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Antiphospholipid Antibodies and Recurrent Thrombotic Events: Persistence and Portfolio

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Abstract

Background—There are very limited prospective data on the significance of persistent of antiphospholipid antibodies (aPL) and recurrent thrombo-occlusive events (TOEs). We investigated the prognostic value of (1) two newer aPL assays, (2) an aPL portfolio, and (3) persistent aPL positivity following stroke.

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Methods—1,770 subjects from the APASS-WARSS study underwent further aPL testing for antibodies to phosphatidylserine (aPS) and β_2 -glycoprotein-I (anti- β_2 GPI) from stored sera. Follow-up aPL status was also tested in a subset of subjects. Primary analysis was based on time to any TOE (ischemic stroke, MI, TIA, DVT, PE, or systemic arterial occlusion)/death at 2 years. Cox proportional hazard analyses assessed whether aPL independently related to outcome.

Results—Persistent anti- β_2 GPI decreased the *time* to TOE/death after adjustment for potential confounders (HR=2.86, CI 1.21-6.76, $p=0.017$). When persistent anti- β_2 GPI was combined with another persistently positive aPL, time to TOE/death was also reduced (HR=3.79, CI 1.18-12.14, $p=0.025$). Neither persistent aCL, persistent aPS alone, nor a single positive anti- β_2 GPI or aPS was associated with decreased time to TOE/death. No single positive aPL, portfolio of baseline aPL, or any persistent aPL increased the *rate* of TOE/death.

Conclusions—Rates of TOE/death were not influenced by aPL results at baseline or follow-up. Persistent anti- β_2 GPI alone and with persistent second aPL were independently associated with decreased *time* to TOE/death. Persistent aPL, an aPL portfolio, and newer aPL in ischemic stroke patients are not helpful in predicting an increased rate of recurrent TOEs.

Keywords

antiphospholipid antibodies; ischemic stroke; thrombosis; coagulation; outcomes; epidemiology; recurrent stroke risk

Introduction

Antiphospholipid antibodies (aPL) have been associated with venous and arterial thrombo-occlusive events (TOE)¹⁻⁵. A single aPL has been associated with initial stroke⁶⁻⁹ but was not predictive of recurrent TOE/death¹⁰. Persistent aPL in young stroke patients was predictive of recurrent stroke¹¹.

The WARSS-APASS collaboration¹⁰ found that ischemic stroke patients positive for *both* lupus anticoagulant (LA) and anticardiolipin antibodies (aCL) at baseline had higher risk for TOE/death than subjects without aPL. Since the WARSS-APASS study, standardized assays for two new aPL have been developed potentially more biologically relevant for predicting TOE¹²⁻²⁵. β_2 -glycoprotein-I (β_2 GPI) has been found to be the most likely antigenic target of the aPL antibodies²³. In addition, antiphosphatidylserine antibodies (aPS) have been independently associated with ischemic stroke²⁵. Persistence of aPL over time may confer a higher risk for TOE^{11, 26} as does positivity on more than one aPL assay (“portfolio”)²⁷.

We therefore re-tested stored sera of WARSS-APASS study subjects. In particular, we looked for the presence of anti- β_2 GPI and aPS at baseline, and for persistence of these and aCL in predicting subsequent TOE/death. We also sought to determine whether an aPL portfolio increased the risk of TOE/death.

Methods

Subjects

2,206 patients were recruited into WARSS²⁸. Of these 1,770 of 1,954 (91%) APASS-eligible WARSS patients consented to participate in APASS-WARSS. The consent form was written and approved with knowledge that additional “antibodies/proteins” could be tested apart from aCL/LA. All patients who participated in APASS were eligible for further testing. Stored serum samples were approved for further aPL testing by all but one of the APASS Centers' Institutional Review Boards (Appendix).

Sera Samples

aPL testing was performed from previously unfrozen sera stored at -70°C. Samples from baseline, 12, and 24 months post-stroke had been stored. Plasma (LA testing) was not drawn/stored at follow-up for concern of unmasking treatment arm (warfarin) of the primary WARSS study.

aPL Assays

Baseline sera were tested for anti- β_2 GPI and aPS. Also, follow-up aPL status for all aPL was assessed from sera at 12 and 24 months post-stroke.

As previously reported¹⁰, aPL cut-off values were established by the assay manufacturers. Positive cut-off values were: aCL: IgG>21 GPL (IgG phospholipids units) and IgM>12 MPL. Anti- β_2 GPI were measured via commercially available ELISA (Innova Diagnostics, USA), and previously used¹⁵. Cutoffs for normal/negative were: IgG 20 and IgM 20. aPS (IgG/M isotypes) were measured by commercial kit (REEADS®). Cutoffs for normal/negative were: IgG 16 and IgM 22.

Persistence

aPL Persistence was defined as positive at all time-points for which that assay was tested. Conversely, transient positivity was defined as positive at baseline in patients who then had at least one subsequent negative assay.

Primary Outcome

TOE as previously defined^{10,28} occurrence of death (any cause), or any TOE - ischemic stroke, MI, TIA, deep venous thrombosis, pulmonary embolism, systemic visceral or peripheral arterial embolism. All TOEs were independently adjudicated, blinded to treatment and aPL status.

Pre-Specified Secondary Analyses

We hypothesized (1) positive aPL would correlate with decreased time to TOE/death, (2) persistent aPL would confer a stronger correlation, (3) increasing number of positive aPL assays would increase TOE/death risk, and (4) positivity on one aPL would correlate with positivity on other aPL assays.

Statistical Analysis

The primary analysis was time to TOE/death at two years. Cox proportional hazard analyses assessed the association between time to TOE and aPL. Multivariate analyses assessed whether aPL independently relates to outcome (TOE) after adjustment for potential confounders and are presented as odds ratios (OR) and their 95% confidence intervals (CIs). Baseline demographic and traditional stroke risk factors that relate to both aPL status and outcome were considered for incorporation into the model. Potential confounders were entered simultaneously into a discrete-time Cox proportional hazards model to obtain the effect of aPL positivity (log relative hazard) adjusted for the potential confounding effects of these covariates.

Treatment differences were tested with an interaction term to assess whether significant differences existed for the effect of aPL in patients treated with warfarin or aspirin. An interaction between aPL and treatment was considered significant when the *p* value for interaction was ≤ 0.10 . (In all analyses of interactions, the main effects that are combined to form the interaction are included in the models.) If no significant interaction was found, the two treatment arms were combined. If the treatment interaction was significant, separate analyses were performed for each treatment arm. All statistical analyses were conducted using SAS software (SAS/STAT software, version 9.2; SAS Institute, Cary, North Carolina).

To test if missing data at 12 and 24 months after stroke were independently associated with TOE status, baseline anti- β_2 GPI, or other observed covariates, a logistic regression model with a missing indicator as an outcome was estimated^{29,30}. Association between missingness/dropping out and these variables was measured using ORs and corresponding 95% CI.

We assessed concordance of aPL positivity over time and agreement among the three assays (aCL, aPS and anti- β_2 GPI) using Kappa Statistics.

Results

Baseline Demographics

Baseline demographics of subjects assayed for anti- β_2 GPI are presented in Table 1, along with months 12 and 24 results. While the number of subjects assayed for aPS and aCL are slightly different the percentages are essentially identical. Subjects with follow-up assays were similar to those without follow-up testing at month 12; in the month 24 follow-up groups, subjects not tested were less likely to have a sedentary lifestyle, be married, and more likely to have diabetes.

Baseline demographic factors associated with aPL status

For each of the three aPL assays, we found: for anti- β_2 GPI being white; for aCL they were age, white, alcohol, ever smoked, obese, history of hypertension and systolic blood pressure; and for aPS they were age, white, college education, alcohol, and systolic blood pressure.

aPL Persistence

Percentage of subjects testing positive on each aPL at each time point is presented in Table 2: 38/147 subjects anti- β_2 GPI positive at baseline (25.9%) showed persistence, while 24/147 subjects (16.3%) were transiently positive. The remaining 85 subjects positive at baseline did not have follow-up testing. One subject with a missing anti- β_2 GPI baseline assay was positive at both months 12 and 24, and was therefore persistently positive. No subjects negative at baseline became positive at both months 12 and 24.

For aPS, 36/115 (31.3%) demonstrated persistence while 20/115 (17.4%) were transiently positive. One patient missing aPS baseline was positive at both follow-ups. One subject aPS negative at baseline became positive at both follow-ups.

For aCL, 143/485 subjects (29.5%) showed persistence while 171/485 (35.3%) were transiently positive. Fifteen aCL negative at baseline subjects were positive at both follow-ups; however none missing the baseline assay were positive at both follow-ups. Twenty patients were persistently anti- β_2 GPI positive and persistently positive for either aPS or aCL.

Clinical Endpoints

There were 394 TOE among 1,624 patients tested for anti- β_2 GPI, 394 among 1,625 patients tested for aPS, and 422 among 1,753 patients tested for aCL. There were 9 TOE in 39 patients persistently positive for anti- β_2 GPI: 5 cerebrovascular (1 recurrent stroke and 4 TIAs), 3 MIs, and one death. Of 20 patients persistently anti- β_2 GPI positive and either persistently aPS or aCL positive, there were 5 TOEs: one stroke, 2 TIAs, and 2 MIs.

There were no single aPLs nor any persistently positive aPLs associated with occurrence of TOE/death. Persistently positive anti- β_2 GPI (on any 2 assays performance consecutively of the 3 time points combined with positive on all 3 assays) showed a trend [adjusted OR 2.09, (95% CI 0.94-4.65), $p = 0.07$]. Positive on any one aPS measurement and aCL conferred an increased risk of TOE/death over being positive on zero measurements. However, positive on two or three aCL measurements conferred a *decreased* risk over zero positive measurements. Increasing numbers of positive anti- β_2 GPI were not associated with either increased or decreased risk of TOE/death (Table 3). There was no significant difference between IgG and IgM isotypes for recurrence risk for any of the 3 aPLs. There were too few high positive titers of any aPL for analyses. Table 4 shows the frequency of subjects who had positive aPL at baseline and negative at the two follow-up time points.

Persistent anti- β_2 GPI decreased the time to TOE (adjusted HR 2.86, 95% CI 1.21-6.76, $p=0.02$). With a second persistent aPL (aCL or aPS), the HR increased to 3.79 (95% CI 1.18-12.14, $p=0.025$). Otherwise, there were no single aPL, no persistent aPL, and no combinations of aPL that decreased time to TOE. (Figures 1 and 2).

Patients with TOE/death were more likely to have missing 12 and 24-month aPL measurements ($p<0.0001$). However, our sensitivity analysis found that baseline anti- β_2 GPI positive did not correlate with missing aPL assays at 12 or 24 months ($p > 0.5$). Kappa scores did not correlate between positivity for anti- β_2 GPI, aPS, or aCL (Kappas all < 0.3).

However, Pearson correlation coefficients of individual aPL assay titers over individual time points (from baseline to 12 months to 24 months) ranged from 0.66 to 0.81.

Discussion

We were unable to demonstrate consistent positive relationships between any aPL positivity, persistent or otherwise, and TOE/death rate over 2 years post index stroke. While one positive titer showed an increased risk when compared to zero titers for both aPS and aCL, having more than one positive titer for aCL was actually associated with an unexpected decreased risk of TOE or death. One possible explanation is that aCL may not be as specific for risk of TOE as other aPLs³¹. It may also be explained by the average age and stroke risk factor prevalence of the cohort and relative lack of aCL pathogenicity compared to other risk factors. However, other studies have suggested that eliminating aCL testing may be premature^{17, 22, 23}.

We found decreased *time* to TOE/death with persistent anti- β_2 GPI. The shorter time to event occurs in the second, not first year of follow-up. If those patients also had a second persistent aPL, the time to TOE/death was further shortened (shorter event-free survival time). These subgroups are a very small percentage of the cohort: 39/1,624 tested for anti- β_2 GPI (2.4%); but of those testing positive at baseline with follow-up testing, the percentage was considerably higher: 38/62 (61.3%). Reasons for TOE events occurring earlier without an increased rate are uncertain and may reflect small numbers among other reasons. The phenomenon may be analogous to the modulating effect of corticosteroids on exacerbations of multiple sclerosis – it gets the patient back to baseline quicker without any overall change in functional status.

Baseline anti- β_2 GPI+ did not predict whether or not a patient would have missed their 12 or 24 follow-up appointment but the presence of the clinical trial (WARSS) endpoint of stroke/death did predict missing follow-up data. This is most likely because the missing follow-up data at 12 or 24 months are consequences of dropping out due to the occurrence of a WARSS primary endpoint of stroke/death, given that there were no differences in TOE at baseline in any of the assays. The absence of an aPL assay performed does not necessarily mean that that assay is positive or more likely to be positive than non-missing aPL assays. While the mechanism that led to missing follow-up aPL data is associated with TOE/death, we do not have evidence in the observed data to say that aPL is associated with an increased rate of TOE/death.

The hypothesis that aPL persistence is more specific than a single positive aPL has some, but not uniform support. Transient aPL are seen in up to 10% of healthy blood donors, much greater than the incidence of stroke in the general population^{32, 33}. Persistent aPL were more strongly associated with thrombotic events in children with systemic lupus erythematosus than transiently aPL, although transient LA and transient anti- β_2 GPI remained associated²⁶. However, in a relatively small cohort of 34 children with first stroke/TIA, persistent IgG aCL did not predict recurrent TOE over a 2.8 year median follow-up³⁴. Using binary partition evaluation, no titer criteria for aCL positivity (range, 0-60 GPL units) predicted recurrent events.

Persistent aPL independently predicted recurrent stroke in a large, young (age 18-45, mean 36), Italian stroke cohort¹¹ followed prospectively for a median of 42 months – 18 months longer than our cohort. Persistent aPL may confer a greater risk because of a longer exposure time. In 194 consecutive patients (with or without prior TOE) followed prospectively persistent IgG anti- β 2GPI was an independent risk factor for recurrent TOE³. A recent case-control study failed to demonstrate increase risk of adverse pregnancy outcomes due to persistent aPL³⁵. Persistence of multiple, but not single aPL, may increase thrombosis risk in asymptomatic individuals, particularly with underlying autoimmune diseases³⁶.

Recent guidelines recommend that aPL detected acutely should persist to diagnose the syndrome³¹. The revised Sapporo criteria for antiphospholipid syndrome calls for 12 weeks between assays (level C evidence), to increase specificity without sacrificing sensitivity³¹. The optimal waiting time remains unclear.

In a small, retrospective study³⁷, patients positive on more than one aPL had higher TOE rates, although aPL persistence was not studied. Identifying those patients at higher risk for earlier recurrent thrombosis could help direct treatment³⁸⁻⁴¹.

Based on APASS-WARSS¹⁰, the most recent guidelines for the secondary prevention of aPL-associated, non-cardioembolic stroke recommend anti-platelets^{42,43}. However, as the initial APASS-WARSS Study only looked at one-time aPL, some continue to treat patients with stroke fulfilling criteria for antiphospholipid syndrome with anticoagulation⁴⁴. It is not possible to differentiate treatment effects (warfarin versus aspirin) in this study, as group sizes are too small and underpowered.

Strengths of our study include prospective design and the relatively large stroke cohort studied serially for aPL (largest sample to date), independent blinded assessments of TOE and aPL status, a single, expert aPL research laboratory with over 20 years of experience, and standardized therapy arms⁴⁵.

Our study has limitations. A number of subjects were not tested at follow-up. The 257 APASS subjects with a primary end-point in WARSS (recurrent stroke or death) would not have follow-up. Other missing aPL are attributable to uncollected samples, unusable samples, and insufficient quantity of serum. Despite these reasons, typically occurring randomly, the missing data are not biased with respect to baseline anti- β 2GPI positivity, as demonstrated by the missing-at-random analysis. Further, this is the largest prospective study to date for determining the significance of persistent aPL after stroke.

Another potential limitation is that samples were stored for several years; however, many important prospective studies in this field used previously unfrozen sera stored for several to many years⁴⁶, even decades longer than our study. We used only previously unfrozen sera, minimizing this potential problem. Also, the definition of persistence of aPL was based on only three aPL determinations over two years and the follow-up period could be too short to detect thrombo-occlusive events. We did not collect data on the presence of specific autoimmune diseases (e.g. SLE) in the cohort. A major limitation was that LA was not been tested in the follow up of this study (to avoid potential unblinding of treatment arm). The

population in WARSS²⁸ may not represent those likely to have a stroke from persistent aPL. Median age was 63 years, and due to exclusion criteria, there were a much larger proportion of small-vessel strokes than seen generally. Conversely, there was a smaller proportion of large vessel stroke and no proven cardioembolic strokes. In daily clinical practice, physicians should not think of aPL as a main cause of stroke in this subset of patients. In fact, other vascular risk factors such as tobacco and hypertension were present in more than 60% of patients, diabetes mellitus in 30% and sedentary lifestyle in 40%. Assessing a younger group of aPL persistently positive patients with stroke/TIA who have relatively lower chances of having other risk factors is necessary. One study failed to show a difference in aPL between patients with cryptogenic and strokes of known etiology⁴⁷. Another study found demographic characteristics, other risk factors, history of prior thrombotic events and distribution of aetiopathogenic types of cerebral ischaemia to not differ in young patients with or without aPL suggesting that with the exception of a clinical context of antiphospholipid syndrome or other autoimmune diseases, the usefulness of aPL in the management of cerebral ischaemia remains limited⁴⁸.

Our results, taken together, appear to conform to the known paradoxical observations when studying recurrent risk – that thrombophilias, while predisposing to a first episode of DVT, do not increase the risk for recurrence⁴⁹. This may be due to index event bias⁵⁰ based on selection of subjects who had a first event. As risk factors for index and recurrent events are often similar, there is a biasing of studies towards the null resulting in risk factor contributions to be substantially underestimated or reversed. This is also seen in the relationship between patent foramen and cryptogenic stroke⁵⁰.

In conclusion, our study suggests persistent aPL may be a marker for increased risk for an earlier TOE/death. We failed to find increased TOE risk with persistent or multiple positive aPL.

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Appendix: Co-Investigators at Participating APASS Centers

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Figure 1. Kaplan-Meier Curve for time to TOE or death with persistently positive β_2 -GPI assays

Figure 2. Kaplan-Meier curves for time to TOE or death with persistently positive β_2 -GPI assays and at least one other persistently positive aPL assay

Table 1
Demographics of Subjects Tested for anti- β_2 -glycoprotein-I

	Baseline		Month 12		Month 24	
	Tested (N=1624)	Missing (N=146)	Tested (N=575)	Missing (N=1195)	Tested (N=475)	Missing (N=1295)
Mean age $\pm$ SD	62.4 $\pm$ 11.3	63.2 $\pm$ 10.8	62.5 $\pm$ 10.9	62.5 $\pm$ 11.5	62.2 $\pm$ 10.6	62.7 $\pm$ 11.5
Male	57.5	62.3	61.6	56.1	59.6	57.2
Caucasian	56.9	61.6	57.2	57.3	58.3	56.9
Married	56.2	61.0	57.9	55.9	62.5	54.4**
Sedentary lifestyle ⁺	43.1	47.3	43.5	43.4	48.0	41.8****
Obese	49.6	48.6	46.9	49.3	49.1	49.7
Tobacco (ever)	64.7	71.2	66.8	64.5	64.8	65.4
Alcohol (heavy)	12.2	12.3	12.9	11.9	11.4	12.5
College	27.0	23.3	27.3	26.4	26.7	26.6
Hypertension	62.5	65.8	67.1	68.4	67.8	68.0
BP sys>160	13.7	15.8	14.6	13.5	14.8	13.5
BP dias>95	9.0	8.2	9.0	8.9	8.6	9.0
Diabetes	31.8	30.1	30.1	32.4	27.8	33.1****
MI	11.8	13.0	11.1	12.2	10.7	12.3
Cardiac disease	23.4	21.2	23.3	23.2	21.1	24.0
IschCard	19.4	17.8	19.3	19.3	17.9	19.8
HistCoag	10.6	10.3	10.1	10.8	11.0	10.4
Treatment with ASA	50	52	52.2	49.3	52.0	49.6
TOE	24.3	21.9	15.3	28.3*	10.7	29.0*

* p < .0001

** p = .01

*** p = .0008

**** p = .02

⁺ defined as walk < 1 mile/day for the past year on average

TOE = thrombo-occlusive events; ASA = aspirin; IschCard = ischemic cardiomyopathy; HistCoag = history of coagulopathy

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Table 2
Percent Positivity of aPL at Each Time Period

	Baseline	Month 12	Month 24	P-value [*]
β_2 -GPI	9	7	9	0.49
aPS	7	8	7	0.98
aCL	28	37	29	<0.001

* difference between visits (Chi-Square test)

Table 3
Association Between TOE and Number of Positive Assays Adjusted for Baseline Factors

	Odds Ratio	95% CI	p-value
anti- β_2 -GPI*			
One positive vs zero	1.05	0.63-1.77	0.84
Two positive vs zero	1.02	0.44-2.34	0.97
Three positive vs zero	0.77	0.29-2.03	0.59
Sedentary lifestyle	1.43	1.13-1.81	0.003
aPS [†]			
One positive vs zero	2.28	1.18-4.41	0.015
Two positive vs zero	1.22	0.51-2.91	0.65
Three positive vs zero	0.27	0.06-1.26	0.1
Sedentary lifestyle	1.41	1.12-1.79	0.004
aCL [‡]			
One positive vs zero	1.81	1.38-2.37	<0.0001
Two positive vs zero	0.67	0.45-0.98	0.04
Three positive vs zero	0.57	0.33-0.97	0.04
Medicaid	1.49	1.13-1.96	0.004

* Baseline variables included in model: history of coagulation, marital status, heavy alcohol use, moderate alcohol use, sedentary lifestyle, caucasian ethnicity, diastolic hypertension

[†] age, history of coagulation, marital status, heavy alcohol use, moderate alcohol use, sedentary lifestyle, Caucasian ethnicity, diastolic hypertension

[‡] age, Medicaid, heavy smoking, Caucasian ethnicity, male gender, systolic hypertension

Table 4
Frequency of positive aPL at baseline and negative at follow-up

aPL assay	positive at baseline	negative at M12	negative at M24
aCl	279	123	105
anti- β 2	49	17	15
aPS	45	14	8