 34: 569-74.
2. HANSSON GK, HERMANSSON A: The immune system in atherosclerosis. *Nat Immunol* 2011; 12: 204-12.
3. DENNY MF, THACKER S, MEHTA H *et al*.: Interferon-alpha promotes abnormal vasculogenesis in lupus: a potential pathway for premature atherosclerosis. *Blood* 2007; 110: 2907-15.
4. MCMAHON M, GROSSMAN J, SKAGGS B *et al*.: Dysfunctional proinflammatory high-density lipoproteins confer increased risk of atherosclerosis in women with systemic lupus erythematosus. *Arthritis Rheum* 2009; 60: 2428-37.
5. KIM JY, PARK YJ, KIM KJ, CHOI JJ, KIM WU, CHO CS: Osteoprotegerin causes apoptosis of endothelial progenitor cells by induction of oxidative stress. *Arthritis Rheum* 2013; 65: 2172-82.
6. KATAKAMI N, KANETO H, MATSUHISA M *et al*.: Association of soluble CD40 ligand with carotid atherosclerosis in Japanese type 1 diabetic patients. *Diabetologia* 2006; 49: 1670-6.
7. HEESCHEN C, DIMMELER S, HAMM CW *et al*.: Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med* 2003; 348: 1104-11.
8. VARO N, DE LEMOS JA, LIBBY P *et al*.: Soluble CD40L: risk prediction after acute coronary syndromes. *Circulation* 2003; 108: 1049-52.
9. BALLA J, MAGYAR MT, BERECZKI D *et al*.: Serum levels of platelet released CD40 ligand are increased in early onset occlusive carotid artery disease. *Dis Markers* 2006; 22: 133-40.
10. BLAKE GJ, OSTFELD RJ, YUCEL EK *et al*.: Soluble CD40 ligand levels indicate lipid accumulation in carotid atheroma: an *in vivo* study with high-resolution MRI. *Arterioscler Thromb Vasc Biol* 2003; 23: e11-4.
11. GARLICHS CD, KOZINA S, FATEH-MOGHADAM S *et al*.: Upregulation of CD40-CD40 ligand (CD154) in patients with acute cerebral ischemia. *Stroke* 2003; 34: 1412-8.
12. ANTONIADES C, BAKOGIANNIS C, TOUSOULIS D, ANTONOPOULOS AS, STEFANADIS C: The CD40/CD40 ligand system: linking inflammation with atherothrombosis. *J Am Coll Cardiol* 2009; 54: 669-77.
13. ELGUETA R, BENSON MJ, DE VRIES VC, WASIUK A, GUO Y, NOELLE RJ: Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* 2009; 229: 152-72.
14. VOGEL JD, WEST GA, STURM A, LEVINE AD, FIOCCHI C: Essential role of the CD40 pathway in T-cell-mediated induction of chemokines and cell adhesion molecules by human intestinal fibroblasts (HIF) and microvascular endothelial cells (HIMEC). *Gastroenterology* 2001; 120: A192.
15. CHO CS, CHO ML, MIN SY *et al*.: CD40 engagement on synovial fibroblast up-regulates production of vascular endothelial growth factor. *J Immunol* 2000; 164: 5055-61.
16. SANGUIGNI V, FERRO D, PIGNATELLI P *et al*.: CD40 ligand enhances monocyte tissue factor expression and thrombin generation via oxidative stress in patients with hypercholesterolemia. *J Am Coll Cardiol* 2005; 45: 35-42.
17. SLUPSKY JR, KALBAS M, WILLUWEIT A, HENN V, KROCZEK RA, MULLER-BERGHAUS G: Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. *Thromb Haemost* 1998; 80: 1008-14.
18. ANDRE P, PRASAD KS, DENIS CV *et al*.: CD40L stabilizes arterial thrombi by a beta3 integrin--dependent mechanism. *Nat Med* 2002; 8: 247-52.

19. PIETRAVALLE F, LECOANET-HENCHOZ S, BLASEY H *et al*.: Human native soluble CD40L is a biologically active trimer, processed inside microsomes. *J Biol Chem* 1996; 271: 5965-7.
20. ALLANORE Y, BORDERIE D, MEUNE C *et al*.: Increased plasma soluble CD40 ligand concentrations in systemic sclerosis and association with pulmonary arterial hypertension and digital ulcers. *Ann Rheum Dis* 2005; 64: 481-3.
21. TAMURA N, KOBAYASHI S, KATO K *et al*.: Soluble CD154 in rheumatoid arthritis: elevated plasma levels in cases with vasculitis. *J Rheumatol* 2001; 28: 2583-90.
22. FERRO D, PIGNATELLI P, LOFFREDO L *et al*.: Soluble CD154 plasma levels in patients with systemic lupus erythematosus: modulation by antiphospholipid antibodies. *Arthritis Rheum* 2004; 50: 1693-4.
23. KATO K, SANTANA-SAHAGUN E, RASSENTI LZ *et al*.: The soluble CD40 ligand sCD154 in systemic lupus erythematosus. *J Clin Invest* 1999; 104: 947-55.
24. DANESE S, KATZ JA, SAIBENI S *et al*.: Activated platelets are the source of elevated levels of soluble CD40 ligand in the circulation of inflammatory bowel disease patients. *Gut* 2003; 52: 1435-41.
25. MUNROE ME, VISTA ES, GUTHRIDGE JM, THOMPSON LF, MERRILL JT, JAMES JA: Pro-inflammatory adaptive cytokine and shed tumor necrosis factor receptor levels are elevated preceding systemic lupus erythematosus disease flare. *Arthritis Rheumatol* 2014; 66: 1888-99.
26. CIFERSKA H, HORAK P, HERMANOVA Z *et al*.: The levels of sCD30 and of sCD40L in a group of patients with systemic lupus erythematodes and their diagnostic value. *Clin Rheumatol* 2007; 26: 723-8.
27. GOULES A, TZIOUFAS AG, MANOUSAKIS MN, KIROU KA, CROW MK, ROUTSIAS JG: Elevated levels of soluble CD40 ligand (sCD40L) in serum of patients with systemic autoimmune diseases. *J Autoimmun* 2006; 26: 165-71.
28. URQUIZU-PADILLA M, BALADA E, CORTES F, PEREZ EH, VILARDELL-TARRES M, ORDIROS J: Serum levels of soluble CD40 ligand at flare and at remission in patients with systemic lupus erythematosus. *J Rheumatol* 2009; 36: 953-60.
29. HOCHBERG MC: Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
30. VARO N, NUZZO R, NATAL C, LIBBY P, SCHONBECK U: Influence of pre-analytical and analytical factors on soluble CD40L measurements. *Clin Sci* (Lond) 2006; 111: 341-7.
31. BOMBARDIER C, GLADMAN DD, UROWITZ MB, CARON D, CHANG CH: Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum* 1992; 35: 630-40.
32. MIYAKIS S, LOCKSHIN MD, ATSUMI T *et al*.: International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295-306.
33. HALLDORSDOTTIR AM, STOKER J, PORCHE-SORBET R, EBY CS: Soluble CD40 ligand measurement inaccuracies attributable to specimen type, processing time, and ELISA method. *Clin Chem* 2005; 51: 1054-7.
34. WEBER M, RABENAU B, STANISCH M *et al*.: Influence of sample type and storage conditions on soluble CD40 ligand assessment. *Clin Chem* 2006; 52: 888-91.
35. FERRONI P, MARTINI F, RIONDINO S *et al*.: Soluble P-selectin as a marker of *in vivo* platelet activation. *Clin Chim Acta* 2009; 399: 88-91.
36. RIONDINO S, MARTINI F, LA FARINA F, SPILA A, GUADAGNI F, FERRONI P: Increased plasma levels of soluble CD40 ligand correlate with platelet activation markers and underline the need for standardized pre-analytical conditions. *Clin Biochem* 2010; 43: 666-70.
37. ANDRE P, NANNIZZI-ALAIMO L, PRASAD SK, PHILLIPS DR: Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. *Circulation* 2002; 106: 896-9.
38. ANTONIADES C, TOUSOULIS D, VASILIADOU C, STEFANADI E, MARINOU K, STEFANADIS C: Genetic polymorphisms of platelet glycoprotein Ia and the risk for premature myocardial infarction: effects on the release of sCD40L during the acute phase of premature myocardial infarction. *J Am Coll Cardiol* 2006; 47: 1959-66.
39. MALARSTIG A, LINDAHL B, WALLENTIN L, SIEGBAHN A: Soluble CD40L levels are regulated by the -3459 A>G polymorphism and predict myocardial infarction and the efficacy of antithrombotic treatment in non-ST elevation acute coronary syndrome. *Arterioscler Thromb Vasc Biol* 2006; 26: 1667-73.
40. NANNIZZI-ALAIMO L, ALVES VL, PHILLIPS DR: Inhibitory effects of glycoprotein IIb/IIIa antagonists and aspirin on the release of soluble CD40 ligand during platelet stimulation. *Circulation* 2003; 107: 1123-8.
41. BRILI S, TOUSOULIS D, ANTONIADES C *et al*.: Effects of ramipril on endothelial function and the expression of proinflammatory cytokines and adhesion molecules in young normotensive subjects with successfully repaired coarctation of aorta: a randomized cross-over study. *J Am Coll Cardiol* 2008; 51: 742-9.
42. VARO N, VICENT D, LIBBY P *et al*.: Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients: a novel target of thiazolidinediones. *Circulation* 2003; 107: 2664-9.
43. HARDING SA, SARMA J, JOSEPHS DH *et al*.: Upregulation of the CD40/CD40 ligand dyad and platelet-monocyte aggregation in cigarette smokers. *Circulation* 2004; 109: 1926-9.
44. CIPOLLONE F, MEZZETTI A, PORRECA E *et al*.: Association between enhanced soluble CD40L and prothrombotic state in hypercholesterolemia: effects of statin therapy. *Circulation* 2002; 106: 399-402.
45. VIALLARD JF, SOLANILLA A, GAUTHIER B *et al*.: Increased soluble and platelet-associated CD40 ligand in essential thrombocythemia and reactive thrombocytosis. *Blood* 2002; 99: 2612-4.
46. MERONI PL, BORGHI MO, RASCHI E *et al*.: Inflammatory response and the endothelium. *Thromb Res* 2004; 114: 329-34.
47. RODRIGO R, GONZALEZ J, PAOLETTO F: The role of oxidative stress in the pathophysiology of hypertension. *Hypertens Res* 2011; 34: 431-40.
48. CHEN C, CHAI H, WANG X *et al*.: Soluble CD40 ligand induces endothelial dysfunction in human and porcine coronary artery endothelial cells. *Blood* 2008; 112: 3205-16.
49. YACOUB D, HACHEM A, THEORET JF, GILLIS MA, MOURAD W, MERHI Y: Enhanced levels of soluble CD40 ligand exacerbate platelet aggregation and thrombus formation through a CD40-dependent tumor necrosis factor receptor-associated factor-2/Rac1/p38 mitogen-activated protein kinase signaling pathway. *Arterioscler Thromb Vasc Biol* 2010; 30: 2424-33.
50. PRASAD KS, ANDRE P, HE M, BAO M, MANGANELLO J, PHILLIPS DR: Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. *Proc Natl Acad Sci U S A* 2003; 100: 12367-71.
51. HRISTOV M, GUMBEL D, LUTGENS E, ZERNECKE A, WEBER C: Soluble CD40 ligand impairs the function of peripheral blood angiogenic outgrowth cells and increases neointimal formation after arterial injury. *Circulation* 2010; 121: 315-24.
52. KEELING D, MACKIE I, MOORE GW, GREER IA, GREAVES M: Guidelines on the investigation and management of antiphospholipid syndrome. *Br J Haematol* 2012; 157: 47-58.