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Annexin A5 anticoagulant activity in children with systemic lupus erythematosus and the association with antibodies to domain I of β 2-glycoprotein I

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Abstract

Children with systemic lupus erythematosus (SLE) have a high prevalence of antiphospholipid (aPL) antibodies and are at increased risk for aPL-related thrombosis. We investigated the association between annexin A5 anticoagulant activity and antibodies to the domain I portion of β 2-glycoprotein I (anti-DI antibodies), and propose a potential mechanism for the pathogenesis of aPL-related thrombosis. Using samples from 183 children with SLE collected during the Atherosclerosis Prevention in Pediatric Lupus Erythematosus (APPLE) trial, we examined resistance to the anticoagulant effects of annexin A5, using the annexin A5 resistance (A5R) assay, and evaluated for anti-DI IgG antibodies. Children with SLE had higher frequency of anti-DI antibodies ($p=0.014$) and significantly reduced A5R compared to pediatric controls: mean A5R = 172 ± 30 % versus 242 ± 32 % ($p<0.0001$). Children with SLE and positive anti-DI antibodies had significantly lower mean A5R levels compared to those with negative anti-DI antibodies: mean A5R = 155 ± 24 % versus 177 ± 30 % ($p<0.0001$). In multivariate analysis, anti-DI antibodies ($p=0.013$) and lupus anticoagulant (LA) ($p=0.036$) were both independently associated with reduced A5R. Children with SLE have significantly reduced annexin A5 anticoagulant activity that is associated with the presence of LA and anti-DI antibodies.

Antiphospholipid antibody syndrome (APS) is an autoimmune disease characterized by the presence of antiphospholipid (aPL) antibodies in close association with vascular thrombosis and miscarriage. In children, this syndrome can occur in isolation (primary APS) or more commonly, in association with other diseases such as systemic lupus erythematosus (SLE). The presence of aPL antibodies in these children often raises significant concern regarding the potential risk for future thrombotic events. Prior studies have reported wide ranges in the prevalence of lupus anticoagulant (LA) and anti-cardiolipin antibodies (aCL) in pediatric SLE, occurring in 6–62% and 19–87% of patients respectively; with the rate of thrombosis ranging from 12–17%^{1–7}. Predicting the true risk of thrombosis, however, has thus far been difficult to quantitate; in part, due to a lack of a comprehensive understanding regarding the mechanism by which aPL antibodies result in the clinical manifestations of APS.

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One mechanism that has been proposed to explain the underlying pathophysiology of APS involves antibody-mediated resistance to a potent anticoagulant protein, annexin A5. Annexin A5 proteins crystallize over potentially thrombogenic cell surfaces, forming an anticoagulant shield, thereby blocking availability of anionic phospholipids from phospholipid-dependent coagulation reactions^{8–10}. In the presence of aPL antibodies, the binding of annexin A5 to phospholipids is disrupted, exposing anionic phospholipids; thereby allowing for accelerated coagulation reactions and increased clotting to occur. This mechanism can be detected through a novel functional assay, the annexin A5 resistance assay (A5R). This assay measures coagulation times in the presence and absence of annexin A5 and expresses the results as an anticoagulant ratio¹¹. A reduction of this ratio, as compared to healthy controls, reflects resistance to annexin A5 anticoagulant activity and has been demonstrated in both adults and children with persistently positive aPL antibodies^{7, 11}.

Additionally, it has become increasingly evident that the phospholipid binding protein, β 2-glycoprotein I (β 2GPI), is a major antigenic target for thrombogenic aPL antibodies^{12–14}. In particular, the presence of anti- β 2GPI antibodies in association with a positive LA test appears to demonstrate the greatest association with clinical manifestations¹². It has been shown that this population of anti- β 2GPI antibodies binds specifically to an epitope on the domain I portion of β 2GPI and correlates strongly with thrombosis^{15, 16}. In addition, the presence of IgG antibodies to epitope including glycine40-arginine43 of the domain I portion of β 2GPI (anti-DI antibodies) has been associated with significantly reduced annexin A5 anticoagulant activity in adults with aPL antibodies¹⁷. In the current study, we investigated the association between annexin A5 anticoagulant activity and anti-DI antibodies in a cohort of children with SLE; reasoning that a high association may aid in our understanding about the underlying pathophysiology of aPL antibodies and provide a platform for further investigation into which pediatric patients with SLE may be at greatest future risk of thrombosis.

Patients and Methods

Study population

We used available data from 183 children and adolescents from the Atherosclerosis Prevention in Pediatric Lupus Erythematosus (APPLE) trial; a prospective, multi-center, randomized, placebo controlled trial of atorvastatin in pediatric SLE¹⁸. Enrollment for this trial was completed in 2006. As previously described, this well characterized cohort of children, aged 10 to 21 years, all met American College of Rheumatology (ACR) revised criteria for SLE, weighed ≥ 25 kg, were able to complete questionnaires in English or Spanish, were willing to comply with the American Heart Association Therapeutic Lifestyle Changes diet and were on approved methods of birth control¹⁹. Exclusion criteria included active nephrotic syndrome, myositis, liver disease, renal insufficiency and hypercholesterolemia (total cholesterol level > 350 mg/dl). In this study, all participants were treated with folate, hydroxychloroquine (HCQ) and low dose aspirin. For our sub-analysis of this cohort, clinical variables of interest included a baseline assessment of demographic features (age, gender, race, and ethnicity), family history, randomly assigned

treatment status (atorvastatin versus placebo), medication usage prior to enrollment (corticosteroids, HCQ, aspirin), disease duration, SLE disease activity index (SLEDAI), history of aPL associated clinical manifestations (confirmed arterial/venous thrombosis, miscarriage, Raynaud's phenomenon, thrombocytopenia, livedo reticularis, neurologic manifestations, heart valve disease and nephropathy), and evidence of other underlying risk factors for thrombosis (renal disease, hormonal contraception, smoking, and family history). Data were additionally collected from a follow-up visit that occurred, on average, 14 months from the initial baseline visit and included current medication usage and SLEDAI score. Any aPL associated clinical manifestations that were recorded throughout the trial as adverse events were additionally included for evaluation.

Laboratory testing

As part of baseline, 15-, 27- and 36-month assessments in the APPLE trial, blood samples were collected to evaluate for the presence of LA or aCL IgG, IgM and IgA using various assays, performed locally, at multiple clinical centers. For our analysis, data were available for these antibodies at baseline and from one of the follow up visits. Due to the fact that this cohort was not designed for the study of APS, data on these conventional assays were limited. We additionally obtained stored plasma samples that were drawn in close proximity to each participant's respective follow up visit. These plasma samples were frozen and stored at the time of the study visit. Frozen specimens were shipped to our clinical laboratory at Montefiore Medical Center.

Using the plasma samples from the APPLE cohort, we examined annexin A5 anticoagulant activity using a 2-stage method as previously described^{10, 11, 20}. This assay (A5R) measures coagulation times in the presence and absence of annexin A5 [Annexin A5 anticoagulant ratio = (coagulation time in the presence of annexin A5/coagulation time in the absence of annexin A5) × 100%]. Resistance to the anticoagulant effects of annexin is expressed as a reduction in this ratio²¹. The reference range for abnormal A5R with the lot of reagent that was used for this study has been determined as < 203%, which is 3 SD from the mean in a population of disease-free donors (mean age: 35 ± 10 years) (Reagents: Actin FSL and Innvin: from Dade Behring (SIEMENS); EDTA: from Sigma Aldrich; 0.02 M CaCl₂: Hemosil calcium chloride solution, from Beckman Coulter).

Additionally, using the specimens from the APPLE cohort, we performed enzyme-linked immunosorbent assays to measure anti-β2GPI IgG antibodies with reactivity toward domain I (anti-D1 antibodies), as previously described^{15, 17}. Plasma samples were regarded as having elevated levels of anti-DI IgG when the absorbed value exceeded a previously established cutoff value (mean + 3 SD in an adult population of disease free donors).

Laboratory data on A5R and anti-D1 antibodies from children with SLE were compared with results from 22 previously obtained pediatric controls within our institution with no known autoimmune or thrombophilic disorders (mean age 12.4 years, 63.6% female). Personnel performed the laboratory assays for A5R and anti-D1 IgG on coded samples and were blinded to the clinical status of the patient and to prior aPL testing.

Statistical analysis

Statistical analysis was conducted using STATA software, version 11.2 (College Station, Texas). All data analyses were preceded by extensive data checking and verification to identify and resolve the reasons for missing values, inconsistencies, and out-of-range values. Descriptive statistics were computed to summarize each variable, including the mean, median, standard deviation and interquartile range (IQR) for continuous variables; and frequencies (percentages) for categorical variables. All tests were 2-sided and p-values less than 0.05 were considered statistically significant. We performed bivariate analyses comparing the results of the A5R assay with our clinical variables of interest. When evaluating the association between aPL antibodies and A5R, we chose to use the antibodies drawn in closest proximity to the date plasma specimens were drawn for the A5R assay. Continuous variables were compared using Pearson correlation and categorical variables were compared using Student's t-test and chi-square analysis. In cases of non-normally distributed data, Spearman correlation and Wilcoxon signed-rank test were additionally performed. Variables with biologic relevance or statistically significant associations (defined as a p value ≤ 0.25) were selected for inclusion in our full multivariate linear regression model examining the independent association between anti-DI antibodies and the A5R assay. Age, gender, race and ethnicity were determined *a priori* to be included in our final model. A manual backward elimination procedure was performed and the model was assessed and adjusted for the presence of confounders and effect modifiers.

Regression diagnostics were performed on the final model, with no evidence of major violations or potentially influential data points.

Results

Patient characteristics

We received clinical data and plasma samples on 183 participants from the APPLE cohort. A summary of the clinical and laboratory variables collected at baseline and during the follow up visit are shown in Table 1. In this sub-cohort, the mean age was 15.6 ± 2.7 years and the majority of participants were female gender (82.5%). Race and ethnicity were self-reported, with half the participants reporting white race (49.7%) and one quarter reporting Hispanic or Latino ethnicity (25.7%). At entry into the study, the majority of participants were on HCQ (97.8%) and corticosteroids (81.9%). Participants had a mean disease duration of 29.5 months with a median SLEDAI score of 2 (IQR: 1–4) at the follow up visit. In terms of laboratory variables, data were available for LA at the baseline assessment and follow-up visit on 147 and 87 participants respectively. For assessment of the presence of overall aCL antibodies (IgM, IgG and/or IgA), limited data were available at the baseline and follow-up visit (41 and 19 participants respectively). Using plasma samples from each participant's respective follow up visit, we additionally found that 25.1% had positive anti-DI antibodies (25.1%) (Table 1). In comparison, no pediatric controls were positive for anti-DI antibodies ($p=0.014$).

Thrombotic events

Among this cohort, 7 of 183 children with SLE (3.8%) were found to have a history of a thrombotic event, including 3 participants with history of arterial thrombosis, 2 with history of deep vein thrombosis and 2 with history of other venous thrombosis. Of these 7 participants with thrombosis, only one child had a positive LA at the follow-up visit and one had positive anti-DI antibodies. In addition, one participant had a single spontaneous abortion; however the gestational timing of this event is unknown. There were no thrombotic events reported as adverse events throughout the duration of the 3-year period of observation.

Bivariate analyses with A5R

Using plasma samples from the follow up visit, the mean A5R in our sub-cohort of children with SLE was $172\% \pm 30\%$, which was significantly lower than pediatric controls (mean A5R: 242 ± 32 ; mean age 12.4 years, 63.6% female), indicating greater resistance to annexin A5 anticoagulant activity ($p < 0.0001$) (Figure 1). In addition, when referring to the reference range for abnormal A5R $< 203\%$, we found that 84% of the children with SLE had an abnormal A5R.

When evaluating the impact of aPL antibodies on A5R levels, we found that children with positive aPL antibodies had significantly lower mean A5R as compared to those with negative antibodies (Table 2). In addition, when examining participants with both LA and anti-DI antibodies, we found that mean A5R was further reduced to 133%. Due to lack of sufficient data available regarding aCL antibodies ($n=19$), this group was excluded from analysis. Despite the small number of APPLE participants who experienced a thrombotic event, we found that all 7 had reduced A5R with mean A5R = $155\% \pm 29\%$ versus $172\% \pm 30\%$ in those without history of thrombosis ($p=0.124$) (A5R levels in participants with thrombosis are additionally reflected in Figure 1).

Additional bivariate analyses with A5R demonstrated no significant difference with regard to age ($p = 0.327$), gender ($p = 0.909$), race ($p = 0.894$), treatment group ($p = 0.489$), baseline HCQ ($p = 0.860$), baseline aspirin ($p > 0.999$), or baseline steroid use ($p = 0.493$). Both ethnicity ($p = 0.096$) and SLEDAI score ($p = 0.152$) met criteria for inclusion into our full multivariate linear regression model using our cutoff of $p = 0.25$.

Correlation between A5R and anti-DI antibodies

We found a weak but statistically significant inverse correlation between annexin A5 anticoagulant activity and anti-DI IgG (Figure 2: $\rho = -0.35$, $p=0.0001$). Interestingly, of the 46 participants with positive anti-DI antibodies, 44 (96%) had abnormal A5R $< 203\%$ (the remaining 2 participants had borderline A5R levels of 204% and 211% respectively); whereas, of the 153 participants with abnormal A5R, only 44 (29%) had positive anti-DI antibodies ($p=0.011$).

Multivariate analysis

Using a backward elimination procedure and an *a priori* decision to adjust for age, gender, race and ethnicity, a model was constructed demonstrating a statistically significant

independent association between anti-DI antibodies and reduced mean A5R (model not shown: $p < 0.001$). After a decision to adjust for LA as a potential confounder in this model; our final model is presented in Table 3. In this model, anti-DI antibodies ($p=0.013$) and LA ($p=0.036$) were both independently associated with reduced mean A5R after adjusting for the other variables in the model. When examining this final model for interactions, we found no statistical significant interaction between LA and anti-DI antibodies ($p = 0.504$).

Discussion

There is accumulating evidence that antibody mediated disruption of an annexin A5 anticoagulant shield plays a role in the pathogenesis of APS^{8–10} and that antibodies to the domain I portion of β 2GPI (anti-DI antibodies) confers the highest risk of clinically significant thrombosis^{15–17}. In a prior study, we found that children with SLE and persistently positive aPL antibodies had significantly reduced annexin A5 anticoagulant activity, particularly in those with anti- β 2GPI antibodies⁷. In our current study, we confirmed these results, demonstrating that children with SLE have markedly reduced annexin A5 anticoagulant activity as compared to pediatric controls; and have additionally shown the association of reduced annexin A5 anticoagulant activity in patients with antibodies that specifically recognize an exposed epitope in domain I of β 2GPI.

β 2GPI is a relatively abundant plasma protein, whose biologic function remains poorly understood. It has been shown to exist in a circular unbound conformation and suggested to play a role in the clearance of apoptotic cells and microparticles^{22, 23}. In the presence of anionic phospholipids, the domain V portion of the protein uncoils and inserts into the phospholipid bilayer, thereby exposing the domain I epitope for antibody recognition and enhancing the immunogenicity of this protein^{23, 24}. It has been theorized that although multiple subpopulations of anti- β 2GPI antibodies exist, anti-DI antibodies may be within a subset among the most clinically relevant^{12, 15, 25}. A multi-step mechanism has been proposed to help explain the underlying pathophysiology of APS, whereby anti-DI antibodies recognize and bind 2 molecules of β 2GPI yielding dimers that have an even greater affinity for negatively charged anionic phospholipids (Figure 3)¹⁷. This dimerized complex can then compete with annexin A5 proteins, disrupting the anticoagulant shield and potentially allowing for thrombosis to occur. In this study, we have shown that 25% of children with SLE had positive anti-DI antibodies, among which were associated with a highly significant reduction in annexin A5 anticoagulant activity. We have additionally shown that the majority patients with anti-DI antibodies had reduced annexin A5 anticoagulant activity, whereas the converse was not true. We postulate that the mechanistic A5R assay may reflect the presence of hemostatic factors and antibodies to several different epitopes important in the underlying pathogenesis of APS; and among these include recognition of DI.

LA has also been shown to highly correlate with thrombosis in multiple studies²⁶. Furthermore, a positive LA test in association with the presence of anti- β 2GPI antibodies may additionally confer even greater risk of thrombosis¹². We found that children and adolescents with SLE demonstrated the greatest reduction in annexin A5 anticoagulant activity when both LA and anti-DI antibodies were positive. However, in our multivariate

analysis we additionally found that both LA and anti-DI antibodies were independently associated with reduced annexin A5 anticoagulant activity and the presence of LA did not appear to statistically modify the significance of the association between anti-DI and annexin A5R. This model suggests that although the presence of multiple aPL antibodies may predict the greatest risk of potential future thrombosis, the clinical significance of positive anti-DI antibodies alone warrants further investigation.

We had hoped to determine the association of thrombosis with anti-DI antibodies and annexin A5 anticoagulant activity in children with SLE; however due to the relatively low rate for histories of thrombosis (3.8%) in this population, our study was not sufficiently powered to do so. It is unclear why the rate of thrombosis in this population of children with SLE appears to be lower than previous reports. Perhaps it is attributable to the majority of participants being on aspirin and HCQ prior to baseline evaluation and throughout the duration of the study, the potential beneficial role of statins within the trial or the relatively low disease activity in these participants.

Finally, using available data previously collected on pediatric controls in our institution, we were able to compare the results of the A5R assay and anti-DI antibodies in children with SLE of comparable age and gender status. Subjects in the APPLE cohort demonstrated higher frequency of anti-DI antibodies and remarkably lower mean A5R levels as compared to pediatric controls. This is in accordance with the results obtained in our prior study, in which children with SLE and SLE-like illnesses demonstrated significantly lower mean A5R levels as compared to children with other autoimmune diseases typically classified as low risk of aPL related manifestations⁷. It is worth noting that the findings in this current study were obtained with the overwhelming majority of SLE participants on HCQ both at baseline and as per study protocol. Prior studies have suggested the HCQ may be protective in reducing the risk of thrombosis in patients with aPL antibodies and could potentially increase annexin A5 anticoagulant activity^{27–30}. It is unclear what impact HCQ use may have had on our results and controlled studies are needed to further examine the potential role of this medication.

Several limitations of this study need to be mentioned. The first is that this is a cross-sectional analysis of the APPLE cohort and thus the true temporal association between aPL antibodies, alterations in annexin A5 anticoagulant activity and the occurrence of thrombosis cannot be fully assessed. In addition, due to the fact that this cohort was not designed for the study of APS, there were missing values of LA and aCL antibodies in this cohort, which further limited our statistical interpretations. Finally, due to the limited number of thrombotic events, this study was underpowered to fully examine the clinical implications of our findings.

In conclusion, we have proposed a potential mechanism by which anti-DI antibodies may play a role in reducing the protective anticoagulant effects on annexin A5. We have shown that children and adolescents with SLE have increased frequency of anti-DI antibodies and markedly reduced annexin A5 anticoagulant activity as compared to pediatric controls. We have additionally shown that the presence of LA and anti-DI antibodies both collectively and independently further reduce annexin A5 activity. To better understand the clinical

significance of these findings, larger prospective trials and observational studies are warranted to further evaluate the associations between anti-DI antibodies and A5R, as they relate to the clinical manifestations of APS.

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			p-value
Age (years)	15.6 ± 2.7	12.4 ± 6.1	0.080
Female	151 (82.5%)	14 (63.6%)	0.046

Figure 1. Annexin A5 anticoagulant activity in children with SLE (within the APPLE cohort) versus pediatric controls. Dark triangles are representative of participants with history of a thrombotic event ($n=7$).

Figure 2.
Inverse correlation between annexin A5 anticoagulant activity (%) and anti-DI IgG (µg/mL).

Figure 3.

Schematic depiction of a proposed mechanism for the association of anti-DI antibodies and annexin A5 in the pathophysiology of APS. (A) When vascular injury occurs in a healthy individual, annexin A5 proteins rapidly crystallize over exposed phospholipids forming an anticoagulant shield. (B) In an individual with aPL antibodies, the exposure of anionic phospholipids allows for anti-DI antibodies to bind two molecules of β 2GPI yielding dimers with high enough affinity to compete with and (C) disrupt annexin A5 proteins¹⁷ [Figures designed by Andrea Watt].

Table 1Clinical and laboratory characteristics of participants in the APPLE cohort (n=183)^a

Baseline characteristics	
Age (years)	15.6 ± 2.7
Female gender	151 (82.5%)
White race	91 (49.7%)
Ethnicity (Hispanic or Latino)	47 (25.7%)
Treatment group (atorvastatin)	90 (49.2%)
Baseline hydroxychloroquine	179 (97.8%)
Baseline aspirin	122 (66.7%)
Baseline corticosteroid	149 (81.9%)
Self reported history of smoking	5 (2.7%)
Family history of cardiovascular disease	66 (37.9%)
Positive LA (n=147) ^b	21 (14.3%)
Positive aCL antibodies (n=41) ^b	5 (12.2%)
Characteristics at follow up visit (mean = 430 days from baseline)	
SLEDAI score	2 (1–4)
Hydroxychloroquine	173 (94.5%)
Aspirin	131 (71.6%)
Positive LA (n=87) ^b	16 (18.4%)
Positive aCL antibodies (n=19) ^b	2 (10.5%)
Positive anti-DI antibodies (n=183)	46 (25.1%)
Annexin A5 anticoagulant ratio (%)	172 ± 30

^aContinuous variables expressed as mean ± standard deviation or median (interquartile range). Categorical variables expressed as frequency (percentages).

^bLA = Lupus anticoagulant, aCL = anti-cardiolipin; Data not available on the remainder of the cohort

Table 2

Mean A5R (%) in relation to the presence of aPL antibodies in children with SLE

	Negative	Positive	p-value
Lupus anticoagulant (n=87) ^a	169 ± 30 (n=71)	150 ± 24 (n=16)	0.021
Anti-DI antibodies (n=183)	177 ± 30 (n=137)	155 ± 24 (n=46)	<0.0001
Lupus anticoagulant and anti-DI (n=87) ^a	167 ± 29 (n=81)	133 ± 20 (n=6)	0.005

^aData not available on the remainder of the cohort

Table 3

Final multivariate linear regression assessing annexin A5 resistance in children with systemic lupus erythematosus ($R^2=0.15$; $n=87$)

Predictor variable	β	Confidence Interval	p-value
Positive anti-DI antibodies	-17.83	[-31.75, -3.90]	0.013
Positive lupus anticoagulant	-17.18	[-33.20, -1.15]	0.036
Age (years)	-0.48	[-3.03, 2.07]	0.711
Female gender	3.64	[-13.03, 20.30]	0.665
White race	-2.27	[-14.80, 10.25]	0.719
Ethnicity (Hispanic or Latino)	-6.71	[-20.10, 6.69]	0.322