High plasma C5a and C5b-9 levels during quiescent phases are associated to severe antiphospholipid syndrome subsets

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Abstract

Objective

High plasma C5a and C5b-9 levels are considered a clear sign of complement activation. We aimed to evaluate the clinical significance of these two complement activation products during quiescent phases of thrombotic antiphospholipid syndrome (APS) by comparing their plasma levels in the different clinical subsets and relating them to the clinical characteristics and antiphospholipid antibody profile of the patients.

Methods

The three patient subsets studied were: i) thrombotic patients responsive to anti-vitamin K therapy (TAPS); ii) patients with refractory to vitamin K antagonists recurrent thrombosis (RAPS); iii) patients diagnosed with catastrophic APS (CAPS). Plasma C5a and C5b-9 levels were assessed using commercial ELISA assays.

Results

Sixty-two quiescent APS patients were recruited: 40 were affected by TAPS, 13 by RAPS and 9 by CAPS. Data analysis showed that the TAPS patients had significantly lower levels of both complement activation products with respect to the RAPS and CAPS patients. In addition, C5a and/or C5b-9 significantly prevailed in the patients with small-vessel thrombosis, just as C5b-9 did in the triple antiphospholipid antibody positive patients. The ROC curve showed that the best cut-offs for C5a and C5b-9 levels had a higher sensitivity, specificity and likelihood ratio in the CAPS and RAPS groups than they did in the TAPS subset.

Conclusion

These results suggest that the persistence of high plasma C5b-9 and C5a levels during quiescent phases identifies APS patients with more severe disease who may develop rethrombosis and benefit from complement inhibition treatment during an acute disease phase.

Key words

antiphospholipid syndrome, complement activation, complement membrane attack complex, complement system proteins, thrombosis

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Introduction

The findings of several animal and human studies support the hypothesis that complement plays a role in antiphospholipid (aPL) antibody mediated thrombosis (1). The presence in the plasma and/or on cell membranes of complement activation products such as anaphylatoxin C5a and terminal complement complex C5b-9 is considered a clear sign of complement activation and consumption (2).

Generated from C5 cleavage, C5a is a potent bioactive molecule that can act on a wide variety of cell types. Although elevated circulatory C5a levels have been identified in thrombotic APS (3, 4), one study failed to uncover higher plasma levels in patients with primary APS (5). The final step of complement activation is the assembly of the C5b-9 complex and its insertion into the cell membrane, which causes cell death. According to some studies, terminal C5b-9 complex is increased in plasma (3, 4, 6-8) and deposited on the walls of the occluded vessels of thrombotic APS patients (4, 8).

Our research group recently uncovered for the first time significantly high plasma C5a and C5b-9 levels during quiescent phases in catastrophic APS (CAPS) patients long before the onset of an acute phase and long after disease remission (9). The finding led us to speculate that high plasma C5a and C5b-9 levels could identify quiescent APS patients at risk of a CAPS episode who could benefit from complement inhibitor therapy (9). Following in the footsteps of our previous study, we set out to investigate the clinical significance of C5a and C5b-9 by analysing plasma levels during quiescent phases in three subsets of thrombotic APS and their correlation with the patients' clinical characteristics and aPL antibody profiles.

Materials and methods

Study and control populations

Primary APS patients with arterial, venous or small-vessel thrombosis diagnosed in accordance with the Sydney clinical criteria for APS classification constituted our study population (10). At least two consecutive positive aPL antibody results obtained more than 12 weeks apart were required to meet the Sydney laboratory criteria for APS (10). The patients with APS associated with systemic autoimmune diseases or with other diseases affecting the complement system were excluded from the study. The common treatment for all patients was antithrombotic therapy which included anti-vitamin K and/or antiplatelet therapy. The three clinical APS subsets studied were constituted by: i) thrombotic patients responsive to anti-vitamin K therapy, generally affected with a single thrombotic event (TAPS); ii) patients with rethrombose despite seemingly adequate treatment with a vitamin K antagonist, *i.e.* patients refractory to standard anticoagulant therapy (RAPS) (11). No patients with RAPS received concurrent medications affecting anticoagulation; iii) patients with definite or probable CAPS diagnosed in accordance with Asherson *et al.'s* criteria (12). One plasma sample from each eligible APS patient attending the outpatient clinic of the Rheumatology Unit of the University Hospital of Padua was collected from January 2009 to January 2021 during a quiescent disease phase at least one year after APS diagnosis. Some of the patients belonging to the RAPS or CAPS subsets developed further thrombotic events after diagnosis. In order to compare the C5a and C5b-9 levels before and after a thrombotic event, a plasma sample was collected from these patients at least one year before or after a recurrent thrombosis and in any case during a quiescent disease phase. In addition, to evaluate C5a and C5b-9 levels over time, two or three plasma samples were collected from ten patients with quiescent APS during the trial period.

The control population was constituted by: i) TAPS, RAPS and CAPS patients tested once during the active disease phase, *i.e.* when the thrombosis was ongoing; ii) healthy subjects matched for sex and age with the quiescent APS patients. All the samples were stored at -80° C until they could be analysed. Data of the following baseline clinical and laboratory parameters were recorded in all patients included in the three clinical APS subsets studied: extra-criteria manifestation (superficial vein

Table I. Demographic, clinical and laboratory characteristics of the study and control populations.

Study and control populations	n.	Age (years) Median (IQR)	Gender F	Gender M	Thrombosis type* number (%)	Triple aPL+ number (%)
Quiescent APS groups						
Thrombotic APS	40	44 (15.7)	30	10	venous 22 (55.0) arterial 20 (50.0) small-vessel 4 (10.0)	25 (62.5)
Refractory APS	13	55 (22.5)	9	4	venous 7 (53.8) arterial 11 (84.6) small-vessel 10 (76.9)	9 (69.2)
Catastrophic APS	9	39 (13.5)	8	1	venous 1 (11.1) arterial 1 (11.1) small-vessel 9 (100)	9 (100)
APS patients with rethrombosis[$]	13	53 (20.5)	9	4	venous 3 (23.1) arterial 4 (30.8) small-vessel 7 (53.8)	10 (76.9)
APS patients followed over time	10	41 (20.0)	8	2	venous 3 (30.0) arterial 5 (50.0) small-vessel 4 (40.0)	10 (100)
Control groups						
APS patients during active phase of disease	13	42 (29.0)	7	6	venous 2 (15.4) arterial 6 (46.1) small-vessel 10 (76.9)	12 (92.3)
Healthy subjects	30	45 (20.2)	25	5	-	-

IQR: interquartile range; F: Females; M: Males; aPL+: antiphospholipid antibody positivity; APS: antiphospholipid syndrome.
*the association of more types of thrombosis was detected in 31 patients.
[$]patients who developed further thrombotic events after the diagnosis.

thrombosis, thrombocytopenia, renal microangiopathy, heart valve disease, livedo reticularis, migraine, chorea, seizures and myelitis) according to Abreu *et al*. (13), cardiovascular disease risk factors (cigarette smoking, body mass index, hypertension, diabetes mellitus), C3/C4 complement fractions, markers of inflammation (erythrocyte sedimentation rate and high-sensitivity C-reactive protein), drugs potentially affecting the complement system (prednisone at an average dosage of 7.5 mg/day, no. 7, hydroxychloroquine at a dosage of 400 mg alternating at 200 mg/day, no. 7, prednisone plus hydroxychloroquine, no. 6, hydroxychloroquine plus methotrexate 15 mg/week, no. 1 and azathioprine 100 mg/day, no. 1, medium and high levels of aPL, IgG and IgM isotypes of both anticardiolipin and anti-β2 Glycoprotein I antibodies and lupus anticoagulant. The study was approved by the Ethics Committee for Clinical Trials of the Provinces of Verona and Rovigo (Italy) (CE: 399CESC) and was carried out in accordance with the 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards. Written informed consent was obtained from all the participants.

C5a and C5b-9 detection

Plasma C5a and C5b-9 levels were assessed by ELISA assays using the MicroVue C5a Plus EIA (QUIDEL®, San Diego, CA, USA) and the MicroVue C5b-9 Plus EIA (QUIDEL®), respectively. In accordance with the manufacturer's suggestions, plasma samples were assayed in duplicate for C5a and C5b-9 detection using 1:20 and 1:10 dilutions, respectively. The optical density was measured using a TECAN Sunrise III microplate reader at 450 nm (Tecan, Männedorf, Switzerland). Expressed as ng/ml, the concentrations were calculated based on the standard curves generated following the specific instructions provided by the manufacturer. The ELISA kits utilised were all from a single production lot to minimise potential biases, confounders, and sources of variability. The intra-and inter-assay coefficients of variation were <10% for both tests.

Antiphospholipid antibody detection

Anticardiolipin and anti-β2Glycoprotein I antibodies were determined by ELISA assays using a home-made method described elsewhere (14). Anticardiolipin antibody values were expressed in IgG phospholipid (GPL) and IgM phospholipid (MPL) units, respectively. The results of anti-β2Glycoprotein I antibodies were expressed in arbitrary units. The cut-off values for the medium-high levels of anticardiolipin and anti-β2Glycoprotein I antibodies were calculated as >the 99th percentile. Lupus anticoagulant was assessed using a three-step procedure carried out utilising platelet-poor plasma samples following updated guidelines and utilising diluted Russell Viper Venom and diluted Activated Partial Thromboplastin Times as screening tests (15).

Statistical analysis

The data were summarised as counts (percent), median and interquartile range (IQR). Spearman's rank coefficient was used to evaluate the correlation between the C5a and C5b-9 lev-

Table II. Comparisons of mean C5a and C5b-9 levels between positive and negative patients for the different baseline clinical and laboratory parameters.

Clinical and laboratory parameters	Patients n.	C5a levels Median (IQR) ng/ml	Significance *p*	C5b-9 levels Median (IQR) ng/ml	Significance *p*
Men *vs*.. women	15 *vs*. 47	14.9 (11.5) *vs*. 14.2 (8.1)	0.5376	459.8 (255.1) *vs*. 448.5 (376.7)	0.8180
Extra-criteria manifestations (presence *vs*. absence)[$]	16 *vs*. 46	15.1(4.3) *vs*. 15.0 (17.3)	0.3385	460.2 (407.8) *v*. 427.1 (481.8)	0.7783
Cardiovascular disease risk factors (presence *vs*. absence) [§]	43 *vs*. 19	14.2 (8.3) *vs*. 14.9 (7.4)	0.8307	448.1 (458.9) *vs*. 471.1 (441.6)	0.3136
Drugs potentially affecting the complement system (yes *vs*. no) [¥]	22 *vs*. 40	12.6 (7.3) *vs*. 14.9 (9.7)	0.2667	495.6 (384.8) *vs*. 386.1 (521.1)	0.1009
C3/C4 complement fractions (positive *vs*. negative)	21 *vs*. 41	14.5 (8.5) *vs*. 14.2 (8.4)	0.7832	522.5 (586.6) *vs*. 405.6 (431.5)	0.1450
Inflammation markers (ESR/hsCRP) (positive *vs*. negative)	7 *vs*. 55	15.3 (33.4) *vs*. 14.2 (8.1)	0.9823	448.5 (985.1) *vs*. 449.3 (443.3)	0.9823
Antiphospholipid medium levels (>99%) *vs*. high levels (≥80 U)	35 *vs*. 27	10.2 (8.7) *vs*. 15.6 (26.6)	0.0074*	306.7 (290.1) *vs*. 710.7 (977.2)	<0.0001*
IgG anticardiolipin positive *vs*. IgG anticardiolipin negative	52 *vs*. 10	14.7 (9.5) *vs*. 9.6 (8.7)	0.1595	470.0 (498.5) *vs*. 245.6 (350.7)	0.0076*
IgM anticardiolipin positive *vs*. IgM anticardiolipin negative	34 *vs*. 28	12,5 (9.2) *vs*. 15.2 (8.1)	0.1511	464.3 (611.1) *vs*. 448.3 (482.5)	0.4663
IgG anti-β2Glycoprotein I positive *vs*. IgG anti-β2Glycoprotein I negative	53 *vs*. 9	14.5 (9.3) *vs*. 10.7 (8.4)	0.4476	468.8 (474.7) *vs*. 207.2 (298.1)	0.0266*
IgM anti-β2Glycoprotein I positive *vs*. IgM anti-β2Glycoprotein I negative	32 *vs*. 30	13.8 (9.2) *vs*. 15.1 (7.7)	0.2151	464.5 (451.6) *vs*. 448.3 (490.4)	0.6170
Lupus anticoagulant positive *vs*. Lupus anticoagulant negative	47 *vs*. 15	14.7 (9.9) *vs*. 10.6 (8.3)	0.1480	468.8 (464.7) *vs*. 323.1 (292.4)	0.1624

IQR: interquartile range; ESR: erythrocyte sedimentation rate; hsCRP; high-sensitivity C-reactive protein.
*Statistically significant value ($p<0.05$).
[$]Extra-criteria manifestations included: superficial vein thrombosis, thrombocytopenia, renal microangiopathy, heart valve disease, livedo reticularis, migraine, chorea, seizures and myelitis.
[§]Cardiovascular disease risk factors included: cigarette smoking, body mass index, hypertension and diabetes mellitus.
[¥]Drugs potentially affecting the complement system included: prednisone, hydroxychloroquine, methotrexate and azathioprine.

els. A non-parametric Mann-Whitney test was used to compare of mean C5a and C5b-9 levels between positive and negative patients for the different baseline clinical and laboratory parameters and between the different study groups. Fisher's exact test was used to compare the prevalence of small-vessel thrombosis in the different quiescent APS subsets. A receiver operating characteristic (ROC) curve analysis was performed to evaluate the ability of C5a and C5b-9 to discriminate between the different quiescent APS subsets and the healthy controls and between the less severe (TAPS) and more severe APS subsets (RAPS+CAPS). The positive and negative predictive values of the cut-offs were then calculated. A $p<0.05$ value was considered statistically significant. Statistical analyses were performed using GraphPad Prism statistical software (San Diego, CA, USA).

Results

The study population was constituted by sixty-two quiescent APS patients: 40 were affected by TAPS, 13 by RAPS and 9 by CAPS (as far as the last subset was concerned, the diagnosis was definite in four cases and probable in five). A plasma sample was taken from all quiescent APS patients at different times after APS diagnosis (median=7 years, IQR=11). In 8 patients with RAPS (61.5%) and 5 (55.5%) with CAPS the following thrombotic events were registered after diagnosis (rethrombosis): 2 relapsing CAPS with small-vessel involvement, 3 arterial, 3 venous, 4 small-vessel and 1 arterial + small-vessel thrombosis. In 7 of these patients a plasma sample was assayed before (median=3 years, IQR=4) and in 6 after (median=4 years, IQR=8.25) the recurrent thrombotic event. The trend of C5a and C5b-9 levels over time in the quiescent APS was traced based on the results of 22 plasma samples collected 8 from 4 TAPS patients, 8 from 3 RAPS patients and 6 from 3 CAPS patients. The samples were collected at irregular intervals during the trial period (median=8.5 years; IQR=7.75) during which the disease was always in a quiescent phase.

The control population was constituted by 30 healthy blood donors and by 13 subjects with APS in an active phase. Two of the latter were affected with TAPS, 7 with RAPS and 4 with CAPS, and they were tested once during a thrombotic episode. The demographic, clinical and laboratory characteristics of the quiescent APS patients and the control population are outlined in Table I.

Comparison of mean C5a and C5b-9 levels between the different study groups

Spearman's rank correlation coefficient uncovered a significant correlation between C5a and C5b-9 levels: r=0.7313, 95% CI=0.635-0.805, $p<0.0001$. The comparisons of mean C5a and C5b-9 levels between positive and negative patients for the different baseline clinical and laboratory parameters are reported in Table II. While, the comparisons of the mean C5a and C5b-9 levels in the different study groups are outlined in Table III Data of this analysis showed that both the C5a and C5b-9 values in the healthy controls were

Table III. Comparison of mean C5a and C5b-9 levels between the different study groups.

Study groups	Patients n.	C5a levels Median (IQR) ng/ml	Significance *p*	C5b-9 levels Median (IQR) ng/ml	Significance *p*
Healthy controls *vs*. Quiescent APS	30 *vs*. 62	4.1 (4.7) *vs*. 14.3 (8.3)	<0.0001*	165.9 (114.5) *vs*. 448.9 (463.6)	<0.0001*
Active APS *vs*. Quiescent APS	13 *vs*. 62	41.2 (67.3) *vs*. 14.3 (8.3)	0.0055*	1691.0 (1788.3) *vs*. 448.9 (463.6)	0.0027*
Thrombotic APS *vs*. Refractory APS	40 *vs*. 13	11.6 (9.3) *vs*. 15.6 (51.8)	0.0249*	309.6 (291.7) *vs*. 814.6 (2808.4)	0.0002*
Thrombotic APS *vs*. Catastrophic APS	40 *vs*. 9	11.6 (9.3) *vs*. 15.2 (75.1)	0.0332*	309.6 (291.7) *vs*. 734.0 (4402.6)	0.0002*
Refractory APS *vs*. Catastrophic APS	13 *vs*. 9	15.6 (51.8) *vs*. 15.2 (75.1)	0.5478	814.6 (2808.4) *vs*. 734.0 (4402.6)	0.3498
Venous thrombosis *vs*. Arterial thrombosis	30 *vs*. 32	12.5 (8.0) *vs*. 14.8 (8.6)	0.5261	432.4 (370.7) *vs*. 432.4 (514.8)	0.8162
Venous thrombosis *vs*. Small-vessel thrombosis	30 *vs*. 23	12.5 (8.0) *vs*. 15.3 (39.7)	0.0166*	432.4 (370.7) *vs*. 623.5 (2749.7)	0.0037*
Arterial thrombosis *vs*. Small-vessel thrombosis	32 *vs*. 23	14.8 (8.6) *vs*. 15.3 (39.7)	0.0832	432.4 (514.8) *vs*. 623.5 (2749.7)	0.0088*
Triple aPL antibodies + *vs*. Triple- aPL antibodies -	43 *vs*. 19	14.7 (9.9) *vs*. 10.6 (8.6)	0.1791	522.5 (502.1) *vs*. 306.7 (290.4)	0.0248*
Samples before *vs*. Samples after rethrombosis	7 *vs*. 6	15.3 (62.2) *vs*. 14.9 (52.7)	0.9452	522.5 (5030.2) *vs*. 1141.0 (3581.3)	0.2949
Samples at beginning *vs*. Samples during follow-up	10 *vs*. 12	15.4 (60.2) *vs*. 25.0 (39.9)	0.8175	573.0 (3054.1) *vs*. 604.0 (1035.0)	0.6682

IQR: interquartile range; APS: antiphospholipid syndrome; aPL: antiphospholipid.
*p<0.05: statistically significant.

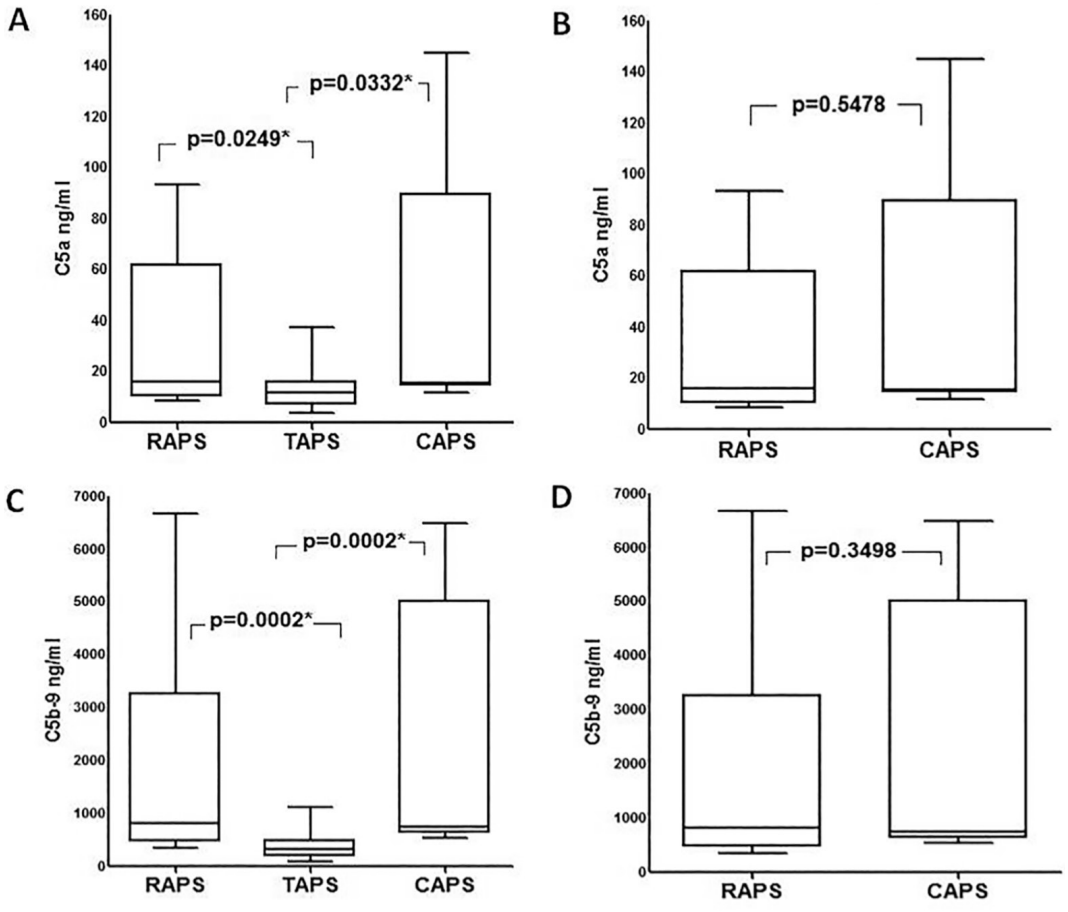

Fig. 1. Comparison of C5a and C5b-9 levels in the different subsets of quiescent APS.
A: Results of the comparison of plasma C5a levels between refractory antiphospholipid syndrome (RAPS), thrombotic antiphospholipid syndrome (TAPS) and catastrophic antiphospholipid syndrome (CAPS) patients and **B:** between RAPS and CAPS patients.
C: Results of the comparison of plasma C5b-9 levels between RAPS, TAPS and CAPS patients and **D:** between RAPS and CAPS patients.
*p<0.05: statistically significant.

significantly lower than those in the quiescent APS patients. They were instead significantly higher in the active APS patients with respect to the quiescent ones. Data analysis also uncovered that the TAPS patients had significantly lower C5a and C5b-9 levels with respect to the RAPS and CAPS patients (Fig. 1A and C); the differences in the two complement activation products were not significant between RAPS and CAPS patient groups (Fig. 1B and D). When the type of thrombosis was related to the levels of C5a and C5b-9 there were no significantly different levels of C5a and C5b-9 between venous and arterial involvement, while the levels of C5a significantly prevailed in small-vessel thrombosis with respect to the venous one (Fig. 2A) and those of C5b-9 in small-vessel thrombosis with respect to both the venous and arterial one (Fig. 2B). Small-vessel thrombosis is a severe vascular involvement associated with CAPS and, to a lesser extent, with large-vessel thrombotic APS; it is often refractory to conventional antithrombotic therapy (16-18). Both the RAPS and CAPS patients had significantly higher frequencies of small-vessel thrombosis with respect to that in the TAPS patients (p<0.0001 for both); but there was no significant difference

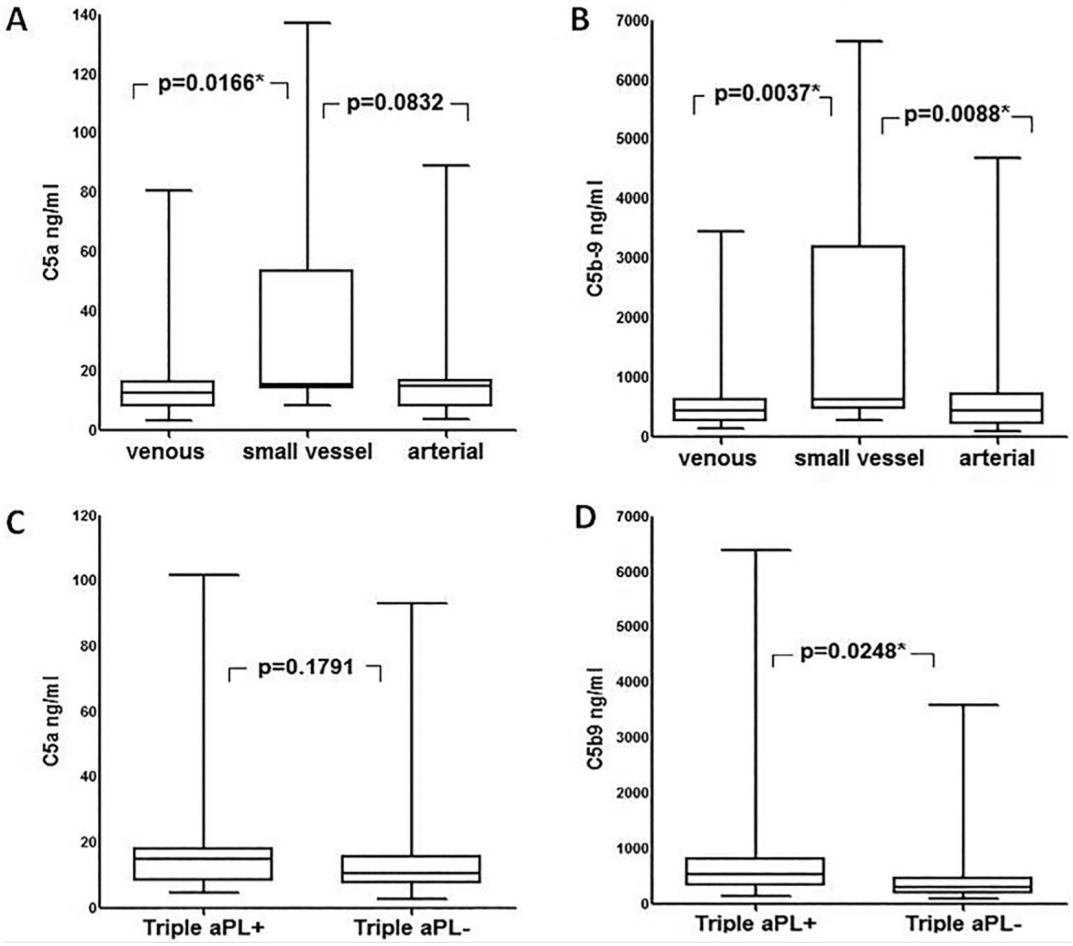

Fig. 2. Relationship between C5a and C5b-9 levels and the type of thrombosis and triple antiphospholipid antibody profile.
A: Results of comparison of plasma C5a levels between venous, small-vessel and arterial thrombosis.
B: Results of the comparison of plasma C5b-9 levels between venous, small-vessel and arterial thrombosis.
C: Comparison of plasma C5a levels between triple antiphospholipid positive (aPL+) and triple antiphospholipid negative (aPL-) patients.
D: Comparison of plasma C5b-9 levels between triple aPL+ and triple aPL- patients. *$p<0.05$: statistically significant.

in the frequency of small -essel thrombosis in the RAPS and CAPS patients. In accordance with data from the literature, triple aPL antibody positivity defined as IgG and/or IgM anticardiolipin antibody positivity plus IgG and/or IgM anti-β2Glycoprotein I antibody positivity plus lupus anticoagulant positivity, was considered a laboratory risk factor for thrombosis (19, 20). An analysis of the relationship between triple aPL positivity and C5a and C5b-9 levels showed that the triple aPL positive patients had higher medians and IQR than the single or double aPL positive patients (Table II). Only C5b-9 levels were, however, significantly higher in the triple aPL positive with respect to the single or double aPL positive patients (Fig. 2C-D). There were no significant differences in C5a and C5b-9 levels between the 7 plasma samples collected before and the 6 collected after a recurrent thrombotic event nor were there any difference between the 10 plasma samples collected at the beginning and the 12 collected during the follow-up period.

Calculating the best cut-offs

A ROC curve was constructed in order to calculate the best cut-off for the C5a (Fig. 3A-B-C) and the C5b-9 (Fig. 4A-B-C) levels between the healthy subjects and the patients falling in the three quiescent APS subsets. The curves showed that the most appropriate cut-offs for the TAPS were: C5a >11.6 ng/ml, C5b-9 >291.8 ng/ml. For the RAPS they were: C5a >14.0 ng/ml, C5b-9 >328.8 ng/ml, and for the CAPS they were: C5a >13.8 ng/ml, C5b-9 >401.4 ng/ml. As demonstrated by the area under curve values, the accuracy was good for all the curves although they showed a higher sensitivity, specificity and positive likelihood ratio for both the RAPS and CAPS with respect to the TAPS patients. Since RAPS and CAPS, which are the most severe clinical subsets of APS, produced similar ROC curves for the C5a and C5b-9 levels (Fig. 3B-C and Fig. 4B-C), the ROC curves were constructed using the sum of the RAPS+CAPS levels as the true positive values and the TAPS levels as the false positive ones in the effort to produce severity cut-offs that were able to discriminate between the more severe and less severe clinical subset. The best severity cut-offs resulted >39.0 ng/ml for C5a and >733.0 ng/ml for C5b-9. Notwithstanding a modest sensitivity, the area under the curve value, specificity, and positive likelihood ratio for both were good (Fig. 3D and 4D). The C5a and C5b-9 positive predictive values of severity cut-offs were 87.50% and 85.71%, respectively and the negative predictive values were 72.22% and 79.17%, respectively.

Discussion

Some investigators have found elevated plasma C5b-9 (4, 6-8) and C5a (4) levels during active CAPS (7, 8) and active thrombotic APS phases (4, 6). The finding that complement-dependent cell

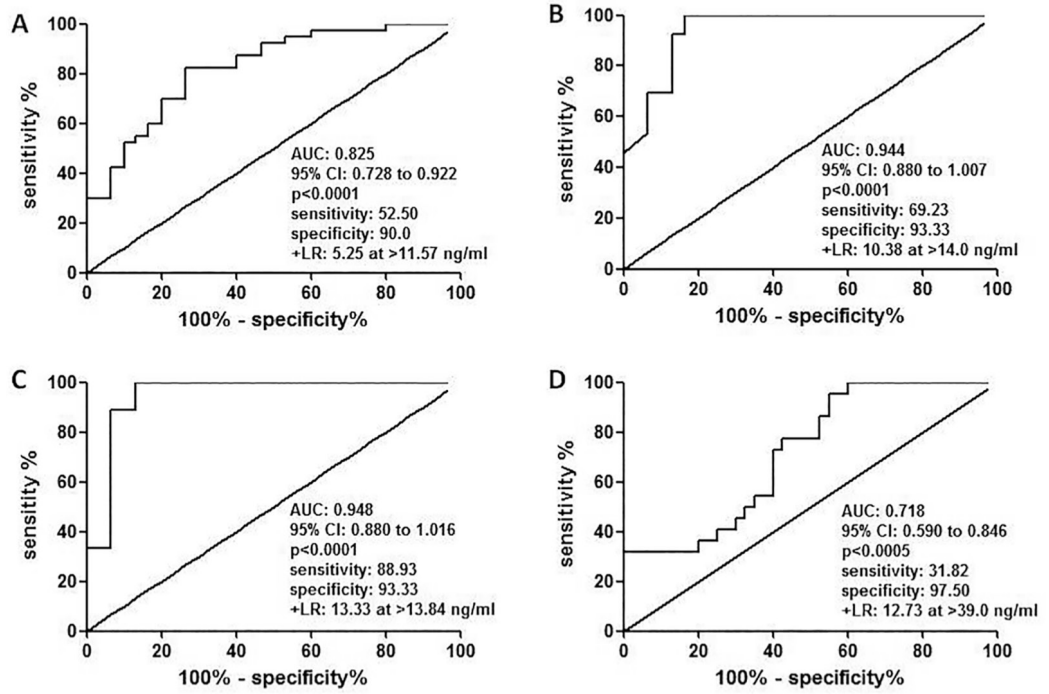

Fig. 3. Data from receiver operating characteristics (ROC) curve analysis to evaluate the ability of the C5a assays to discriminate the levels of patients with the different subsets of antiphospholipid syndrome from those of healthy controls and the levels of the more severe antiphospholipid syndrome subsets from those of the less severe.
A: Characteristics of the best cut-off of thrombotic antiphospholipid syndrome (TAPS) patients, **B:** of refractory antiphospholipid syndrome (RAPS) patients, and **C:** of catastrophic antiphospholipid syndrome (CAPS) patients.
D: The best severity cut-off obtained by using RAPS + CAPS C5a levels as true positive values and TAPS C5a levels as false positive values.
AUC: area under curve;
CI: confidence interval;
+LR: positive likelihood ratio.

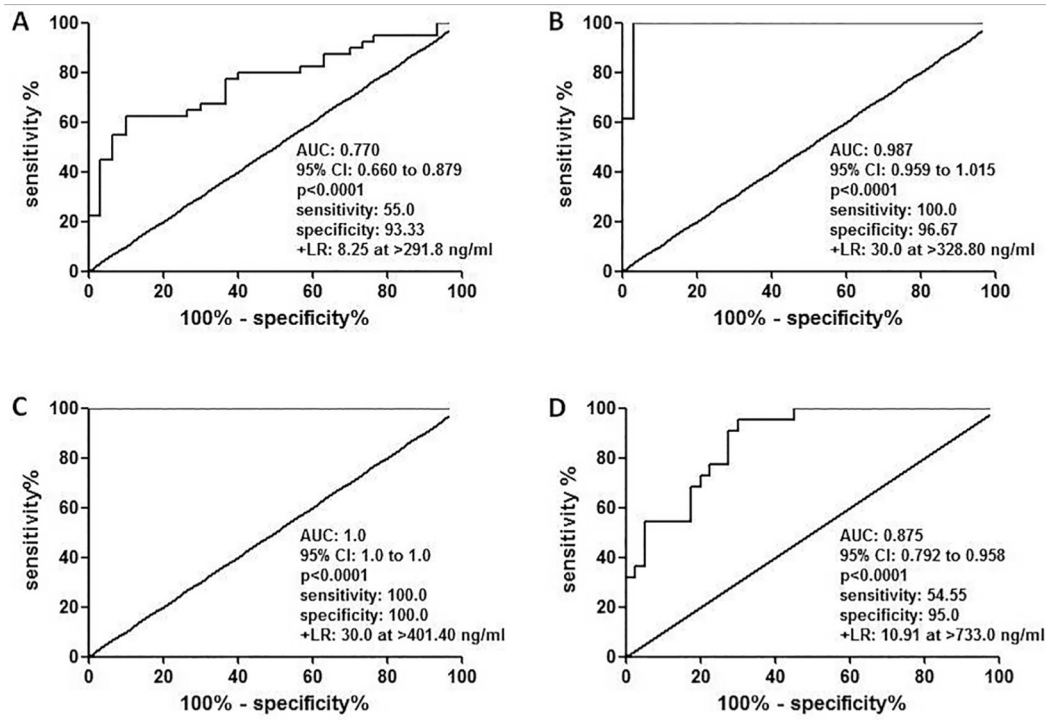

Fig. 4. Data from receiver operating characteristics (ROC) curve analysis to evaluate the ability of the C5b-9 assays to discriminate the levels of patients with the different subsets of antiphospholipid syndrome from those of healthy controls and the levels of the more severe antiphospholipid syndrome subsets from those of the less severe one.
A: Characteristics of the best cut-off of thrombotic antiphospholipid syndrome (TAPS) patients, **B:** of refractory antiphospholipid syndrome (RAPS) patients, and **C:** of catastrophic antiphospholipid syndrome (CAPS) patients.
D: The best severity cut-off obtained by using RAPS + CAPS C5b-9 levels as true positive values and TAPS C5b-9 levels as false positive values.
AUC: area under curve;
CI: confidence interval;
+LR: positive likelihood ratio.

killing and the cell-surface deposition of C5b-9 on PIGAnull TF-1 cells have frequently resulted positive during an active CAPS phase and in APS thrombosis occurring within 1 year prior to testing, further supports the hypothesis that the complement system plays a role in the pathogenesis of thrombotic manifestations of APS (21), although the mechanism involving aPL antibodies in complement activation has not been so far clarified (1). The current study confirms the previously reported findings by proving that C5a and C5b-9 levels are very high in active APS phases. In addition, our findings demonstrate, for the first time, that also in all three APS subsets the levels are significantly higher with respect healthy controls, over long term quiescent APS phases. In accordance with our recently published data, the current study found significantly higher C5a and C5b-9 levels in the quiescent CAPS with respect to quiescent TAPS patients (9). In addition, significantly higher C5a and C5b-9 levels were found in the quiescent RAPS with respect to the quiescent TAPS patients, but the difference in those complement components between the

CAPS and RAPS patients was not significant. Notably, the CAPS and RAPS subsets of APS share several common features such as clinical severity, high likelihood of suffering recurrent thrombosis, being refractory to anti-vitamin K therapy, showing a high prevalence of small-vessel thrombosis and a high frequency of triple aPL antibody positivity (Table I) (11, 18, 22, 23).

The comparison of mean C5a and C5b-9 levels between positive and negative patients for the different baseline clinical and laboratory parameters showed significantly higher levels of C5a and C5b-9 in patients with high aPL antibody titres with respect to those with medium titres and significantly higher levels of C5b-9 in those positive for IgG isotype of anticardiolipin and anti-β2 Glycoprotein I antibodies than in the negative ones. To note that high titres and IgG isotype of aPL antibodies are considered robust diagnostic tools and markers of severity in patients with APS (24, 25). Instead, the comparison of all the other clinical and laboratory parameters was not significant. However, probably due to the low number of treated patients, the true effect of steroids and/or hydroxychloroquine on C5a and C5b-9 levels cannot be assessed in this study.

When C5a and C5b-9 levels in the different clinical and laboratory characteristics were analysed, we found that: i) there were significantly higher levels of both complement activation products in small-vessel with respect to venous thrombosis, ii) there were significantly higher levels of C5b-9 in small-vessel thrombosis also with respect to the arterial one, iii) there were significantly higher C5b-9 terminal complex levels in the triple aPL positive patients with respect to their triple negative counterparts, thus, confirming that high C5a and C5b-9 levels are markers of severity in quiescent APS patients. The 22 samples collected on different time points during the follow-up from 10 patients affected with the different subsets of quiescent APS showed no significant difference in C5a and C5b-9 levels. While it is true that only a small number of samples were collected at irregular intervals, the finding seems to indicate that C5a and C5b-9 levels tend to remain stable over time. The absence of a significant difference between C5a and C5b-9 levels before and after a recurrent thrombotic event further supports this hypothesis.

ROC curve analysis, which was used to identify optimal cut-off values capable of distinguishing between patients affected by different APS subsets and healthy subjects, found that the best cut-offs for C5a and C5b-9 had higher sensitivity, specificity and likelihood ratio in the CAPS and RAPS than they did in the TAPS patients. This would indicate that a positive C5a and C5b-9 result would more likely identify patients suffering from the two most severe subsets of quiescent APS than from the less severe one.

There was, as explained above, a significant correlation between C5a and C5b-9 levels. However, the C5b-9 levels not only agreed with the C5a levels but showed in addition a significant association with small-vessel thrombosis with respect to arterial thrombosis and with triple aPL positivity and a greater accuracy with higher indicators of performance of the ROC curve than those of C5a levels. These findings make C5b-9 a very sensitive and specific marker of complement activation in severe APS subsets during quiescent phases.

In the presence of aPL, the higher C5b-9 and C5a levels in RAPS and CAPS than in TAPS during the quiescent phase may predispose these patients to new thrombotic events such as relapsing CAPS or recurrent thrombosis at occurrence of a precipitating factor capable of triggering thrombosis such as infections, surgery or pregnancy (26). This hypothesis is in agreement with the findings of the study by Fischetti *et al*. demonstrating that human polyclonal immunoglobulin G with anti-β2Glycoprotein I activity triggers clotting in the microcirculation of rat mesentery in the presence of a priming proinflammatory stimulus such as a bacterial lipopolysaccharide from Escherichia coli (27). Those authors also observed that complement activation is required for aPL antibody-dependent thrombus formation and that C5b-9 terminal complex is the primary mediator of the coagulation process (27). Moreover, the persistence of high C5a and C5b-9 levels during the quiescent phase of disease in patients affected with severe clinical APS subsets suggests that there may be a defective complement control in these patients. In fact, a high prevalence of rare germline variants in complement regulatory genes was recently identified in CAPS patients and although a dysregulation of the complement system has been hypothesised in these patients, no functional data concerning the pathogenic role of these germline variants have as yet been provided (21).

One of the limits of the study is its retrospective nature, but the rarity of these types of primary APS patients complicates performing prospective studies with a sufficient number of cases. The fact that the study used to determine C5b-9 and C5a levels a non-standardised ELISA method based on commercial kits, could be considered another its limit. However, due to the current lack of a standardised method, other authors cited in this study (3, 5, 6) also tested plasma levels of C5b-9 and C5a with commercial kits, something that could, nevertheless, have the advantage of being possible in most laboratories. Furthermore, the periods of time from a recurrent thrombotic event to sampling were variable, and we did not have large number of serial samples collected prospectively at regular intervals, thus, we are unable to draw any certain conclusions regarding the trend of C5b-9 and C5a levels in quiescent APS over time.

Chaturvedy *et al*. stated in a recent review that complement related biomarkers would ideally be able to identify patients who are more likely to be refractory to standard therapy and who would benefit from complement inhibition treatment as an addition to conventional antithrombotic therapy. Complement activation products have not as yet been shown to correlate with or predict the development of thrombosis (28). The current findings of this study suggest that high levels of C5b-9 and C5a during quiescent APS phases could help clinicians to stratify the risk in APS patients. In the future, if these results are confirmed by further larger scale studies, it will become possible to

identify the patients who may develop further thrombotic events as well as benefit from complement inhibitors in the event of an acute episode unresponsive to conventional treatment.

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