Antiphospholipid Antibodies and Recurrent Thrombotic Events: Persistence and Portfolio

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Key Words
Antiphospholipid antibodies · Ischemic stroke · Thrombosis · Coagulation · Outcomes · Epidemiology · Recurrent stroke risk

Abstract
Background: There are very limited prospective data on the significance of persistent antiphospholipid antibodies (aPL) and recurrent thrombo-occlusive events (TOEs). We investigated the prognostic value of (1) 2 newer aPL assays, (2) an aPL portfolio and (3) persistent aPL positivity following stroke. Methods: A total of 1,770 subjects from the APASS-WARSS study underwent further aPL testing for antibodies to phosphatidylserine (aPS) and anti-β2-glycoprotein-I (anti-β2GPI) 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, myocardial infarction, transient ischemic attack, deep vein thrombosis, pulmonary embolism or systemic arterial occlusion)/death at 2 years. Cox proportional hazard analyses assessed whether aPL independently related to outcome. Results: Persistent anti-β2GPI decreased the time to TOE/death after adjustment for potential confounders (hazards ratio (HR) 2.86, 95% CI 1.21–6.76, p = 0.017). When persistent anti-β2GPI was combined with another persistently positive aPL, time to TOE/death was also reduced (HR 3.79, 95% CI 1.18–12.14, p = 0.025). Neither persistent anticardiolipin antibodies nor persistent aPS alone nor a single positive anti-β2GPI nor aPS was associated with decreased time to TOE/death. No single positive aPL, portfolio of baseline aPL or any persistent aPL increased

C.F.A. and S.R.L. contributed equally to the manuscript.
the rate of TOE/death. **Conclusions:** Rates of TOE/death were not influenced by aPL results at baseline or follow-up. Persistent anti-β2-GPI alone, and with persistent second aPL, was 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.

**Introduction**

Antiphospholipid antibodies (aPL) have been associated with venous and arterial thrombo-occlusive events (TOEs) [1–5]. A single aPL has been associated with initial stroke [6–9] but was not predictive of recurrent TOE/death [10]. Persistent aPL in young stroke patients was predictive of recurrent stroke [11].

The WARSS-APASS collaboration [10] 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 2 new aPL have been developed potentially more biologically relevant for predicting TOE [12–25]. β2-Glycoprotein-I (β2GPI) has been found to be the most likely antigenic target of the aPL antibodies [23]. In addition, antibodies to phosphatidylserine (aPS) have been independently associated with ischemic stroke [25]. Persistence of aPL over time may confer a higher risk for TOE [11, 26] as does positivity on more than 1 aPL assay (‘portfolio’) [27].

We therefore re-tested stored sera of WARSS-APASS study subjects. In particular, we looked for the presence of anti-β2-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**

A total of 2,206 patients were recruited into WARSS [28]. Of these, 1,770 of 1,954 (91%) APASS-eligible WARSS patients consented to participate in the APASS-WARSS study. 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 unthawed sera stored at −70°C. Samples from baseline as well as 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.

**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 2 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 OR and their 95% 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 independent of the other main effects that are combined to form the interaction.)
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 3 aPL assays, we found the following associated factors: for anti-β2GPI 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 of 147 subjects anti-β2GPI positive at baseline (25.9%) showed persistence, while 24 of 147 subjects (16.3%) were transiently positive. The remaining 85 subjects who were positive at baseline did not have follow-up testing. One subject with a missing anti-β2GPI baseline assay was positive at both months 12 and 24, and was therefore persistently positive.

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**Results**

**Baseline Demographics**

Baseline demographics of subjects assayed for anti-β2GPI 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.

**Table 1. Demographics of subjects tested for anti-β2GPI**

<table>
<thead>
<tr>
<th></th>
<th>Baseline tested (n = 1,624)</th>
<th>Baseline missing (n = 146)</th>
<th>Month 12 tested (n = 575)</th>
<th>Month 12 missing (n = 1,195)</th>
<th>Month 24 tested (n = 475)</th>
<th>Month 24 missing (n = 1,295)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD</td>
<td>62.4±11.3</td>
<td>63.2±10.8</td>
<td>62.5±10.9</td>
<td>62.5±11.5</td>
<td>62.2±10.6</td>
<td>62.7±11.5</td>
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<tr>
<td>Male</td>
<td>57.5</td>
<td>62.3</td>
<td>61.6</td>
<td>56.1</td>
<td>59.6</td>
<td>57.2</td>
</tr>
<tr>
<td>Caucasian</td>
<td>56.9</td>
<td>61.6</td>
<td>57.2</td>
<td>57.3</td>
<td>58.3</td>
<td>56.9</td>
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<tr>
<td>Married</td>
<td>56.2</td>
<td>61.0</td>
<td>57.9</td>
<td>55.9</td>
<td>62.5</td>
<td>54.4</td>
</tr>
<tr>
<td>Sedentary lifestyle†</td>
<td>43.1</td>
<td>47.3</td>
<td>43.5</td>
<td>43.4</td>
<td>48.0</td>
<td>41.8</td>
</tr>
<tr>
<td>Obese</td>
<td>49.6</td>
<td>48.6</td>
<td>46.9</td>
<td>49.3</td>
<td>49.1</td>
<td>49.7</td>
</tr>
<tr>
<td>Tobacco (ever)</td>
<td>64.7</td>
<td>71.2</td>
<td>66.8</td>
<td>64.5</td>
<td>64.8</td>
<td>65.4</td>
</tr>
<tr>
<td>Alcohol (heavy)</td>
<td>12.2</td>
<td>12.3</td>
<td>12.9</td>
<td>11.9</td>
<td>11.4</td>
<td>12.5</td>
</tr>
<tr>
<td>College</td>
<td>27.0</td>
<td>23.3</td>
<td>27.3</td>
<td>26.4</td>
<td>26.7</td>
<td>26.6</td>
</tr>
<tr>
<td>Hypertension</td>
<td>62.5</td>
<td>65.8</td>
<td>67.1</td>
<td>68.4</td>
<td>67.8</td>
<td>68.0</td>
</tr>
<tr>
<td>BP systolic &gt;160</td>
<td>13.7</td>
<td>15.8</td>
<td>14.6</td>
<td>13.5</td>
<td>14.8</td>
<td>13.5</td>
</tr>
<tr>
<td>BP diastolic &gt;95</td>
<td>9.0</td>
<td>8.2</td>
<td>9.0</td>
<td>8.9</td>
<td>8.6</td>
<td>9.0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>31.8</td>
<td>30.1</td>
<td>30.1</td>
<td>32.4</td>
<td>27.8</td>
<td>33.1</td>
</tr>
<tr>
<td>MI</td>
<td>11.8</td>
<td>13.0</td>
<td>11.1</td>
<td>12.2</td>
<td>10.7</td>
<td>12.3</td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>23.4</td>
<td>21.2</td>
<td>23.3</td>
<td>23.2</td>
<td>21.1</td>
<td>24.0</td>
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<td>Ischemic cardiomyopathy</td>
<td>19.4</td>
<td>17.8</td>
<td>19.3</td>
<td>19.3</td>
<td>17.9</td>
<td>19.8</td>
</tr>
<tr>
<td>History of coagulopathy</td>
<td>10.6</td>
<td>10.3</td>
<td>10.1</td>
<td>10.8</td>
<td>11.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Treatment with aspirin</td>
<td>50</td>
<td>52</td>
<td>52.2</td>
<td>49.3</td>
<td>52.0</td>
<td>49.6</td>
</tr>
<tr>
<td>TOE</td>
<td>24.3</td>
<td>21.9</td>
<td>15.3</td>
<td>28.3†</td>
<td>10.7</td>
<td>29.0†</td>
</tr>
</tbody>
</table>

* p < 0.0001, ** p = 0.01, *** p = 0.0008, **** p = 0.02. † Defined as walk <1 mile/day for the past year on average.
No subjects negative at baseline became positive at both months 12 and 24.

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

For aCL, 143 of 485 subjects (29.5%) showed persistence while 171 of 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-β2-GPI positive and persistently positive for either aPS or aCL.

Clinical End Points

There were 394 TOEs among 1,624 patients tested for anti-β2-GPI, 394 among 1,625 patients tested for aPS and 422 among 1,753 patients tested for aCL. There were 9 TOEs in 39 patients persistently positive for anti-β2-GPI: 5 cerebrovascular (1 recurrent stroke and 4 TIs), 3 MIs, and one death. Of 20 patients persistently anti-β2-GPI positive and either persistently aPS or aCL positive, there were 5 TOEs: 1 stroke, 2 TIs, and 2 MIs.

There were no single aPLs nor any persistently positive aPLs associated with the occurrence of TOE/death. Persistently positive anti-β2-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 1 aPS measurement and aCL conferred an increased risk of TOE/death over being positive on zero measurements. However, positive on 2 or 3 aCL measurements conferred a decreased risk over zero positive measurements. Increasing numbers of positive anti-β2-GPI were not associated with either increased or decreased risk of TOE/death (table 3). There was no significant difference between IgG and IgM isoforms 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 follow-up.

Patients with TOE/death were more likely to have missing 12- and 24-month aPL measurements (p <
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 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 aPLs [31]. It may also be explained by the average age and stroke risk factor prevalence of the cohort – 39 of 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 of 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-month follow-up appointment but the presence of the clinical trial (WARSS) end point 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 end point 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 persistent aPL and Recurrent TOE.
Persistent aPL independently predicted recurrent stroke in a large, young (age 18–45 years, mean 36), Italian stroke cohort [11] followed prospectively for a median of 42 months, that is, 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 aCL did not predict recurrent TOE over a 2.8-year median follow-up [34]. 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 years, mean 36), Italian cohort [11] followed prospectively for a median of 42 months, that is, 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-β2GPI remained associated [26]. 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-up [34]. Using binary partition evaluation, no titer criteria for aCL positivity (range 0–60 GPL units) predicted recurrent events.

Recent guidelines recommend that aPL detected acutely should persist to diagnose the antiphospholipid syndrome [31]. The revised Sapporo criteria for antiphospholipid syndrome calls for 12 weeks between assays (level C evidence), to increase specificity without sacrificing sensitivity [31]. The optimal waiting time remains unclear.

In a small, retrospective study [37], 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 treatment [38–41].

Based on the APASS-WARSS study [10], the most recent guidelines for the secondary prevention of aPL-associated, non-cardioembolic stroke recommend antiplatelets [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 anticoagulation [44]. It is not possible to differentiate treatment effects (warfarin vs. 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 arms [45].

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 further 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-β2GPI positivity, as demonstrated by the missing-at-random analysis. Furthermore, this is still 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 unthawed sera stored for several to many years [46], even decades longer than our study. We used only previously unthawed sera, minimizing this potential problem. Also, the definition of persistence of aPL was based on only 3 aPL determinations over 2 years and the follow-up period could have been too short to detect TOEs. We did not collect data on the presence of specific autoimmune diseases (e.g. SLE) in the cohort. A major limitation was that LA had not been tested in the follow-up of this study (to avoid potential unblinding of treatment arm). The population in WARSS [28] 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 strokes 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 etiology [47]. Another study found demographic characteristics, other risk factors, history of prior throm-
thrombotic events and distribution of etiopathogenic types of cerebral ischemia 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 ischemia remains limited [48].

Our results, taken together, appear to conform to the known paradoxical observations when studying recurrent risk—that thrombophilia, while predisposing to a first episode of deep vein thrombosis, does not increase the risk for recurrence [49]. This may be due to index event bias [50] based on selection of subjects who had a first event. As risk factors for index and recurrent events are often similar, there is a bias of studies toward 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 stroke [50].

In conclusion, our study suggests that 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.

**Appendix: Co-Investigators at Participating APASS Centers**

Louis R. Caplan, Beth Israel and New England Medical Center; Carlos S. Kase, Boston University; Patrick Pullicino, Buffalo General Hospital; Cathy Sila, Cleveland Clinic; Ralph L. Sacco, Columbia University; Constance Johnson, Johns Hopkins University; Fenwick Nichols, Medical College of Georgia; Cathryn Helsagag, University of Illinois; Harold P. Adams Jr, University of Iowa; L. Creed Pettigrew, University of Kentucky; Percy Karkanji, Marshfield Clinic; J. Philip Kistler, Massachusetts General Hospital; Stanley Tuhrim, Mount Sinai School of Medicine; Gary Friday, Lankenau Medical Center; Joshua Hollander (deceased), Rochester General Hospital; Glen Fischberg, University of Southern California; Gregory W. Albers, Stanford University; Antonio Culebras, SUNY-Syracuse; James C. Grotta, University of Texas; Jonathan Dissin, Albert Einstein/PA and University of Vermont; E. Clarke Haley Jr, University of Virginia; Lawrence M. Brass (deceased), Yale University; David Anderson, Hennepin County Hospital; Richard Libman, Long Island Jewish Medical Center; Aaron Miller, Maimonides Medical Center; Anne N. Nafziger, Bassett Hospital; Jose Biller, Indiana University; Alejandro Fortezza, University of Miami; Howard Kirshner, Vanderbilt University; Panayiotis Mitsias, Henry Ford Hospital; Mohammed Yaseen, Georgetown University; Dara Jamieson, Pennsylvania Hospital; B.K. Dandapani, Cleveland Clinic – FL; Laura Lennihan, Helen Hayes Hospital; Christy Jackson, UCSD; Seemant Chaturvedi, Wayne State University; John Rothrock, University of Southern Alabama; James Frey, Barrow Neurological Institute/St. Joseph’s Phoenix.

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**Disclosure Statement**


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