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Acquired activated protein C resistance is associated with lupus anticoagulants and thrombotic events in pediatric patients with systemic lupus erythematosus

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Acquired activated protein C resistance (APCR) has been hypothesized as a possible mechanism by which antiphospholipid antibodies (APLAs) cause thrombotic events (TEs). However, available evidence for an association of acquired APCR with APLAs is limited. More importantly, an association of acquired APCR with TEs has not been demonstrated. The objective of the study was to determine, in pediatric patients with systemic lupus erythematosus (SLE), whether (1) acquired APCR is associated with the presence of APLAs, (2) APCR is associated with TEs, and (3) there is an interaction between APCR and APLAs in association with TEs. A cross-sectional cohort study of 59 consecutive, nonselected children with SLE was conducted. Primary clinical outcomes were symptomatic TEs, confirmed by objective radiographic tests. Laboratory testing included lupus anticoagulants (LAs), anticardiolipin antibodies (ACLAs), APC ratio, protein S, protein C, and factor V Leiden. The results revealed that TEs occurred in 10 (17%) of 59 patients. Acquired APCR was present in 18 (31%) of 58 patients. Acquired APCR was significantly associated with the presence of LAs but not ACLAs. Acquired APCR was also significantly associated with TEs. There was significant interaction between APCR and LAs in the association with TEs. Presence of both APCR and LAs was associated with the highest risk of a TE. Protein S and protein C concentrations were not associated with the presence of APLAs, APCR, or TEs. Presence of acquired APCR is a marker identifying LA-positive patients at high risk of TEs. Acquired APCR may reflect interference of LAs with the protein C pathway that may represent a mechanism of LA-associated TEs. (Blood. 2001;97: 844-849)

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by the presence of multiple autoantibodies. About one-third of patients with SLE have antiphospholipid antibodies (APLAs).[1,2] APLAs are a heterogenous group of autoantibodies directed at phospholipid-bound plasma proteins.[3] There are 2 major types of APLAs, lupus anticoagulants (LAs), which are detected by phospholipid-dependent coagulation tests, and anticardiolipin antibodies (ACLAs), which are quantitated by solid-phase immunoassays.[4] In patients with SLE, a significant association between the presence of APLAs and thromboembolic events (TEs) is well described.[5]

Although the association between APLAs and TEs is established, the mechanism of APLA-associated TEs remains unclear. One plausible mechanism hypothesized in the literature relates to acquired abnormalities of the protein C pathway associated with APLAs. The protein C pathway is one of the most important physiologic anticoagulant systems,[6] and congenital abnormalities of the protein C pathway are associated with an increased risk of TEs. Reported abnormalities associated with APLAs include acquired activated protein C resistance (APCR)[7-18] and decreased plasma concentrations of protein S.[19-26]

However, the association of acquired APCRs or decreased protein S concentrations with APLAs has not been clearly established because studies to date were performed in small patient populations selected on the basis of APLA positivity. More importantly, no study has determined whether acquired abnormalities of the protein C pathway in patients with APLAs are associated with TEs. Finally, it remains to be elucidated whether there is an interaction between these abnormalities and APLAs in association with TEs, ie, whether the risk of TEs associated with APLAs is increased in the presence of acquired APCR and/or protein S deficiency.

Previously, we reported a highly significant association between the presence of LAs with TEs in a cohort of pediatric patients with SLE.[2] An important observation from our study was that no pediatric SLE patients who were negative for LAs had TEs. In contrast, approximately 40% of adult SLE patients negative for APLAs have TEs.[27] Therefore, when studying potential mechanisms of APLA-associated TEs, a pediatric cohort is advantageous, as other risk factors that confound the association of APLAs with TEs in adult populations are rare in children.

The objectives of the current study were to determine, in pediatric patients with SLE, (1) whether acquired APCR and/or protein S deficiency are associated with the presence of APLAs, (2) whether acquired APCR and/or protein S deficiency are associated with TEs, and (3) whether there is an interaction between acquired APCR and/or protein S deficiency and APLAs in association with TEs.

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Patients, materials, and methods

Study population

The study design was a cross-sectional cohort study of consecutive nonselected pediatric patients with SLE. Details of this cohort have been published previously.[2] In brief, patients were managed at the rheumatology clinics of 2 tertiary care centers, Hospital for Sick Children, Toronto, and Hôpital Ste. Justine, Montreal, Canada. All patients fulfilled the American Rheumatism Association criteria for SLE.[28] There were no exclusions of eligible patients. A disease activity index was determined at each study visit, using a validated scoring system.[29] A control population consisted of concurrent age-matched healthy children undergoing minor elective surgery. These children had an additional 5 mL of blood drawn at the time of venipuncture for preoperative blood work. Informed consent was obtained from all patients and control subjects or their guardians.

Evaluation for thromboembolic events

Patients with SLE and/or their parents were interviewed by the nurse study coordinator who had no knowledge of the laboratory results. A detailed history of previous TEs was obtained, and a standardized questionnaire was completed. A retrospective chart review was also performed, and, if a TE was noted, the radiographic test employed to diagnose the TE was reviewed by an independent panel of experts. Patients were followed prospectively for TEs over a period of 2 years during the study. All symptomatic TEs were confirmed by objective radiographic test. Acceptable radiographic tests were defined as venography or ultrasound for deep venous thrombosis, a ventilation perfusion scan for pulmonary embolism, and either a computerized tomography scan or magnetic resonance imaging for TEs in the central nervous system.

Laboratory testing

All study patients had blood samples taken at study entry and on a separate occasion during a follow-up visit at least 3 months after the initial visit. Patients who developed acute TEs during the study period had blood drawn at the time of the event and at a later visit at least 3 months from the acute event. Normal control subjects had blood samples drawn on one occasion.

Venous blood was drawn into Vacutainer tubes (Becton Dickinson, New Jersey) containing 0.105 M buffered citrate solution for a final ratio of 1 part anticoagulant to 9 parts of blood. Plasma was immediately separated from cellular elements by double centrifugation at 1500*g* for 15 minutes at 4°C. Samples to be tested for LAs were filtered through a 0.02-micron screen (Acrodisk, Gelman Sciences, Ann Arbor, MI) to achieve complete platelet removal. Plasma was subsequently aliquoted and frozen at −70°C. All assays were performed in one central laboratory at the Hamilton Civic Hospitals Research Centre by the same technologist who had no knowledge of the patients' clinical status. Lot numbers of all reagents were constant during the study.

Testing for LAs was based on 3 steps according to the criteria of the subcommittee on APLAs of the International Society of Thrombosis and Haemostasis[30] as previously described in detail[2]: (1) screening assays included the kaolin clotting time, dilute activated partial thromboplastin time (APTT), dilute prothrombin time, and 2 dilute Russel viper venom times; (2) all tests were repeated in a 1:1 mix with normal plasma; (3) positive tests were confirmed with the same reagent in the presence of excess phospholipids. At least one test system had to be positive in all 3 steps for a patient to be considered LA positive. ACLAs were measured by enzyme-linked immunosorbent assay (ELISA; Advanced Biological Products, Mississauga, Ontario, Canada).

The APC ratio was measured, using an APTT-based system (Biopool, Ventura, CA) on the ACL coagulometer (Instrumentation Laboratory, Milan, Italy). The test was performed in undiluted patient plasma. The APC ratio was determined by the clotting time obtained in the presence of added human APC divided by the APTT in the absence of APC. In LA-positive patients with a largely prolonged baseline APTT, the APTT in the presence of APC is frequently not proportionally prolonged. The resulting decreased APC ratio is a laboratory artifact.[31] In this study, the APTT reagent used for the APC ratio (micronized silica) was insensitive to LAs, resulting in baseline APTTs in patients with LAs that were normal or minimally prolonged (≤ 7 seconds) that would not cause this artifact. Consequently, a decreased APC ratio (ie, an inadequate APTT prolongation after addition of APC) was considered reflective of inhibition of the effect of APC. The normal cutoff value for the APC ratio was 2.2 as derived from the 2.5th percentile of values measured in age-matched healthy control subjects.

Factor V Leiden status was determined by extracting genomic DNA from plasma of patients in whom cellular material was not available. Plasma DNA extraction was done with the use of DNA purification columns (Gentra System Capture Columns, Minneapolis, MN). DNA from buffy coat samples was extracted, using the Bio Rad InstaGene Whole Blood Kit (Bio Rad, Missisauga, Ontario). DNA was amplified by using the polymerase chain reaction method as previously described.[32] Samples containing DNA in insufficient amounts were further amplified, using a nested polymerase chain reaction system as described by Ryan et al.[33] PCR products were digested with Mnl1 and subjected to electrophoresis, using 3.5% agarose gels.

Protein S, total and free (Affinity Biologicals, Hamilton, Ontario), and protein C (Asserachrom, Diagnostica Stago, France) were measured by commercial ELISA kits. To assay free protein S, the plasma was treated with polyethylene glycol to precipitate the bound protein S, leaving free protein S to be measured. C4b binding protein was measured by radial immunodiffusion, using a rabbit anti-human antibody (Diagnostica Stago). Normal ranges were derived from the mean ± 2 SD of values measured in age-matched healthy control subjects. When patients were on warfarin treatment, the data on APC ratio, protein C, and protein S were excluded from analysis (10 samples in 7 patients).

von Willebrand factor (vWF) antigen levels were measured by ELISA (Affinity Biologicals). vWF was tested to account for a potential association of vWF or factor VIII with APCR and the risk of TEs as previously reported in the literature.[34] As study samples had been stored for several years, analysis of VIII:C by functional assay was not considered valid. vWF measured by ELISA is less affected by sample storage. There is a well-described correlation between plasma VIII:C and vWF levels; therefore, vWF was measured as a surrogate for VIII:C levels. Normal ranges were derived from the mean ± 2 SD of values measured in healthy controls.

Statistical analysis

Statistical analysis was performed, using the Minitab Statistical package (version 12.1). For comparisons between groups, results of samples from different time points were summarized for each individual patient. For continuous variables (eg, the APC ratio), results are reported as the mean of each patient's samples. For dichotomous outcome variables (eg, the presence of APCR), results were categorized as abnormal if at least one of the patient's samples was abnormal.

Continuous variables were compared between groups by *t* test. Because of skewed distributions, APC ratios were log-transformed. Dichotomous variables were organized into 2 × 2 tables. Risk differences (RD) with 95% confidence intervals (CI) were calculated (eg, the difference in proportions of TEs between groups with or without APCR). The RD was used as a summary outcome measure because some subgroups had zero outcome events that did not allow the calculation of odds ratios. Statistical comparison was done using chi-square or Fisher's exact test. Multivariable analysis was performed to simultaneously test for the associations of APCR, LAs, and APCR × LA interaction with TEs. A binomial additive excess model was fitted, using Epicure (version 2.07, Program GMBO), essentially fitting a linear model to the RD. The score test was used to assess the fits of the components of the model.

Results

Study population

Fifty-nine patients with SLE were studied, 49 females and 10 males. Their median age was 14 years with a range from 4 to 19

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years. Disease activity scores overall were 2.2 ± 2.3 (mean, SD), on a scale ranging from 0 to 10. Disease activity scores were 2.6 ± 2.5 at study entry and 1.9 ± 2.1 during follow-up. Scores did not differ between patients with or without TEs. The control population consisted of 35 concurrent healthy children, 18 females and 17 males. Their median age was 10 years with a range from 4 to 19 years.

Thrombotic events

Thirteen TEs occurred in 10 (17%) of the 59 patients with SLE. Six TEs occurred before patients entered the study. Seven TEs occurred during the prospective follow-up, 3 of which were recurrent in patients with previous TEs following discontinuation of their warfarin therapy. Four events were deep venous thromboses in the lower extremities, 2 were pulmonary emboli, 1 was a portal vein thrombosis, and 6 involved the central nervous system of which 3 events were venous (sagittal sinus thrombosis) and 3 were arterial strokes.

All TEs were symptomatic. All deep venous TEs were proximal in location, causing significant swelling and pain. Both patients with pulmonary embolism presented with pleuritic chest pain and ventilation perfusion scans that showed high probability of a pulmonary embolism. Central nervous system TEs caused a variety of neurologic symptoms, depending on their location. There were no deaths. All TEs were managed with heparin, followed by warfarin for at least 3 months.

Activated protein C resistance

Frequency of APCR in patients with SLE. APCR was present in 18 (31%) of 58 evaluable patients (95% CI, 20%-45%) with SLE. Median APC ratios were significantly decreased in patients with SLE (median, 2.58; minimum [min]-maximum [max], 1.86-4.47) compared to normal control subjects (median, 3.04; min-max, 2.18-4.26; $P < .001$) (Figure 1). APC ratios in samples taken during warfarin treatment were significantly increased (median, 4.13; min-max, 2.90-5.0) compared to APC ratios in other samples of patients with SLE and normal control subjects and were excluded from the analysis (Figure 1). APC ratios were evaluable in 2 or 3 samples in 53 of 59 patients. Because of warfarin therapy, 5 patients with TEs had APC ratio evaluable from only their sample at study entry, and one patient had no evaluable sample.

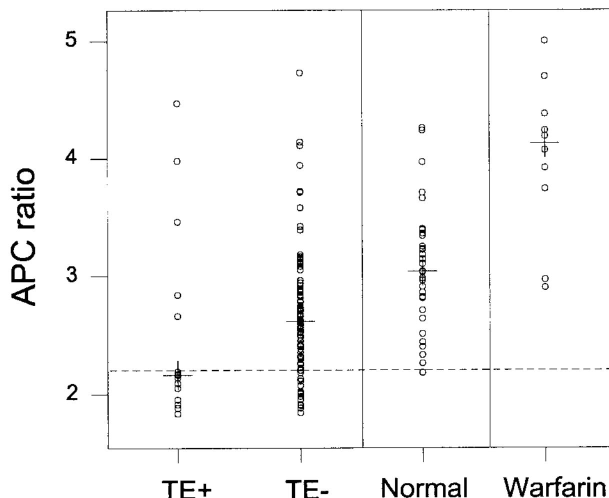

Figure 1. Activated protein C ratios. APC ratios in samples of SLE patients with thrombotic events (TE+) or without (TE−) and normal control subjects. Samples of patients on warfarin had significantly increased APC ratios and were excluded from the analysis. ○ indicates individual APC ratios; +, median values; and –, normal cutoff level.

Table 1. Association between the presence of lupus anticoagulants and activated protein C resistance

LA	APCR positive	APCR negative	Risk (%)	RD (%) (95% CI)	P
Positive	10	10	50	29 (4-53)	.03
Negative	8	30	21		

LA indicates lupus anticoagulants; APCR, activated protein C resistance; RD, risk difference; CI, confidence interval.

Acquired APCR. For the purposes of this study, acquired APCR was defined as APCR not associated with factor V Leiden. Factor V Leiden was tested in the 18 patients with APCR. Sixteen patients tested negative for factor V Leiden. Genetic testing was unsuccessful in 2 patients, one patient with and one patient without TEs. Because APCR was transient in both patients, the patients were assumed to be factor V Leiden negative. Therefore, all 18 patients were considered to have acquired APCR.

Association of APCR with APLAs. Acquired APCR was significantly more frequent in LA-positive patients (10 of 20, 50%) than in LA-negative patients (8 of 38; 21%) (RD, 29%; 95% CI, 4%-53%; $P = .03$) (Table 1). There was no significant association between APCR and individual LA tests. Median APC ratios did not differ significantly between LA-positive (median, 2.55; min-max, 1.91-4.47) and LA-negative patients (median, 2.65; min-max, 1.86-3.84; $P = .96$). APCR was not significantly associated with the presence of ACLAs (RD, 20%; 95% CI, −3% to 43%; $P = .16$).

Association of APCR with TEs. A significant association was observed between the presence of acquired APCR and TEs (Table 2). Seven (39%) of 18 patients with APCR had TEs compared to 2 (5%) of 40 patients without APCR (RD, 34%; 95% CI, 13%-57%; $P = .001$). APCR was present in 5 of the 7 patients who had samples obtained prior to TEs, in 3 of 3 samples taken at the time of event, and in one of 2 patients with evaluable follow-up samples after TEs. Median APC ratios tended to be decreased in patients who had TEs (median, 2.18; min-max, 1.91-4.47) compared to patients without TEs (median, 2.67; min-max, 1.86-3.48; $P = .73$) (Figure 1).

Interaction between APCR and LAs in association with TEs. Table 3 shows the associations of both APCR and LAs with TEs. In APCR-positive patients, 7 of 10 LA-positive patients had TEs, compared to none of the LA-negative patients (RD, 70%; 95% CI, 28-90; $P = .004$). In APCR-negative patients, only 2 of 10 LA-positive patients had TEs, compared to none of the LA-negative patients (RD, 20%; 95% CI −6 to 52; $P = .06$). There was a statistically significant interaction between APCR and LAs in association with TEs as tested by a risk difference model (chi-$\text{square}_{\text{score}}$ 4.2; $P = .04$). These results indicate that the presence of both APCR and LAs was associated with the highest risk of TEs.

Table 2. Association between the presence of activated protein C resistance and thrombotic events

APCR	TE positive	TE negative	Risk (%)	RD (%) (95% CI)	P
Positive	7	11	39	34 (13-57)	.001
Negative	2	38	5		

APCR indicates activated protein C resistance; LA, lupus anticoagulants; RD, risk difference; CI, confidence interval; TE, thrombotic event.

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Table 3. Interaction between activated protein C resistance and lupus anticoagulants in their association with thrombotic events

APCR	LA	TE positive	TE negative	Risk (%)	RD (%) (95% CI)	*P*
Positive	Positive	7	3	70	70 (28-90)	.004
	Negative	0	8	0		
Negative	Positive	2	8	20	20 (−6-52)	.06
	Negative	0	30	0		

Risk difference model: APCR × LA interaction: $\chi^2_{score(1)}$ 4.2; $P = .04$.

APCR indicates activated protein C resistance; LA, lupus anticoagulants; TE, thrombotic event; RD, risk difference; CI, confidence interval.

Protein S, C4b binding protein, and protein C

Plasma concentrations of total and free protein S were similar in patients with SLE compared to normal controls. Total protein S levels (mean ± SD, 0.98 ± 0.12 U/mL) and free protein S concentrations (1.04 ± 0.28 U/mL) in LA-positive patients tended to be increased compared to LA-negative patients (total protein S, 0.91 ± 012 U/mL; $P = .16$; free protein S, 0.92 ± 0.22; $P = .11$). Protein S (total and free) concentrations were not associated with either the presence of ACLAs or APCR. Total protein S levels were similar in patients with or without TEs. Free protein S levels tended to be lower in patients with TEs (0.88 ± 0.29 U/mL) than patients without TEs (0.97 ± 0.24 U/mL; $P = .44$). Total protein S values were all within normal range. Free protein S levels were decreased below normal range in 3 patients (5% of all patients with SLE) who did not have TEs.

Mean C4b binding protein concentrations were similar in patients with SLE (1.13 ± 0.26 U/mL) compared to normal control subjects (1.06 ± 0.18 U/mL) and were similar in the presence or absence of APLAs, APCR, or TEs. Twenty (36%) SLE patients had abnormally increased C4b binding protein concentrations that were equally distributed between patients with or without APLAs, APCR, or TEs.

Protein C concentrations were increased in patients with SLE (1.10 ± 0.28 U/mL) compared to normal controls (0.83 ± 0.18 U/mL; $P < .001$). Protein C concentrations were not different in the presence or absence of APLAs, APCR, or TEs.

von Willebrand factor

Forty patients (69%) had vWF plasma concentrations increased above the upper normal limit. There was no association between increased vWF and the presence of APCR. Also, there was no association between increased vWF and the risk of TEs. Of 3 patients who had samples taken at the time of TEs, 1 had increased and 2 patients had normal vWF levels.

Discussion

The present study is the first to show, in a nonselected cohort of pediatric SLE patients, that (1) a significant association between acquired APCR and LAs, (2) a significant association between acquired APCR and TEs, and (3) a significant interaction between acquired APCR and LAs in association with TEs. These results provide the first clinical evidence that the interference of LAs with the protein C pathway may represent a mechanism of TEs.

Resistance to APC is defined as a decreased anticoagulant response to APC. Hereditary APCR because of the factor V Leiden mutation is clearly associated with an increased risk of thrombosis.[35] Acquired APCR has been reported in patients with APLAs,[7-18] in pregnancy,[36] and with use of oral contraceptives.[34] Although the mechanisms of acquired APCR are not well understood, autoantibodies directed against components of the protein C pathway[37,38] and increased factor VIII levels[34,39] have been suggested. Acquired APCR was associated with TEs in case–control studies of patients who experienced a stroke[40-42] or venous TEs[39]; however, whether there is a causal relationship between acquired APCR and TEs has yet to be determined.

Acquired APCR induced by LAs has been hypothesized to be a possible mechanism of LA-associated TEs.[43] There is good evidence based on in vitro laboratory experiments that plasma or purified immunoglobulin G from LA-positive patients impairs APC-mediated factor Va and factor VIIIa inactivation.[7-11,15,16] Furthermore, studies reported decreased APC ratios in subsets of LA-positive patients negative for factor V Leiden. However, clinical evidence of acquired APCR is limited to small retrospective studies in patient populations selected on the basis of LA positivity.[12-14,17,18] Because of design limitations, these studies could neither clearly demonstrate an association between APCR and LAs nor establish an association between acquired APCR and TEs. The current study of a nonselected cohort of pediatric SLE patients was designed to address these questions.

Acquired APCR was present in 31% of SLE patients, none of whom had factor V Leiden. APCR was significantly associated with LAs, being present in 50% of LA-positive patients. These findings support the hypothesis that LAs induce an acquired APCR. In agreement with previous reports,[13,44] no significant association between APCR and ACLAs was observed in our study.

The current study is the first to show an association between acquired APCR and objectively documented TEs in SLE patients. Patients who had APCR had a 34% (95% CI, 13%-57%) increased risk of TEs compared to APCR-negative patients. These findings highlight the clinical relevance of acquired APCR. Notably, a significant interaction was observed between APCR and LAs in their associations with TEs. Therefore, presence of both APCR and LAs was associated with the highest risk of TEs.

Conclusions from these data need to be drawn cautiously. First, because the study was cross-sectional in design, some patients had TEs identified retrospectively. However, 70% of patients had TEs during prospective follow-up. Second, longitudinal testing of APC ratios was not available from some patients with TEs, as they were on warfarin therapy. Therefore, the temporal relationship between the presence of APCR and TEs cannot fully be established. Consequently, a causal relationship cannot be proven. Finally, the data have been generated from a pediatric population, and whether the findings can be extrapolated to adults has to be determined in future studies.

The most plausible interpretation of the interaction between APCR and LAs is that APCR reflects interference of LAs with the protein C pathway and represents a mechanism for thrombosis. The results of the current study provide the first clinical evidence for this previously hypothesized mechanism.[7-18] An alternative interpretation of the observed interaction is that both factors synergistically increase the risk of TEs by different mechanisms. Such a synergism has previously been suggested for patients with both LAs and the factor V Leiden mutation.[45] However, our finding that APCR and LAs were associated does not support the synergy model. A third interpretation is that APCR may have been the result of TEs in some patients, reflecting an acute phase response.[46] However, this mechanism would not explain the APCR found prior to TEs in the majority of patients.

More in-depth laboratory work, using patient samples from

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well-designed clinical studies, is still needed to accurately define the mechanism of acquired APCR.

Acquired APCR was observed in 21% of LA-negative patients that may have been related to other APLA subtypes not assessed in this study. However, APCR in the absence of LAs was not associated with TEs. Inversely, TEs occurred in 2 LA-positive patients who did not have APCR, suggesting that APCR is not the only mechanism of LA-associated TEs.

For the purposes of this study, the APC ratio was determined by the conventional method, using undiluted patient plasma. In the modified APC ratio, using a dilution of patient plasma in factor V-deficient plasma to improve specificity for factor V Leiden,[47] other factors interfering with APC function (eg, LA antibodies) would be diluted out, and alterations in factor levels would be normalized.[39] A disadvantage of the conventional APC ratio test, however, is that it cannot be used for patients receiving warfarin.[48,49]

In the current study, no significant decrease of the plasma concentrations of total and free protein S, protein C, and C4b binding protein was observed in relation to the presence of APLAs, of APCR, or the occurrence of TEs. These results indicate that APCR is due to functional interference of LA antibodies with the protein C pathway rather than depletion of its components. Also, these observations do not support the hypothesis in the literature that acquired abnormalities in plasma concentrations of these proteins are a mechanism for TEs in APLA-positive patients.[19-26]

In conclusion, pediatric SLE patients positive for both APCR and LAs are at the highest risk of TEs. Acquired APCR may reflect interference of LAs with the protein C pathway that may represent an important mechanism for LA-associated TEs. APCR may serve as a marker to identify LA-positive patients at high risk of TE.

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