PREDICTIVE VALUE OF PERSISTENT VERSUS TRANSIENT ANTIPHOSPHOLIPID ANTIBODY SUBTYPES FOR THE RISK OF THROMBOTIC EVENTS IN PEDIATRIC PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

Christoph Male1,2, Denise Foulon2, Hugh Hoogendoorn2, Patricia Vegh2, Earl Silverman3, Michèle David4, Lesley Mitchell5.
1 Children’s Hospital, Medical University of Vienna, Austria,
2 Henderson Research Centre, McMaster University, Hamilton, Ontario,
3 Hospital for Sick Children, Toronto, Ontario,
4 Hôpital Ste. Justine, Montreal, Quebec,
5 Stollery Children’s Hospital, Edmonton, Alberta, Canada.

Category: Clinical Observations, Interventions, and Therapeutic Trials
Running title: APLA subtypes associated with thrombosis in pediatric SLE

Correspondence: Lesley Mitchell
University of Alberta
Pediatric Thrombosis Program
Stollery Children’s Hospital
Aberhart Centre #1, Room 8213
11402 University Ave
Edmonton, Alberta T6G 2J3
phone 780-439-1071
fax 780-407-1221
LesleyMitchell@cha.ab.ca

Grants: This work was supported by a grant from the Canadian Institute of Health Research (Grant #DOP-51711). C.M. is the recipient of an “Erwin Schroedinger Scholarship” from the Austrian Science Fund. L.M. is a scholar of the Canadian Institutes of Health Research

Authors contribution: C Male and L Mitchell designed the research, analysed data and wrote the paper; D Foulon and H Hoogendoorn provided vital new reagents and designed the research; P Vegh performed the research; E Silverman and M David provided study patients.
ABSTRACT

Study objectives were to determine in children with systemic lupus erythematosus (SLE) the: i) association of antiphospholipid antibody (APLA) subtypes with thrombotic events (TE); ii) predictive value of persistent versus transient antibodies for TE. Cohort study of 58 SLE children in whom lupus anticoagulants (LA), anticardiolipin antibodies (ACLA), anti-β₂-glycoprotein-I (anti-β₂GPI), anti-prothrombin (anti-PT) were assessed on >2 occasions (>3 months apart). Antibodies were classified as persistent (positive >2 occasions) or transient (positive once). Outcomes were symptomatic TE, confirmed by objective radiographic tests identified retrospectively and prospectively.

Seven of 58 patients (12%) had 10 TE; 5 patients had TE during prospective follow-up. Persistent LA showed the strongest association with TE (p<0.0001). Persistent ACLA (p=0.003) and anti-β₂GPI (p=0.002) were significantly associated with TE, anti-PT (p=0.063) showed a trend. Persistent or transient LA and anti-β₂GPI showed similar strength of association, while ACLA and anti-PT were no longer associated with TE. Positivity for multiple APLA subtypes showed stronger associations with TE than for individual APLA subtypes because of improved specificity. Lupus anticoagulant is the strongest predictor of the risk of TE, other APLA subtypes provide no additional diagnostic value. Anticardiolipin antibodies and anti-PT require serial testing, as only persistent antibodies are associated with TE.

Corresponding authors’s email: LesleyMitchell@cha.ab.ca

KEY WORDS: Lupus anticoagulants, anticardiolipin antibodies, anti-β₂-glycoprotein-I, anti-prothrombin, persistent, transient, thrombosis
INTRODUCTION

Antiphospholipid antibodies (APLA) are a heterogeneous group of auto- or alloantibodies directed at plasma protein/phospholipid complexes. APLA frequently occur in autoimmune disease, such as systemic lupus erythematosus (SLE), and there is a well documented association between the presence of APLA and both venous and arterial thromboembolic events (TE). APLA also occur in infectious diseases but these antibodies are usually not associated with TE.

There are two classical subtypes of APLA: the lupus anticoagulants (LA) which are detected by their interference with phospholipid-dependent coagulation tests; and anticardiolipin antibodies (ACLA) which are quantitated by solid-phase immunoassays using phospholipids such as cardiolipin as antigen. Recently, immunoassays have been developed to specifically test for APLA subtypes directed at plasma protein cofactors binding to anionic phospholipids, most importantly anti-β2-glycoprotein (anti-β2GPI) and anti-prothrombin (anti-PT) antibodies. Assays for anti-β2GPI and anti-PT appear to be useful for discriminating APLA found in autoimmune disease from post infectious APLA while the conventional ACLA assay detects both types of antibodies.

A considerable number of clinical studies have investigated the relationship of various APLA subtypes with the occurrence of TE in diverse groups of patients (reviewed in 10,11). The results of these studies are controversial, with some studies suggesting anti-β2GPI or anti-PT to be more closely associated with TE, other studies showing no advantage over testing for LA and ACLA. There are several reasons for these inconsistencies between study results: First, all studies to date were of retrospective design and therefore, were unable to assess the temporal relationship of presence of APLA with the occurrence of TE. There was variable selection of study populations, variable definition of clinical outcomes, and frequently lack of objective diagnosis of TE. Second, there is inherent heterogeneity of APLA and yet unresolved problems with assay standardization for both the classical assays for LA and ACLA as well as for anti-β2GPI or anti-PT. Third, studies did not discriminate between persistent and transient APLA subtypes which is another means of discriminating pathogenic from benign APLA.
We have previously reported a highly significant association of presence of LA with TE in pediatric patients with SLE.\textsuperscript{19} The cohort consisted of non-selected children with SLE who had serial samples taken to test for APLA and prospective outcome assessment for TE. The objectives of the present study, performed in the same cohort, were i) to compare the strengths of association of LA, ACLA, anti-\(\beta_2\)-GPI, anti-PT with the occurrence of TE; and ii) to compare persistent versus transient APLA subtypes in their predictive value for the risk of TE.

**METHODS**

**Study design**
The study design was an ambidirectional (retrospective and prospective) cohort study of non-selected children with SLE.

**Study population**
The study cohort overlaps with the cohorts of previously published studies.\textsuperscript{19-21} Patients were managed at the rheumatology clinics of two tertiary care centers, Hospital for Sick Children, Toronto, and Hôpital Ste. Justine, Montreal, Canada. All patients fulfilled the American Rheumatism Association criteria for SLE.\textsuperscript{22} Consecutive children with SLE were recruited over a period of 2.8 years, except for 3 patients who did not consent. A disease activity index was determined at each study visit using a validated scoring system.\textsuperscript{23} 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. The study was approved by both hospitals institutional review boards.

**Evaluation for thromboembolic events**
All patients with SLE were followed prospectively for TE over a period of 4.5 to 7.5 years for clinically symptomatic TE. All TE were confirmed by objective radiographic test. Additionally, all patients were assessed retrospectively for TE that had occurred prior to study entry. Patients and/or their parents were interviewed by the nurse study
coordinator who was unaware of the laboratory results. A detailed history of previous TE was obtained, and a standardized questionnaire completed. A retrospective chart review was also performed and if a TE was identified, the radiographic test that had been employed to confirm TE was reviewed by an independent panel of experts.

Accepted radiographic tests for TE were 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 TE in the central nervous system.

**Laboratory testing**

All study patients had blood samples taken at study entry and on a separate occasion during follow-up at least three months after the initial visit. Patients who developed acute TE during the study period had blood drawn at the time of the event and a third time at least three months from the acute event. Patients in whom a blood sample was available from one occasion only were excluded (n = 5). Patients in whom the results of a specific APLA subtype were available from only one sample were not included in the analysis for the respective APLA subtype. Normal controls had blood samples drawn on one occasion.

Venous blood was drawn into vacutainer tubes (Becton Dickinson) containing 0.105 M buffered citrate solution for a final ratio of one part anticoagulant to nine parts of blood. Plasma was immediately separated from cellular elements by double centrifugation at 1500g for 15 minutes at 4°C. Samples to be tested for LA were filtered through a 0.2 micron screen (Acrodisk, Gelman Sciences, Ann Arbor, MI) to achieve complete platelet removal. Plasma was subsequently aliquotted and frozen at -70°C. All assays were performed in one central laboratory at the Pediatric Thrombosis Research Laboratory by the same technologist who was blinded to the patients’ clinical status. Lot numbers of all reagents were constant during the study.

**Assays for lupus anticoagulants and anticardiolipin antibodies**

Testing for LA was performed according to the criteria of the Subcommittee on APLA of the International Society of Thrombosis and Haemostasis as previously described in detail: \(^{19}\) i) screening assays included the kaolin clotting time, dilute
APTT, dilute prothrombin time, and two dilute Russel viper venom times; ii) all tests were repeated in a 1:1 mix with normal plasma; iii) positive samples had confirmation assays performed with the same reagent in the presence of excess phospholipids. At least one test system had to be positive in all three steps for a patient to be considered LA positive.

Anticardiolipin antibodies were measured by commercial ELISA (Advanced Biological Products Inc., Mississauga, Ontario, Canada). This assay is calibrated against standards from the Antiphospholipid Standardization Laboratory, University of Louisville, Kentucky, USA. Patient results were considered abnormal if values were three standard deviations above the mean of the age-matched controls.

**Assay for anti-β2-glycoprotein antibodies**

Anti-β2GPI was measured by in-house ELISA adapted from previously described methods.\(^5,9,25-27\) Polystyrene ELISA plates, \(\gamma\)-irradiated by the manufacturer (Immunolon-4, high-binding, Dynex, Chantilly, VA, USA), were coated overnight at 4\(^\circ\)C with 100 \(\mu\)l/well of purified human \(\beta_2\)-glycoprotein (Affinity Biologicals, Hamilton, Ontario, Canada) in a concentration of 5 \(\mu\)g/ml in phosphate buffered saline, pH 7.4 (PBS, 8 g NaCl, 2.9 g Na2HPO4.12H2O, 0.2 KCL, 0.2 Kh2PO4 per litre). Wells were blocked with 150 \(\mu\)l PBS containing 1% bovine serum albumin (PBS-1%BSA; BSA >99% purity, Sigma, St. Louis, MO, USA) for 1 hour at room temperature. Then plates were washed 4 times with PBS containing 0.1% Tween20. Patient samples were diluted 1:100 in PBS-1%BSA-0.1%Tween20, 100 \(\mu\)l applied to each well and incubated for 2 hours at room temperature. Then wells were washed 4 times.

Horseradisch peroxidase conjugated goat anti-human IgG (\(\gamma\)-chain-specific, Sigma) and IgM (\(\mu\)-chain-specific, Sigma) were diluted in PBS-1%BSA-0.1%Tween at 1:4000 and 1:6000, respectively, and 100 \(\mu\)l of detecting antibody solution were applied to each well and incubated for 1 hour at room temperature. Then plates were washed 4 times. Colour development was achieved by adding 5 mg ortho-phenylene-diamine to 12 ml citrate-phosphate buffer, pH 5.0, containing 0.03% \(\text{H}_2\text{O}_2\) (v/v), 100\(\mu\)l to each well and incubated for 10 minutes. The reaction was stopped by the addition of 50 \(\mu\)l 2.5 M \(\text{H}_2\text{SO}_4\) at timed intervals. Absorbances were read at 490 nm on a microplate reader (Molecular Devices, Spectra Max Plus 384, Sunnyvale, CA, USA).
Assay for anti-prothrombin antibodies

Anti-PT were measured by in-house ELISA adapted from previously described methods. The assay was performed as described for the assay for anti-β2GPI with a few modifications: γ-irradiated polystyrene ELISA plates (Immunolon-4) were coated overnight at 4° with 100 µl/well of purified human prothrombin (Enzyme Research Laboratories, South Bend, Indiana, USA), 10 µg/ml in PBS, pH 7.4. Wells were blocked with 200 µl PBS containing 2% for 1 hour at room temperature and then washed 4 times with PBS-0.1%Tween20. Patient plasma samples were diluted to 1:100 in PBS-1%BSA-0.1%Tween20, 100 µl applied to each well in duplicate, and incubated for 2 hours at room temperature. After washing 4 times, hHorseradish peroxidase conjugated goat anti-human IgG and IgM 1:4000 in PBS-1%BSA-0.1%Tween, 100 µl/well, were incubated for 1 hour at room temperature. After washing 4 times, 5 mg ortho-phenylene-diamine in 12 ml citrate-phosphate buffer, pH 5.0, containing 0.03% H2O2 (v/v), 100µl were added to each well and incubated for 20 minutes. The reaction was stopped by the addition of 50 µl 2.5 M H2SO4 at timed intervals. Absorbances were read at 490 nm. Performing the assay in a PBS buffer system containing Ca++ (2 mM) gave similar readings as in the Ca++-free buffer in a series of positive and negative control samples.

For both anti-β2GPI and anti-PT, non-coated wells were run on each plate and absorbances subtracted from antigen-coated wells to control for non-specific binding. All experiments included controls of known samples with high, intermediate, and low antibody levels. For each APLA subtype and isotype, one positive control was chosen as internal standard and the mean absorbance value measured for this standard was set at 100%. Absorbance values measured from unknown samples were expressed as percentage of the internal standard. For anti-β2GPI IgG, external calibrators for anti-β2-GPI IgG were kindly provided by Dr. V. DeBari, Paterson, New Jersey. Values obtained with these calibrators in our assay compared well with those reported by Dr. DeBari’s group and an internal standard with comparable reactivity was chosen. Intra-assay coefficients of variations for anti-β2GPI IgG and IgM were 9.4% and 8.1%, and inter-assay coefficients of variations were 6.5% and 3.4%, respectively. Intra-assay coefficients of variations for anti-PT IgG and IgM were 10.8% and 9.2%, and
inter-assay coefficients of variations were 5.8% and 4.5%, respectively.

Normal reference values were established by testing 20 healthy adults and 36 healthy children ages 1-18 years. Upper normal range cutoffs were derived from the 99\textsuperscript{th} percentile of the reference population. Reference values for anti-β\textsubscript{2}GPI IgG and IgM were slightly lower for children than for adults, and reference values for anti-PT IgG and IgM were higher for children than for adults. Within children, there were no differences between age groups for either antibody subtype or isotype. Cutoffs for children for anti-β\textsubscript{2}GPI IgG and IgM were both 9% of the internal standard. Cutoffs for children for anti-PT IgG and IgM were 20% and 40%, respectively.

### Classification of persistent versus transient positivity of antibodies

Patients were classified as ‘persistently positive’ for an APLA subtype if positive on two or more occasions; ‘transiently positive’ if positive on one occasion only; and negative if negative on all occasions\textsuperscript{32,33}. In the analysis, persistently positive patients were compared with all positive patients (i.e. patients persistently or patients transiently positive) for an APLA subtype in their association with the occurrence of TE.

### Statistical analysis

Statistical analysis was performed using Minitab Statistical package, version 13, and SAS, version 8.2. Values of antibody titres were log-transformed because of skewed distributions. Antibody titres were compared between groups by t-test (first versus follow-up sample). Repeated-measures-analysis of variance was used to compare antibody titres between LA positive and negative and TE positive and negative patients, and mixed models to assess correlations between individual antibody titres while accounting for repeat samples in each patient. Frequencies of antibody positivity were analyzed in relation to frequencies of patients with or without TE using contingency tables. Associations between antibody presence and occurrence of VTE were analyzed using Fisher’s exact test and summarized by odds ratios (OR) and 95% confidence intervals (95\%CI). Multivariable analyses to simultaneously test for the associations of several APLA subtypes with the absolute risk of VTE were performed using exact logistic regression. All tests were two-sided.
RESULTS

Study population
Fifty-eight children with SLE were studied, 48 females and 10 males. Their median age was 14.5 years with a range from 4 to 19 years. All but 2 patients with SLE were receiving corticosteroids during the study period. Oral contraceptives were taken by 2 patients who did not have a thrombotic event. There were no pregnancies. Disease activity scores were similar in patients with thrombotic events compared to other patients. The control population consisted of 36 concurrent healthy children, 18 females and 18 males. Their median age was 10 years with a range from 1 to 19 years.

Thrombotic events
Seven of the 58 patients with SLE (12%) had a total of 10 TE. Three patients had TE (n=4) before they entered the study, 5 patients had TE (6) during prospective follow-up. Five events were deep venous TE or pulmonary embolism, and 5 events involved the central nervous system of which 3 events were sinovenous thromboses and 2 arterial strokes.

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

APLA subtype titres
Median time from initial to last follow-up blood samples was 5.5 months (minimum 2.8; maximum 34.5). In patients who had TE identified retrospectively, the time from TE to initial blood samples was 3.4 and 46 months, respectively, and in patients with TE identified prospectively, the time from the preceding sample to TE was median 10.2 months (4.1; 46).
Titres of ACLA, anti-β_2_2GPI IgG, and anti-PT IgG did not differ in relation to time of sampling. Titres of anti-β_2_2GPI IgM showed a small decrease from initial to follow-up samples, and anti-PT IgM a small increase (data not shown). Titres of ACLA, anti-β_2_2GPI, and anti-PT IgG and IgM showed no correlation with SLE disease activity scores (data not shown). APLA subtype titres were all significantly increased in LA positive compared to LA negative patients accounting for repeat sampling in individual patients. There were significant correlations between most individual APLA subtypes, except for a lack of correlation between anti-PT IgG and the IgM of other subtypes, and between anti-PT IgM and the IgG of other subtypes (data not shown).

Table 1 shows titres of APLA subtypes IgG and IgM comparing patients with and without TE. ACLA, anti-β_2_2GPI, and anti-PT IgG titres were significantly increased in patients with TE. Among patients with TE, samples taken close to a TE tended to have increased titres of APLA subtypes compared to samples taken at other times which reached significance for anti-β_2_2GPI IgM (median 34 versus 18 %, p=0.05). In samples taken during warfarin treatment (n=4), LA screening tests tended to have increased values compared to samples in patients with TE when they were not on warfarin (n=15). However, plasma mixing studies and confirmation tests were not different. All patients with TE were classified as persistently positive for LA, in samples obtained on or off warfarin.

**Frequencies of APLA subtypes**

The figure shows frequencies of presence of APLA subtypes, separate for IgG and IgM isotypes, in children with SLE. Frequencies are given for persistently positive antibodies, transiently positive antibodies, and all positive antibodies. LA were persistent in 26%, transient in 10%, and persistent or transient in 36% of patients. ACLA (IgG or IgM isotypes combined) were persistent in 21%, transient in 29%, and persistent or transient in 50% of patients. Anti-β_2_2GPI (IgG or IgM) were persistent in 31%, transient in 10%, and persistent or transient in 41% of patients. Anti-PT (IgG or IgM) were persistent in 27%, transient in 25%, and persistent or transient in 52% of patients.
Association of presence of APLA subtypes with thrombosis

Table 2 shows the association of presence of APLA subtypes (IgG or IgM combined) with the risk of TE. Associations are compared for persistently positive antibodies versus persistently or transiently positive antibodies. Presence of LA showed the strongest association with TE with an odds ratio that was infinite because no patient negative for LA had TE. Of the LA assays used, the dilute prothrombin time best predicted the risk of TE as previously reported in more detail. Persistently positive ACLA and anti-β2GPI were significantly associated with TE, and there was borderline significance for anti-PT. Considering persistently or transiently positive antibodies, the strength of association remained about the same for LA and anti-β2GPI. In contrast, persistent or transient ACLA and anti-PT were no longer significantly associated with TE.

Table 3 shows sensitivity, specificity, and negative and positive predictive values of presence of APLA subtypes (IgG and IgM combined) for the risk of TE. Persistently positive antibodies are compared with persistently or transiently positive antibodies. Presence of LA had the highest predictive power for TE with a sensitivity and negative predictive value of 100%. The specificity for TE was 84% for persistently positive LA compared to 73% for all positive LA. Persistently positive ACLA and anti-β2GPI, had about equal predictive power. Considering persistent or transient antibodies decreased specificity and positive predictive value, particularly for ACLA, because of a large proportion of transient ACLA that were ‘false positive’, i.e. not associated with TE. Anti-PT had limited predictive value for the risk of TE.

Table 4 shows the association of presence of APLA subtypes, separate for IgG and IgM isotypes, with the risk of TE. Persistently positive antibodies are compared with persistently or transiently positive antibodies. The strongest association was observed for ACLA IgM and anti-β2GPI IgG with significant associations both for persistently positive and persistently or transiently positive antibodies, due to high specificity. In contrast, only persistently positive ACLA IgG were significantly associated with TE. Persistently or transiently positive ACLA IgG were not significantly associated with TE because of low specificity, i.e. a large proportion of ‘false positive’ transient ACLA IgG. Anti-PT IgG and IgM showed no significant association with TE.
In multivariable analysis simultaneously testing for the associations of all individual APLA subtypes with TE, LA remained the only significant predictor of the risk of TE. Multiple positivity for two or more APLA subtypes did not improve the strength of association with TE compared to LA. However, multiple positivity for most APLA subtype combinations showed stronger associations with TE than for individual ACLA, anti-β2GPI, or anti-PT positivity because of improved specificity (table 5).

DISCUSSION

In patients with SLE, there is a significant association between the presence of APLA and both venous and arterial TE. However, APLA are a heterogeneous group of antibodies and not all patients with APLA develop TE. Therefore, there is a need to identify APLA subtypes that best predict the risk of TE. Assays for APLA subtypes directed at specific plasma protein cofactors have been developed, i.e. anti-β2GPI and anti-PT, but the literature thus far is controversial whether these assays provide advantages over the classical assays for LA and ACLA. Moreover, there is no information whether the persistent presence of APLA subtypes strengthens the association with TE.

The present study was performed in a non-selected cohort of pediatric SLE patients who had serial testing of APLA subtypes and prospective assessment for TE which were confirmed by objective tests. The study results demonstrate that i) LA was the strongest predictor of the risk of TE and the only factor remaining significant in multivariable analysis; ii) persistent ACLA and anti-β2GPI, were significantly associated with the risk of TE, and anti-PT showed borderline significance. Considering persistently or transiently positive antibodies did not change the strength of association for LA and anti-β2GPI, but ACLA and anti-PT were no longer associated with TE.

The findings of the current pediatric study with respect to LA are consistent with those of two systematic reviews of the literature. The first was a meta-analysis including 18 studies in adult patients with SLE demonstrating that LA shows stronger association with TE than ACLA. A more recent systematic review included 25 studies in adult
patients with or without SLE.\textsuperscript{10} The review confirmed LA as strong risk factor for TE but did not find unequivocal evidence for a significant association of ACLA with TE. In the present pediatric study, the association of LA with TE was even stronger than that reported for adults. The high negative predictive value (100\%) for LA reflects that no children negative for LA had TE. In contrast, of adult SLE patients negative for APLA, approximately 40\% had TE.\textsuperscript{32} Therefore, when studying the association of APLA subtypes with TE, a pediatric cohort is advantageous, as other confounding risk factors of TE are rare in children, such as contraceptive use, pregnancy, cancer, atherosclerotic disease, age. Since there was no comparison with adult SLE patients in this study, the effect of such risk factors could not directly be assessed. The specificity (84\%) of LA for the risk of TE seen in the present study was also higher than reported in previous studies in adults.

Another recent systematic review summarized studies available to date (n=32) on anti-\(\beta_2\)GPI and/or anti-PT and their association with clinical complications.\textsuperscript{11} The main message from that review is that there is considerable heterogeneity in designs of previous studies that did not allow a formal meta-analysis. All studies to date on anti-\(\beta_2\)GPI or anti-PT have been retrospective with the potential of selection in patient accrual, information bias in the assessment of clinical outcome, and the inability to assess a temporal relationship of presence of APLA with the occurrence of TE. Studies had variable definition of study populations including patients with SLE, with primary antiphospholipid syndrome, or patients selected based on APLA positivity. Moreover, clinical outcomes were poorly defined and frequently not restricted to TE, and many studies lacked objective diagnosis of TE. Not surprisingly, the systematic review revealed large heterogeneity in the results of previous studies, with inconsistent information comparing the relative strength of associations of various APLA subtypes with TE. Only few studies have performed multivariable analysis to assess the joint contribution of individual APLA subtypes for prediction of the risk of TE.

Our discussion of previous studies comparing several APLA subtypes will be restricted to studies that investigated SLE patients only, in non-selected cohorts, and focused on TE as clinical outcomes.\textsuperscript{27,30,34-38} In these studies, each of LA, ACLA, anti-\(\beta_2\)GPI, and anti-PT were in most instances associated with TE in univariate analysis.
Multivariable analysis, performed in only part of studies, revealed inconsistent results, showing LA only, anti-β2GPI only, or LA in combination with ACLA or anti-PT significantly associated with TE.\textsuperscript{27,36-38} The relative strengths of associations varied depending on antibody isotype and whether arterial or venous TE were considered. However, there was no consistent association between a specific APLA subtype or isotype and either arterial or venous TE. One pediatric study assessed LA, ACLA, and anti-β2GPI in a heterogeneous cohort of patients with SLE, SLE-like syndrome, primary antiphospholipid antibody syndrome, or APLA positivity.\textsuperscript{39} The study did not find an association of any APLA subtype with TE, neither in the total cohort nor in the subgroup of children with SLE.

The main advantage of the present study over any previous study on anti-β2GPI and anti-PT is its ambidirectional study design. The majority of patients (71\%) had TE identified during prospective follow-up allowing to establish a temporal relationship with presence of APLA. Each of the APLA subtypes were demonstrated to be associated with TE. Anti-β2GPI and persistent ACLA showed about equal strength of association with TE. In multivariable analysis, LA remained the only significant risk factor for TE. The functional assays for LA may best reflect the pathogenic interference of APLA with the coagulation system. The APLA subtypes tested by immunoassays had no additional diagnostic value to LA. However, immunoassays for APLA subtypes may still be important in instances when coagulation assays for LA are not feasible, such as in patients receiving oral anticoagulants. In this situation, ACLA IgM and anti-β2GPI IgG have the best predictive value because of their high specificity.

The second advantage of the present study was that repeat samples were obtained from all patients allowing to compare persistent versus transient APLA subtypes in their predictive value for the risk of TE. According to current consensus, the antiphospholipid antibody syndrome is defined by the persistent presence APLA in combination with specific clinical complications such as TE.\textsuperscript{40} Persistent APLA positivity is defined as the presence of the antibodies on two occasions a minimum of six weeks apart. Persistent APLA are considered to reflect a chronic autoimmune process and are more likely associated with TE. Transient APLA either reflect low disease activity or represent post infectious antibodies, and are frequently not
associated with TE. Particularly in children, frequent infections may trigger transient APLA that rarely lead to clinical complications. The improved predictive value of persistent APLA has thus far only been shown for LA and ACLA. Studies on anti-β2GPI or anti-PT to date have not provided data to discriminate persistent from transient antibodies. Only one study reported repeat testing of anti-β2GPI in SLE patients but all anti-β2GPI observed in that study were persistent.

In the present study, persistent LA, ACLA, anti-β2GPI, and anti-PT were all associated with the risk of TE. Considering persistently or transiently positive antibodies decreased specificity but the overall strength of association with TE remained about the same for LA and anti-β2GPI. In contrast, ACLA and anti-PT were no longer associated with TE when transient antibodies were included. Thus, anti-β2-GPI are advantageous over ACLA, because a single positive test is associated with TE. Anticardiolipin antibodies and anti-PT require repeat testing as only persistent antibodies are significantly associated with TE.

Another interesting finding of the study was that positivity for multiple APLA subtypes showed stronger associations with TE than for individual ACLA, anti-β2GPI, or anti-PT. These findings correspond with those of a recently published study in adults. The strong associations result from improved specificity of multiple APLA positivity for the risk of TE, but sensitivity is decreased because fewer patients have combinations of several APLA subtypes. In the present cohort, LA as individual test still showed the strongest association with TE.

In conclusion, LA remains the strongest predictor of the risk of TE in patients with SLE, while other APLA subtypes provide no additional diagnostic value. If testing for LA is not feasible, ACLA IgM and anti-β2GPI IgG have the highest predictive value. Lupus anticoagulant and anti-β2GPI require only single testing, but ACLA and anti-PT require repeat testing.
ACKNOWLEDGEMENTS

The authors would like to thank Dr. V. DeBari, Dept. of Medicine, Seton Hall University School of Graduate Medical Education, Paterson, New Jersey, USA, for providing reference calibrators for anti-β2GPI IgG. We also thank Dr. M. Crowther, Dr. J. Denburg, and K. Stewart, Dept. of Medicine, McMaster University, Hamilton, Ontario, Canada, for providing APLA positive sera used as standards and controls in anti-β2GPI and anti-PT ELISAs.
REFERENCES


Table 1. APLA subtype IgG and IgM titres in SLE patients with and without thrombotic events.

<table>
<thead>
<tr>
<th>APLA subtype</th>
<th>Thrombosis positive (n=7)</th>
<th>Thrombosis negative (n=51)</th>
<th>Antibody titres(^1) (median (min – max))</th>
<th>F-value(^2)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACLA IgG</td>
<td>24.0 (4.5 - 65)</td>
<td>11.0 (1.0 - 65)</td>
<td>6.1</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>ACLA IgM</td>
<td>31.0 (2.5 - 100)</td>
<td>12.0 (2.5 - 100)</td>
<td>6.1</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Anti-β(_2)-GPI IgG</td>
<td>10.5 (2.7 – 91)</td>
<td>3.5 (0 - 127)</td>
<td>11.7</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Anti-β(_2)-GPI IgM</td>
<td>21.0 (0.3 - 54)</td>
<td>4.3 (0.4 - 78)</td>
<td>3.9</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>Anti-PT IgG</td>
<td>23.2 (5.9 - 135)</td>
<td>11.7 (1.4 - 134)</td>
<td>3.7</td>
<td>0.059</td>
<td></td>
</tr>
<tr>
<td>Anti-PT IgM</td>
<td>22.0 (3.9 - 92)</td>
<td>13.8 (1.2 - 111)</td>
<td>0.6</td>
<td>0.453</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) ACLA titres are given in GPL and MPL units based on standards from the Antiphospholipid Standardization Laboratory, University of Louisville, Kentucky, USA; anti-β\(_2\)-GPI and anti-PT titres represent percentages of internal standards.

\(^2\) The comparison is based on repeated-measures-analysis-of-variance accounting for repeat sampling in each patient (initial and follow-up samples), performed on log-transformed values.

Abbreviations: APLA, antiphospholipid antibodies; IgG, immunoglobulin G; IgM, immunoglobulin M; ACLA, anticardiolipin antibodies; anti-β\(_2\)-GPI, anti-β\(_2\)-glycoprotein-I antibodies; anti-PT, anti-prothrombin antibodies; min, minimum; max, maximum.
Figure. Frequencies of APLA subtypes, IgG and IgM, in children with SLE.
Frequencies are shown for persistent, transient, and all positive (persistent or transient) antibodies.
Table 2. Association of presence of APLA subtypes, IgG or IgM combined, with thrombotic events; persistent versus persistent or transient antibodies.

<table>
<thead>
<tr>
<th>APLA subtype</th>
<th>Persistent</th>
<th></th>
<th>Persistent or Transient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>p-value ( ^1 )</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td>Lupus anticoagulant</td>
<td>( \infty ) (6.0 - ( \infty ))</td>
<td>&lt;0.0001</td>
<td>( \infty ) (3.2 - ( \infty ))</td>
</tr>
<tr>
<td>ACLA (IgG or IgM)</td>
<td>15.7 (2.5 - 97)</td>
<td>0.003</td>
<td>2.8 (0.5 - 16)</td>
</tr>
<tr>
<td>Anti-( \beta_2 )-GPI (IgG or IgM)</td>
<td>22.0 (2.3 - 207)</td>
<td>0.002</td>
<td>12.0 (1.3 - 110)</td>
</tr>
<tr>
<td>Anti-PT (IgG or IgM)</td>
<td>4.7 (0.9 - 25)</td>
<td>0.063</td>
<td>2.6 (0.5 - 15)</td>
</tr>
</tbody>
</table>

Abbreviations: as in table 1; OR, odds ratio; 95%CI, 95% confidence interval.

\( ^1 \) Fisher's exact test.
Table 3. Predictive value of presence of APLA subtypes, IgG or IgM combined, for the risk of thrombotic events; persistent versus persistent or transient antibodies.

<table>
<thead>
<tr>
<th>APLA subtype</th>
<th>Sens</th>
<th>Spec</th>
<th>NPV</th>
<th>PPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lupus anticoagulant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Persistent</td>
<td>100</td>
<td>84</td>
<td>100</td>
<td>47</td>
</tr>
<tr>
<td>Persistent or transient</td>
<td>100</td>
<td>73</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td><strong>ACLA (IgG or IgM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Persistent</td>
<td>71</td>
<td>86</td>
<td>96</td>
<td>42</td>
</tr>
<tr>
<td>Persistent or transient</td>
<td>71</td>
<td>53</td>
<td>93</td>
<td>17</td>
</tr>
<tr>
<td><strong>Anti-β2-GPI (IgG or IgM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Persistent</td>
<td>86</td>
<td>79</td>
<td>97</td>
<td>40</td>
</tr>
<tr>
<td>Persistent or transient</td>
<td>86</td>
<td>67</td>
<td>97</td>
<td>30</td>
</tr>
<tr>
<td><strong>Anti-PT (IgG or IgM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Persistent</td>
<td>57</td>
<td>78</td>
<td>92</td>
<td>29</td>
</tr>
<tr>
<td>Persistent or transient</td>
<td>71</td>
<td>51</td>
<td>92</td>
<td>19</td>
</tr>
</tbody>
</table>

Abbreviations: as in table 1; sens, sensitivity; spec, specificity; NPV, negative predictive value; PPV, positive predictive value.
Table 4. Association of presence of APLA subtypes, separate for IgG and IgM isotypes, with thrombotic events; persistent versus persistent or transient antibodies.

| APLA subtype | Persistent | | Persistent or Transient |  
| | OR (95%CI) | p-value | OR (95%CI) | p-value |  
| ACLA IgG | 10.0 (1.8 - 56) | 0.013 | 2.8 (0.5 – 16) | 0.211 |  
| ACLA IgM | 32.7 (4.2 - 256) | 0.001 | 10.0 (1.8 – 56) | 0.013 |  
| Anti-β2-GPI IgG | 17.3 (2.6 - 116) | 0.005 | 10.6 (1.7 - 65) | 0.010 |  
| Anti-β2-GPI IgM | 6.7 (1.2 – 37) | 0.036 | 9.2 (1.5 - 55) | 0.015 |  
| Anti-PT IgG | 3.5 (0.7 – 19) | 0.155 | 3.1 (0.6 – 18) | 0.179 |  
| Anti-PT IgM | 3.6 (0.3 - 46) | 0.358 | 2.6 (0.4 - 17) | 0.292 |  

Table 5. Association of presence of multiple APLA subtypes with thrombotic events; persistent versus persistent or transient antibodies.

<table>
<thead>
<tr>
<th>Combination of APLA subtypes (IgG or IgM)</th>
<th>Persistent</th>
<th>Persistent or Transient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>LA + ACLA</td>
<td>29.4 (4.3 – 203)</td>
<td>0.001</td>
</tr>
<tr>
<td>LA + anti-β₂-GPI</td>
<td>40.0 (5.3 – 299)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LA + anti-PT</td>
<td>37.5 (3.1 – 449)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACLA + anti-β₂-GPI</td>
<td>40.0 (5.3 – 299)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACLA + anti-PT</td>
<td>15.7 (2.6 – 96)</td>
<td>0.003</td>
</tr>
<tr>
<td>anti-β₂-GPI + anti-PT</td>
<td>21.3 (3.2 – 142)</td>
<td>0.002</td>
</tr>
<tr>
<td>LA + ACLA + anti-β₂-GPI</td>
<td>32.7 (4.2 – 256)</td>
<td>0.001</td>
</tr>
<tr>
<td>LA + ACLA + anti-PT</td>
<td>37.5 (3.1 – 449)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LA + anti-β₂-GPI + anti-PT</td>
<td>37.5 (3.1 – 449)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACLA + anti-β₂-GPI + anti-PT</td>
<td>32.7 (4.2 – 256)</td>
<td>0.001</td>
</tr>
<tr>
<td>LA + ACLA + anti-β₂-GPI + anti-PT</td>
<td>37.5 (3.1 – 449)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Predictive value of persistent versus transient antiphospholipid antibody subtypes for the risk of thrombotic events in pediatric patients with systemic lupus erythematosus

Christoph Male, Denise Foulon, Hugh Hoogendoorn, Patricia Vegh, Earl Silverman, Michele David and Lesley Mitchell