Complement activation in patients with isolated antiphospholipid antibodies or primary antiphospholipid syndrome

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Summary
The antiphospholipid syndrome (APS) is the association of thrombosis and recurrent pregnancy loss and/or pregnancy morbidity with persistent antiphospholipid antibodies (aPL). Increased complement activation has been implicated in the pathogenesis of APS in animal models. It was our objective to evaluate complement activation in patients with aPL or primary antiphospholipid syndrome (PAPS). We measured complement activation products, fragments Bb and C3a-desArg by ELISA in 186 aPL/PAPS patients and 30 healthy controls. All patients with aPL had significantly increased levels of complement activation products. Fragment Bb levels (mean, 95% CI); (thrombotic APS 0.54 units/ml, 0.31–0.83, obstetric APS 0.60 units/ml,0.39–1.02, isolated aPL 0.48 units/ml, 0.29–0.85, overall 0.39 units/ml, 0.33–0.47) and C3a-desArg levels (mean, 95% CI): (thrombotic APS 261 ng/ml, 219–311, obstetric APS 308 ng/ml, 243–391, isolated aPL 258 ng/ml, 193–337, overall 225 ng/ml, 202–251) were significantly higher compared to controls (fragment Bb 0.06 units/ml, 0.03–0.11, C3a-desArg 69 ng/ml, 50–92). There were correlations between Fragment Bb and C3a-desArg levels in all patients with aPL. Receiver operator characteristic (ROC) analysis showed increased fragment Bb and C3a-desArg levels had strong associations with the presence of persistent lupus anticoagulant (area under ROC: Bb 0.89, and C3a-desArg 0.90), dual and triple aPL positivity (Bb 0.71–0.82, C3a-desArg 0.71–0.80) but not with high titre anti-cardiolipin antibodies (Bb 0.62, C3a-desArg 0.65), or anti β2-glycoprotein 1 antibodies (Bb 0.66, C3a-desArg 0.67). Complement activation is present in all patient groups within this large cohort of patients aPL. This suggests it may have a major role in the pathogenesis of APS and merits further study.

Keywords
Antiphospholipid antibodies, antiphospholipid syndrome, complement activation

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Introduction

The persistence of antiphospholipid antibodies in association with thrombosis and/or obstetric complications is defined as the antiphospholipid syndrome (APS). APS can occur in isolation (primary APS) or in association with other immune disorders such as systemic lupus erythematosus (SLE). Specific classification criteria for the diagnosis of APS are outlined in the recently updated International consensus statement (Sydney criteria) (1). Antiphospholipid antibodies (aPL) can occur in isolation and have a prevalence of up to 5–6% in the general population (2, 3). The exact pathogenic mechanisms underlying the thrombotic and obstetric complications of APS are as yet unclear.

The complement system is initiated through any of three activating mechanisms – the classical, lectin and alternate pathways. Complement activation plays a major role in host defence mechanisms against infection and inflammation, but is also implicated in the pathogenesis of many diseases (4). Complement activation has been proposed to underlie obstetric and thrombotic complications associated with aPL in murine models of APS. Inhibition of complement and complement deficiency has been shown to prevent foetal loss and growth restriction mediated by aPL in mice (5, 6) and is associated with decrease in size of aPL induced thrombus in mice (7, 8).

To date, there have been few studies performed on the role of complement activation in humans with aPL. A study of placentae in patients with aPL demonstrated increased complement deposition in association with placental lesions (9), and mutations in complement regulatory proteins of patients with SLE and/or aPL have been associated with preeclampsia (10). Evidence of increased complement activation in patients with APS has been suggested by two recent studies (11, 12). Both studies included small groups of patients, and one did not include patients with obstetric complications of aPL and used just one marker of complement turnover. Therefore, a large study was needed to determine if increased complement activation plays a role in the pathogenesis of aPL-related complications.

Complement activation can be evaluated by immunochemical assays (including ELISA assays). Fragment Bb is a cleavage product

of Factor B, generated as a result of activation of the alternate complement pathway and C3a-desArg is a stable cleavage product of C3. Both are reliable markers of complement activation and have been validated in other settings such as infection, inflammation, sickle cell disease (13) and preeclampsia (14).

The aim of this study was to assess complement activation in patients with primary APS (PAPS) or isolated aPL by measuring complement fragments, C3a-desArg and fragment Bb.

Materials and methods

Patients

Local ethics committee approval was obtained from Guy's and St. Thomas' Trust Research Ethics Committee. Patients aged between 18–75 years were recruited from our institution, had PAPS classified according to Sydney criteria (1) , or had persistent aPL without associated complications. Estimation of sample size proved difficult since there are no similar studies in humans on which to base power calculations, therefore sampling was opportunistic.

Patients currently pregnant, patients with SLE, intercurrent infection, acute illness or malignancy were excluded because of increased complement activation in these groups. Medical records were reviewed retrospectively, and patients were interviewed to determine clinical details; patients were not recruited if they had an aPL-related event within the previous three months. The control group consisted of healthy hospital staff members who were not known to have aPL, were non-smokers, were not pregnant and not taking oral contraceptives. Blood samples were collected with informed consent. Interleukin (IL)-6 levels were assessed in a subset of patients with aPL/PAPS and controls to rule out evidence of underlying active inflammation.

Blood sample collection and processing

Blood was drawn into Vacutainer tubes prefilled with potassium EDTA, trisodium citrate 0.105 M, but free of anticoagulant and were processed within 3 hours of collection. Samples were double centrifuged at 3,000 g for 15 minutes at 4°C. Serum and plasma were separated and aliquoted into eppendorf tubes and stored in a freezer at - 80°C until testing.

aPL determination

Patients with PAPS or isolated aPL had demonstrated positive testing for aPL (lupus anticoagulant, IgG/IgM anti-cardiolipin antibodies, or IgG/IgM anti-β2-glycoprotein I antibodies) on two or more occasions greater than 12 weeks apart (1).

Solid phase assays

Anticardiolipin antibodies (isotypes IgG and IgM) were quantified by indirect ELISA using AEUSKULISA® Cardiolipin-GM reagents (Grifols UK, Cambridge, UK). Anti-β2-glycoprotein I antibodies (isotypes IgG and IgM) were quantified by indirect ELISA using QUANTA Lite® reagents (INOVA Diagnostics Inc., San Diego, CA, USA). Positive cut-off values for both were determined according to Sydney Criteria (>40 GPL/MPL or >99th percentile) (1).

Lupus anticoagulant detection

Lupus anticoagulant detection in compliance with published guidelines (15) was determined by dilute Russell's viper venom time (DRVVT) and dilute APTT (DAPTT), accompanied by appropriate confirmatory tests. DRVVT was performed with Life Diagnostics LA Screen and LA Confirm reagents (Life Therapeutics, Clarkston, GA, USA). DAPTT was performed using PTT-LA (Diagnostica Stago, Asniéres, France) in the screen with a platelet neutralisation procedure employing Biodata Platelet Extract Reagent (Alpha Laboratories, Hampshire, UK) in the phospholipid-dependence confirmatory test. Patients on oral anticoagulation additionally received screening with Taipan snake venom time (TSVT) employing Diagen Taipan venom (Diagnostic Reagents, Thames, UK) with an Ecarin time confirmatory test using *E. carinatus* venom (Diagnostic Reagents). All elevated screens received the confirmatory test plus a screen and confirmatory test on 1:1 mixing studies with normal plasma. Technoclone Lyophilised Platelet Poor Plasma (Pathway Diagnostics Ltd, Dorking, UK) was used as the normal plasma throughout.

Complement fragment Bb and C3a-desArg ELISA assays

ELISA assays measuring complement fragments Bb and C3a-desArg (Microvue Bb Plus and C3a EIA kits, Quidel, Pathway diagnostics ltd., Dorking, UK) were performed on EDTA plasma samples in two batches by one scientist according to manufacturer's protocol. The intra- and inter-assay variability of C3a DesArg was 2.1% and 5.1%, and for fragment Bb, 2.5% and 7.2%, respectively.

IL-6 analysis

ELISA assays measuring IL-6 levels (R&D systems, Abingdon, Oxford, UK) were performed on plasma samples in two batches by one scientist according to manufacturer's protocol. Intra- and inter-assay variability was 4.2% and 5.0%, respectively.

Statistical analysis

Statistical analysis was performed using Stata-11 (Statacorp, College Station, TX, USA). Data was logarithmically transformed because of a departure from normal distribution. Multiple regression analysis was used to determine differences between groups adjusting for age, ethnicity and medications (aspirin and warfarin). Correlations between fragment Bb and C3a levels were determined. Logistic regression and estimation of the area under the receiver operator curve (ROC) was used to assess the utility of Bb and C3a-desArg assays to predict for the presence of aPL/PAPS, presence of LA, high-titre anticardiolipin antibodies (defined as IgG or IgM aCL u/ml >99th percentile), anti-β2-glycoprotein I antibodies, dual or triple aPL positivity. Although ROC curves are used to compare the diagnostic potential of a variable (clinical or laboratory), they were used in this study to further demonstrate the association of increased complement activation in patients with aPL/PAPS. Complement activation can occur in other disease settings, so can never truly predict for the presence of aPL/PAPS.

To determine the relationship between higher levels of complement fragments and aPL positivity, data was categorised into tertiles. For those with fragment Bb and C3a-desArg values in the upper tertile (Fragment Bb >0.4 units/ml and C3a desArg >200 ng/ml), odds ratios (OR) were calculated for the presence of aPL/PAPS. Exact logistic regression was used to calculate OR when the

Table 1: Patient characteristics.

Variables	Thrombotic APS (n=95)	Obstetric APS (n=52)	Isolated aPL (n=39)	Controls (n=30)	P-value
Age Median (range)	 47 (23–61)	 41 (32–54)	 43.5 (19–73)	 37.5 (20–58)	<0.01
Sex (female: male)	 87:8	 52:0	 35:4	 30:0	0.04
Ethnicity (White:Asian:Black)	 86:7:2	 39:8:5	 30:6:3	 30:6:3	0.34
aPL subtype					
Lupus anticoagulant positive	76 (80%)	35 (67%)	34 (87%)	0 (0%)	
IgG or IgM anticardiolipin antibody positive (% patients)	28 (30%)	12 (23%)	6 (15%)	0 (0%)	
IgG or IgM anti-β2 glycoprotein 1 antibody positive	33 (35%)	21 (40%)	9 (23%)	0 (0%)	
Anticardiolipin and anti-β2 glycoprotein 1 antibody positive	20 (22%)	7 (13%)	3 (8%)	0 (0%)	
Anticardiolipin antibody and lupus anticoagulant positive	12 (13%)	8 (15%)	2 (5%)	0 (0%)	
Anti β2 glycoprotein- 1 antibody and lupus anticoagulant positive	18 (19%)	20 (38%)	8 (21%)	0 (0%)	
aPL triple positivity†	8 (8%)	7 (13%)	2 (5%)	0 (0%)	
aPL complication					
Previous arterial thrombosis	40	0	0	0	
Previous venous thrombosis	41	0	0	0	
Previous arterial and venous thromboses	14	0	0	0	
Obstetric complications (early :late complications:both)† †	7:11:2	44:14:6	0	0	
Medication					
Warfarin	84	0	0	0	
Heparin	0	0	0	0	
Aspirin	8	27	21	0	

†Anticardiolipin, anti β2 glycoprotein1 antibody and lupus anticoagulant positive. †† Early obstetric complications defined according to International Statement Criteria (1).

Table 2: Mean values of Fragment Bb and C3a-desArg.

Plasma complement levels	aPL/PAPS Overall	Thrombotic APS subgroup	Obstetric APS subgroup	Isolated aPL subgroup	Controls
Fragment Bb Mean units/ml † (95% CI)	 0.39 (0.33–0.47)	 0.54 (0.31–0.83)	 0.60 (0.39–1.02)	 0.48 (0.29–0.85)	 0.06 (0.03–0.11)
C3a-DesArg Mean ng/ml † (95% CI)	 225 (202–251)	 261 (219–311)	 308 (243–391)	 258 (197–337)	 69 (50–92)

†Adjusted for age, ethnicity and medications.

Table 3: Values of area under ROC curve for Fragment Bb and C3a-desArg.

Area under ROC	Presence of aPL/PAPS	Presence of LA	Presence of ACL	Presence of anti-β2 GPI	Presence of LA and ACL	Presence of LA and anti-β2 GPI	Presence of ACL and anti-β2 GPI	Presence of all aPL
Fragment Bb (P values)	0.89 (<0.01)	0.78 (<0.01)	0.62 (0.31)	0.66 (<0.01)	0.81 (<0.01)	0.72 (<0.01)	0.71 (0.02)	0.82 (<0.01)
C3a –desArg (P values)	0.90 P<0.01	0.77 (<0.01)	0.65 (0.02)	0.67 (<0.01)	0.77 (<0.01)	0.71 (<0.01)	0.73 (<0.01)	0.80 (<0.01)

Estimation of the area under the ROC curve to assess the utility of Bb and C3a-desArg assays to predict for the presence of aPL/PAPS, presence of LA, high titre anticardiolipin antibodies, anti-β2-glycoprotein I antibodies, dual or triple aPL positivity.

number of false positives were extremely low (16). Estimates are presented with 95% confidence intervals (CI) of the mean.

Results

Demographic details of study subjects are shown in ▶Table 1. A total of 216 participants were recruited, including 186 patients with PAPS and isolated aPL, and 30 healthy controls. Of the patients with PAPS, 95 had thrombotic complications (20 females included in this group also had aPL associated obstetric complications), 52 had obstetric complications. Thirty patients had isolated aPL. The patient groups were of similar average ages.

▶Table 2 demonstrates plasma complement fragment levels in study subjects. Overall, patients with aPL and/or PAPS had significantly higher levels of both complement fragment Bb and C3a-desArg compared to healthy controls. No significant differences were found in fragment Bb or C3a-desArg levels between groups according to clinical phenotype of aPL (thrombotic APS, obstetric APS and isolated aPL), but when each aPL phenotype group was compared to healthy controls, they each had independently significantly greater levels. No significant differences in fragment Bb or C3a-desArg levels were found among groups in relation to age, ethnicity or medications (aspirin and warfarin). No significant differences were found in IL-6 levels in a subset of patients with aPL/PAPS (n=92, mean 4.1 pg/ml, 95% CI 3.7–4.4) compared to a subset of healthy controls (n=20, mean 3.7 pg/ml, 95% CI 3.0–4.4, p=0.5).

There was a significant correlation between Fragment Bb and C3adesArg levels in all patients with APS/aPL (r=0.64, p<0.01). The correlation was reduced but remained significant when patients were grouped according to clinical phenotype of aPL (thrombotic r=0.33, p<0.01, obstetric r=0.61, p<0.01, aPL r=0.64, P<0.01). There was no significant correlation between Fragment Bb and C3adesArg levels in healthy controls (r=0.24, p=0.20).

▶Table 3 demonstrates the values under the ROC curve for the associations of Fragment Bb and C3a-desArg with the presence of aPL/PAPS and for various aPL subtypes. Fragment Bb and C3a-desArg had strong associations with aPL/PAPS, and a combination of Bb and C3a-desArg resulted in a stronger association with aPL/PAPS (▶Fig. 1). Both complement fragments had an association with presence of lupus anticoagulant but had a weak association with high titre anticardiolipin antibodies and anti-β2-glycoprotein I antibodies (▶Table 3).

Fragment Bb and C3a-desArg had a relatively strong association for dual aPL positivity, the strongest association observed was for patients with both lupus anticoagulant and anticardiolipin antibodies. Fragment Bb and C3a-desArg had a strong association with aPL triple positivity (LA, anticardiolipin antibodies and anti-β2-glycoprotein I antibodies).

▶Table 4 shows the odds ratios for aPL/PAPS for patients with Fragment Bb and C3a desArg values in the upper tertile (Fragment Bb >0.4 units/ml and C3a desArg >200 ng/ml). These values were based on the top third of values for Fragment Bb and C3adesArg (rounded to 1 significant figure). 77% of patients with aPL/PAPS had Fragment Bb values in the upper tertile (65% for C3a-desArg) compared to only 3% of controls. Patients with Fragment Bb and

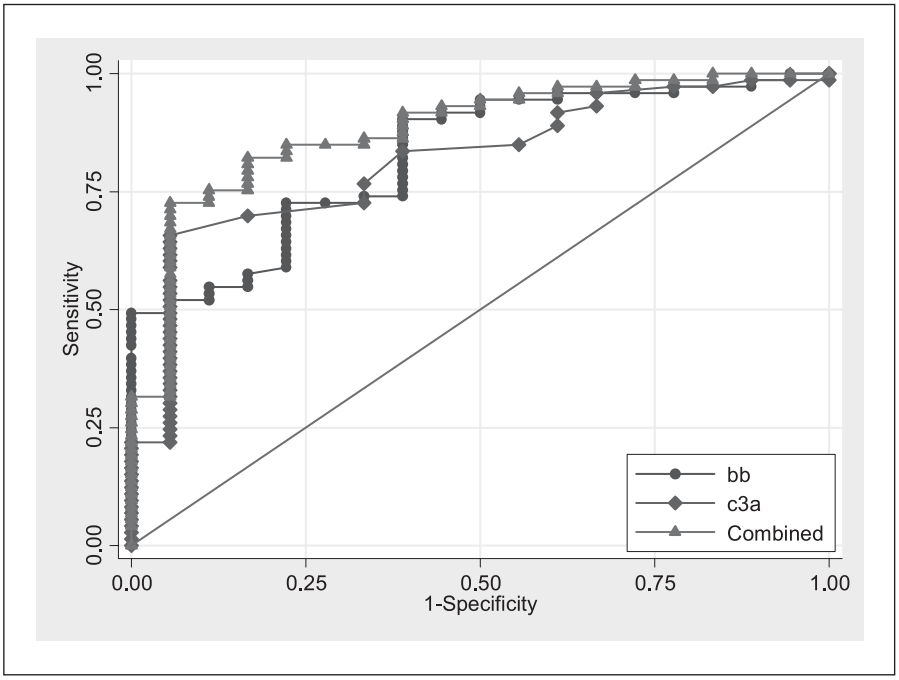

Figure 1: Increased Fragment Bb and C3a-desArg is associated with aPL/PAPS (area under ROC =0.88 and 0.90, respectively). A combined increased level of Bb and C3a-desArg has the highest association with aPL/PAPS (area under ROC=0.93).

C3a-desArg values in the upper tertile had an OR for aPL and/or PAPS of 88 and 53, respectively. For patients with values of both Fragment Bb and C3a-desArg in the upper tertiles (46% of aPL/PAPS), odds ratio for the presence of aPL and/or PAPS was 72.

Discussion

Our results demonstrate increased complement activation in a large cohort of well defined patients with antiphospholipid antibodies or primary antiphospholipid syndrome. Complement activation products, Fragment Bb and C3a-desArg were elevated in all patient groups, irrespective of the presence or absence of aPL-related complication (thrombotic APS, obstetric APS or isolated aPL),compared to healthy controls. Moreover, there were no significant differences in complement levels between groups. There was a significant correlation between elevated Fragment Bb and C3a-desArg levels in all patients with aPL regardless of complication. No difference was observed in IL-6 levels between a subset of patients with aPL/PAPS compared to healthy controls eliminating active inflammation as a mechanism for increased complement activation products.

Increased C3a-desArg levels have previously been described in two small cohorts of patients with aPL (11, 12), one study demonstrated patients with PAPS to have increased levels of C3a-desArg and C4a-desArg in association with low serum complement levels, C3, C4 and CH_{50} (11). However, this study (n=36) included only three patients with pregnancy morbidity and none with isolated aPL (11). The other study included patients positive for lupus anticoagulant only (n=56), and no patients had anticardiolipin antibodies or anti-β2-glycoprotein I antibodies nor had they aPL-related obstetric complications (12).

Presence of lupus anticoagulant, and/or high titres of anticardiolipin antibodies have been associated with a higher risk of aPL associated complications (17, 18) as have the presence of more than one aPL antibody (19). Triple aPL positivity has been associated with the highest risk of complications (20). We found a weak association between increased levels of complement fragments and some aPL subtypes, i.e. high titre anticardiolipin antibodies or anti-β2-glycoprotein I antibodies. However, a stronger association was found between increased levels of complement fragments and presence of lupus anticoagulant as well as dual and triple aPL positivity.

Complement activation has been implicated in obstetric and thrombotic complications of aPL with most evidence to date from murine models of APS. In mice it appears that aPL cause comple-

Table 4: Odds ratios for presence of aPL/PAPS for Fragment Bb and C3a-desArg values in the upper tertile.

Assay (tertile)	Cases (n)	Controls (n)	OR	95%CI
Fragment Bb (units/ml) (3^{rd}:>0.40)	143/186 (77%)	1/30 (3%)	88	13 – 3690
C3a-desArg (ng/ml) (3^{rd}:>200)	120/186 (65%)	1/30 (3%)	52	8 – 2197
Bb & C3a-desArg Combined (3^{rd}:Bb>0.40 & C3a-desArg>200)	85/186 (46%)	0/30 (0%)	72	16–656

Odds ratios (OR) for presence of aPL/PAPS for those patients with fragment Bb and C3a-desArg values in the upper third of values. CI, confidence interval.

ment activation which directly causes placental injury and is associated with foetal loss (5, 6). C3 deficiency or complement inhibition using the C3 inhibitor, Cryy-Ig or a C5a receptor antagonist peptide prevents foetal loss (5, 6). Furthermore, complement compounds (C5–9) co-localise with thrombus generated by anti-ß2-glycoprotein I fractions and lipolysaccharide in murine models of APS and is inhibited by C6 deficiency or complement inhibition with an anti C5 antibody. C3 and C5 inhibition or deficiency decreases the thrombogenic effects of infused aPL (7, 8).

It is still unclear as to how complement activation is occurring in patients with aPL, or how it relates to the pathogenesis of aPL-related complications (recently reviewed by Meroni et al. [21]). Hypocomplementaemia (low C3, C4 and CH50) have been demonstrated in association with elevated C3a levels in patients with APS, so increased activation rather than complement deficiency has been proposed (11). Are aPL triggering complement activation, which in turn lead to the complications of APS, or do the aPL-related complications occur first, which then triggers complement activation? Or is it possible that complement activation is occurring in parallel to aPL-related complications and not directly related to the complications of APS? Since none of the patients included in our study had an aPL-related event within three months of recruitment, and complement activation products were increased in patients with isolated aPL, we hypothesise that complement activation is triggered by aPL binding. It may be that a second hit is needed to produce the aPL associated complications. It is also possible that some patients with aPL have an impaired ability to deal with increased complement activation.

What is known about this topic?

- Most studies on the role of complement activation in the antiphospholipid syndrome (APS) are in murine models of APS.
- Inhibition of complement activation and/or complement deficiency prevents foetal loss in murine models of APS.
- Further studies of murine models of APS have demonstrated complement activation and/or complement deficiency decreases thrombus growth.
- Only two studies have been performed on the role of complement activation in patients with antiphospholipid antibodies (aPL).

What does this paper add?

- Animal models suggest complement activation is an important pathogenic mechanism in the pathogenesis of APS – we have shown there is complement activation in a large cohort of well defined patients with aPL.
- We have demonstrated complement activation in all subgroups of APS: patients with thrombotic complications, obstetric complications, and patients with isolated aPL using two different markers of complement activation.
- Previous studies have been smaller, have used only one marker of complement activation and have not included such large numbers of patients with both thrombotic and obstetric complications as well as a subgroup with aPL and no known complications.

Shortcomings of our study included the preparation of samples and grouping of patients for purposes of analysis. Following venepuncture, blood samples are often stored on ice immediately post sampling. Although this was not the case in our study, all samples were treated similarly, so the significant differences between these groups show this was not a confounding factor. Although C3a-des-Arg has been previously used in two small studies of patients with aPL, Fragment Bb has not been previously used in the setting of APS but has been validated in other settings. Patients with both thrombotic and obstetric complications were included in the thrombotic group for the purposes of analysis, but we would have analysed results in these patients as a separate group had the study been larger. The aim of this study was not to ascertain through which effector pathway complement was activated in patients with aPL. Although we found evidence of alternate pathway activation (elevated fragment Bb levels), this may have been the result of activation of C3 by the other activation pathways.

This study has clearly shown that complement activation is present in patients with aPL. Further studies are required to evaluate the mechanism of complement activation in association with aPL, specifically by separately studying depletion of classical, alternative and ficolin pathways, and in turn, establish how this leads to the thrombotic and obstetric complications of APS. Inhibition of complement in murine models of APS reverses some aPL-associated complications (5, 7), and agents inhibiting complement activation have shown promising results in patients with thrombotic disorders such as paroxysmal nocturnal haemoglobinuria (22) and atypical haemolytic uraemic syndrome (23). It is possible that such agents may prove useful in the future treatment of patients with APS.

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Conflicts of interest

None declared.

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