Antiphospholipid antibodies are persistently positive at high titers. Additive value of platelet-bound C4d

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Background: Classification criteria for antiphospholipid syndrome (APS) require that antiphospholipid antibody (aPL) positivity is confirmed after at least 12 weeks. We tested the hypothesis that aPL at high titers remain positive while low titers fluctuate over time. As both platelet-bound C4d (PC4d) and aPL are associated with thrombosis in systemic lupus erythematosus (SLE), we also evaluated whether PC4d can aid in APS diagnosis.

Methods: Data from serum or plasma sent to Exagen’s laboratory for routine aPL testing were analyzed. Anti-cardiolipin (aCL) and anti-beta2 glycoprotein-1 antibodies (aB2GP1) were measured by chemiluminescence or ELiA fluorescence enzyme immunoassay; anti-phosphatidylserine/prothrombin complex antibodies (aPS/PT) by ELISA; PC4d by flow cytometry. Statistical analysis included descriptive statistics, logistic regression, and Pearson correlation.

Results: More than 80% of positive samples with aCL and aB2GP1 at high titers - but not low titers - were positive at a retest. Non-criteria aPL (aPLs/PTs) followed a similar trend. aCL and aB2GP1 measured with two different technologies were highly correlated. PC4d and IgG of the three aPLs were at best moderately correlated even when only positive aPL samples were analyzed (coefficient: 0.1917 to 0.2649).

Conclusions: High titers aPL are often persistently positive, allowing an earlier diagnosis and risk assessment at the time of the initial screening. Conversely, a retest may be necessary for low titers. The high correlation between two
Introduction

Antiphospholipid antibodies (aPL) are autoantibodies directed against plasma proteins complexed with negatively charged phospholipids on cell membranes (1). They are commonly found in patients with systemic lupus erythematosus (SLE) (2, 3) and are the main autoantibodies in patients with the antiphospholipid syndrome (APS) (4).

Binding of aPL to their autoantigen results in activation of complement and of endothelial cells, platelets, neutrophils, and monocytes (4). Activation of these cells promotes inflammation, clot formation, vasculopathy, thrombosis, and pregnancy complications in patients with APS (4) and SLE (2).

The classification criteria for APS (5) include lupus anticoagulant (LAC), as well as the IgG and IgM isotypes of anti-cardiolipin (aCL) and anti-beta2-glycoprotein 1 (aB2GP1), while some classification criteria for SLE include also the IgA isotypes (6–8). aPL titers are taken into consideration in all criteria.

In addition to LAC, aCL, and aB2GP1, so-called non-criteria aPL are common in APS and SLE (9, 10). IgG and IgM against the phosphatidylserine/prothrombin complex (aPS/PT) are among the best characterized non-criteria aPL (11, 12) and may have clinical significance in increasing risk of thrombosis (13, 14).

Classification criteria for SLE require only one aPL determination (6–8), while criteria for APS (5) require a confirmatory assay performed at least 12 weeks later to establish persistent positivity. In fact, transiently positive aPL, especially at low titer, may be the consequence of infections or medications and may not be clinically relevant (4, 15). Although evaluation of persistent positivity is important, a wait period of 12 weeks may delay risk assessment and adequate patient treatment. Persistent positivity for aPL has been evaluated in patients enrolled in the APS ACTION Registry, which includes patients with or without systemic autoimmune diseases but persistently positive for APS criteria aPL. Approximately 80% of patients with clinically meaningful aPL positivity at baseline remained stable at a median follow-up of 5 years, suggesting that high titer aPL may remain positive over time (15).

Materials and methods

Anti-phospholipid antibodies

All samples analyzed for this study were collected in the United States. All assays were carried out in Exagen’s clinical laboratory following the manufacturers’ instructions and Exagen’s standard operating procedures. aCL and aB2GP1 were measured by chemiluminescence (QUANTA Flash; Werfen, San Diego, CA) or ELiA fluorescence enzyme immunoassay (Phadia; ThermoFisher Scientific, Freiburg, Germany) in serum or plasma from venous blood collected in ethylenediamine-tetraacetic acid (EDTA). aPS/PT were measured by ELISA (QUANTA Lite; Werfen) and were considered positive if > 30 units (U). All the isotypes of aCL and aB2GP1 measured by chemiluminescence were considered positive if > 20 chemiluminescent units (CU); aCL IgG and IgM measured by ELiA were considered positive if > 40 units/ml; aCL methodologies suggests that these findings are independent of assay platform. The low to moderate correlation between PC4d and aPL might suggest a possible additive value to evaluate association with thrombosis in autoimmune diseases.

KEYWORDS
antiphospholipid antibodies, antiphospholipid syndrome, biomarkers, persistency, cell-bound complement activation products, PC4d
IgA measured by ELISA was considered positive if > 20 units/ml; the isotypes of aB2GP1 measured by ELISA were considered positive if > 10 units/ml. Exagen measures aCL and aB2GP1 isotypes IgG and IgM as a panel. Thus, these four biomarkers are measured for all - or most - samples from the same specimen. aCL IgA, aB2GP1 IgA, and aPS/PT IgG and IgM are not part of the panel and are performed separately.

Platelet-bound C4d

PC4d was measured by flow cytometry following Exagen’s standard operating procedures, as described (14, 23). The assay was extensively validated by Exagen before it was made commercially available, and validation was approved by the New York State Department of Health. Briefly, red blood cells from EDTA-anticoagulated blood were lysed and platelets were stained using a mouse monoclonal antibody against human C4d (Quidel, San Diego, CA) or a mouse IgG1 isotype control (MPOC-21). After incubation, samples were stained with a goat anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC). A mouse anti-human monoclonal antibody against human CD42b conjugated to phycoerythrin (PE) was used to identify platelets. FACS analysis was performed using a Gallios flow cytometer (Beckman Coulter, Brea, California). Light scatter (forward and side) gating parameters were used to isolate the platelet population, followed by secondary gating based on positive CD42b PE staining. Quantification of the non-specific (isotype control) and specific (C4d) fluorescence was determined for the CD42b PE gated platelets (5000 events). Net MFI was calculated by subtraction of isotype control MFI on the specific C4d MFI on gated platelets.

Patient population

Data of samples sent to Exagen’s clinical laboratory for routine patient testing were extracted from Exagen database. Approximately 36,000 or 19,000 individuals who had at least two determinations for aCL IgG, aCL IgM, aB2GP1 IgG, and aB2GP1 IgM over a 5-year period (from November 2016 to October 2021) were identified. The number was lower (approximately 19,000) for the IgA isotypes and for aPS/PT. Only the samples for whom the first determination was positive were analyzed. If a patient was tested more than once after a positive determination, only the first subsequent determination was included in the analysis.

Statistical analysis

Descriptive statistics were performed to evaluate persistent positivity of the biomarkers by quartile analysis, performed by dividing the number of observations into four equal parts. Logistic regression analysis was performed to evaluate covariates associated with persistent positivity. Pearson correlation analysis was performed to evaluate correlation between two platforms for aPL measurement, and between aPL and PC4d.

Results

aPL persistency

Of the approximately 36,000 or 19,000 individuals who had at least two aPL determinations, we analyzed only the samples for whom the first aPL determination was positive. The interval of time between the first and the second determination ranged between 0 and 252 weeks. The median time ranged from 54.07 weeks for aB2GP1 IgG (IQR = 24.0 to 95.14 weeks) to 61.00 weeks for aPS/PT IgG (IQR = 30.71 to 106.71 weeks) (Supplementary Table 1). The number of patients who were tested at least twice and for whom a certain aPL isotype was positive at the first determination is reported in Table 1.

Because our analysis was based on real world data and aimed to evaluate persistent positivity regardless of the interval of time required by the classification criteria for APS, all samples were included in the analysis, irrespective of the time between determinations. However, the majority of samples were retested after more than 12 weeks. Depending on the particular analyte, 92% to 95% of samples were tested more than 12 weeks apart. In addition, 98% to 99% of samples were tested more than 30 days apart. Positivity rate of samples tested for the IgG of aCL and aB2GP1 at least 12 weeks, 30 days, or 7 days apart is reported in Supplementary Table 2.

To evaluate aPL persistent positivity over time, we performed a quartile analysis for the isotypes of aCL and aB2GP1 measured by QUANTA Flash and the isotypes of aPS/PT measured by QUANTA Lite. Confirmation rates of each aPL show that, for all biomarkers, percent positivity at the retest increased as the titer of the first positive determination increased. Confirmation rate was the lowest in the first quartile and ranged from 23.1% for aPS/PT IgG to 55.4% for aB2GP1 IgA. On the other hand, confirmation rate was highest in the fourth quartile and ranged from 89.3% for aPS/PT IgM to 98.9% for aB2GP1 IgA (Table 1).

As expected based on literature data (25), a higher percentage of double positive samples (positive for the IgG of aCL and aB2GP1) were positive at retest compared to single positive samples. In fact, 94.4% of double positive samples tested positive at retest for one of these aPL, while 61.4% to 68.8% single positive samples tested positive at retest (Supplementary Table 3). Interestingly, no samples positive for both IgM aCL and IgM aB2GP1 were IgG negative.
Logistic regression analysis showed that the strongest predictor for retest positivity for all the aPL was the initial quantitative positive value of the same analyte, while positivity for a different aPL, gender, age, or time between visits were not associated with probability of testing positive at the retest (data not shown).

Consistent with the high persistent positivity of high aPL titers and low persistent positivity of the low titers, we observed that samples that were negative at the first determination but close to cutoff tended to be positive at a retest more frequently than negative samples that had lower titers. Negative aCL and aB2GPI samples >10 CU were positive at a retest 7.1% to 14.1% of the time, while negative samples >15 CU were positive at a retest 14.0% to 23.1% of the time. A similar trend was observed for aPS/PT (Supplementary Table 4).

**aPL platform comparison**

We compared the QUANTA Flash and ELiA platforms using 73 previously frozen serum or plasma samples and found good correlation between the platforms. Correlation coefficients ranged from 0.6039 for aCL IgG to 0.9091 for aB2GPI IgA (Table 2). Overall, the two platforms were more concordant at high aPL titers than low titers (data not shown).

**Correlation with PC4d**

We evaluated the correlation between PC4d and aPL in the cohort of patients for whom PC4d and aPL were measured. Correlation between PC4d and IgG was moderate even when only samples positive for a certain aPL were analyzed (Person correlation coefficients: 0.2649, 0.1917, and 0.2622 for the IgG of aCL, aB2GPI, and aPS/PT, respectively). Correlation was lower for IgM and was poor for the IgA isotypes (Table 3).

**Discussion**

To our knowledge, this is the first study that evaluated aPL persistent positivity in a large population of patients from the United States for whom aPL were tested for routine clinical assessment. In addition, we evaluated whether a marker of complement activation, PC4d, which is associated with thrombosis in SLE, can aid APS diagnosis.

**Table 1** aPL positivity at retest.

| aPL Platform | Positive samples at retest/total samples | Percent |
|--------------|------------------------------------------|---------|
| aCL IgG N=2,964 | Q1 (20-25 CU) 334/741 45.1% |         |
|               | Q2 (25-32 CU) 514/741 69.4% |         |
|               | Q3 (32-52 CU) 627/741 84.6% |         |
|               | Q4 (52-2024 CU) 709/741 95.7% |         |
| aCL IgM N=1,653 | Q1 (21-27 CU) 196/414 47.3% |         |
|               | Q2 (27-38 CU) 294/413 71.2% |         |
|               | Q3 (38-69 CU) 363/413 87.9% |         |
|               | Q4 (69-774 CU) 398/413 96.4% |         |
| aCL IgA N=553 | Q1 (21-26 CU) 47/139 33.8% |         |
|               | Q2 (26-35 CU) 86/138 62.3% |         |
|               | Q3 (35-64 CU) 122/138 88.4% |         |
|               | Q4 (66-352 CU) 134/138 97.1% |         |
| aB2GPI IgG N=1,664 | Q1 (21-30 CU) 164/416 39.4% |         |
|               | Q2 (30-57 CU) 283/416 68.0% |         |
|               | Q3 (57-200 CU) 368/416 88.5% |         |
|               | Q4 (201-6100 CU) 408/416 98.1% |         |
| aB2GPI IgM N=948 | Q1 (21-29 CU) 115/237 48.5% |         |
|               | Q2 (29-45 CU) 186/237 78.5% |         |
|               | Q3 (45-88 CU) 209/237 88.7% |         |
|               | Q4 (88-841 CU) 228/237 96.2% |         |
| aB2GPI IgA N=368 | Q1 (21-27 CU) 51/92 55.4% |         |
|               | Q2 (27-40 CU) 82/92 89.0% |         |
|               | Q3 (41-80 CU) 89/92 96.7% |         |
|               | Q4 (80-352 CU) 91/92 98.9% |         |
| aPS/PT IgG N=1,073 | Q1 (31-36 CU) 62/269 23.1% |         |
|               | Q2 (36-45 U) 103/268 38.4% |         |
|               | Q3 (46-70 U) 162/268 60.5% |         |
|               | Q4 (70-150 U) 242/268 90.3% |         |
| aPS/PT IgM N=3,217 | Q1 (31-36 U) 285/805 35.4% |         |
|               | Q2 (36-47 U) 390/804 48.5% |         |
|               | Q3 (47-80 U) 555/804 69.0% |         |
|               | Q4 (80-150 U) 718/804 89.3% |         |

Samples positive for the specified isotype (IgG, IgM, and IgA) of anti-cardiolipin antibodies (aCL), anti-beta2 glycoprotein-1 antibodies (aB2GPI), or anti-phosphatidylserine/prothrombin complex antibodies (aPS/PT) and for which a second determination was available were divided in quartiles (Q) based on their titers. The range in each quartile is reported in parenthesis. N indicates the number of samples that were positive at the first test. Percent indicates the percent of positive samples that gave positive results at the first subsequent test in each quartile. All aCL and aB2GPI isotypes were measured by chemiluminescence (QUANTA Flash; Werfen) and were considered positive if > 20 chemiluminescent units (CU); aPS/PT isotypes were measured by ELISA (QUANTA Lite; Werfen) and were considered positive if > 30 units (U).

Logistic regression analysis showed that the strongest predictor for retest positivity for all the aPL was the initial quantitative positive value of the same analyte, while positivity for a different aPL, gender, age, or time between visits were not associated with probability of testing positive at the retest (data not shown).

Correlation with PC4d

We compared the QUANTA Flash and ELiA platforms using 73 previously frozen serum or plasma samples and found good correlation between the platforms. Correlation coefficients ranged from 0.6039 for aCL IgG to 0.9091 for aB2GPI IgA (Table 2). Overall, the two platforms were more concordant at high aPL titers than low titers (data not shown).

**Correlation coefficient**

| aPL | IgG     | 0.6039 |
|-----|---------|--------|
|     | IgM     | 0.6963 |
|     | IgA     | 0.7394 |
| aB2GPI | IgG     | 0.8213 |
|     | IgM     | 0.7962 |
|     | IgA     | 0.9091 |

Pearson correlation coefficients between the isotypes (IgG, IgM, and IgA) of anti-cardiolipin antibodies (aCL) and anti-beta2 glycoprotein-1 antibodies (aB2GPI) measured by chemiluminescence (QUANTA Flash; Werfen) and ELISA fluorescence enzyme immunoassay (Phadia, ThermoFisher Scientific) (N=73).
positive in the vast majority of patients. In fact, when data were stratified by quartiles, over 80% of patients with titers in the two highest quartiles were positive at retest. Persistent positivity rate of the non-criteria aPS/PT was only slightly lower.

The interval of time between determinations in our dataset was variable and ranged from 0 to 252 weeks. This is not surprising, as our analysis was based on real world data. We hypothesized that high titers of aPL are persistently positive and, thus, that repeating aPL determinations after 12 weeks or longer is not necessary when titers are high. To test this hypothesis, we included all samples in the analysis, regardless of whether they were positive or negative for a certain aPL. aPL positive samples N and aPL positive samples Correlation coeff. refer to the analysis that included the subset of samples positive for the respective aPL.

When evaluating predictors for aPL positivity at retest, the strongest predictor was the initial quantitative positive value of the same analyte, reinforcing the concept that aPL at high titers is not necessary when titers are high. This finding is consistent with previous data (25).

### Pearson correlation coefficients between PC4d and the isotypes (IgG, IgM, and IgA) of anti-cardiolipin antibodies (aCL) and anti-beta2 glycoprotein-1 antibodies (aB2GP1) measured by chemiluminescence (QUANTA Flash; Werfen) or anti-phosphatidylserine/prothrombin complex antibodies (aPS/PT) measured by ELISA (QUANTA Lite; Werfen). All samples N and All samples Correlation coeff. refer to the analysis that included all samples, regardless of whether they were positive or negative for a certain aPL. aPL positive samples N and aPL positive samples Correlation coeff. refer to the analysis that included the subset of samples positive for the respective aPL.

|                | All samples | All samples | aPL positive samples | aPL positive samples |
|----------------|-------------|-------------|----------------------|----------------------|
|                | N           | Correlation coeff. | N                  | Correlation coeff.   |
| aCL            |             |               |                     |                      |
| IgG            | 6,912       | 0.1764       | 647                 | 0.2649               |
| IgM            | 6,911       | 0.1049       | 330                 | 0.1600               |
| IgA            | 6,403       | 0.1483       | 198                 | 0.0192               |
| aB2GP1         |             |               |                     |                      |
| IgG            | 6,912       | 0.1841       | 435                 | 0.1917               |
| IgM            | 6,912       | 0.0999       | 201                 | 0.1009               |
| IgA            | 6,402       | 0.1198       | 171                 | 0.0409               |
| aPS/PT         |             |               |                     |                      |
| IgG            | 6,400       | 0.2368       | 316                 | 0.2622               |
| IgM            | 6,400       | 0.2448       | 964                 | 0.1982               |

Pearson correlation coefficients between PC4d and the isotypes (IgG, IgM, and IgA) of anti-cardiolipin antibodies (aCL) and anti-beta2 glycoprotein-1 antibodies (aB2GP1) measured by chemiluminescence (QUANTA Flash; Werfen) or anti-phosphatidylserine/prothrombin complex antibodies (aPS/PT) measured by ELISA (QUANTA Lite; Werfen). All samples N and All samples Correlation coeff. refer to the analysis that included all samples, regardless of whether they were positive or negative for a certain aPL. aPL positive samples N and aPL positive samples Correlation coeff. refer to the analysis that included the subset of samples positive for the respective aPL.
of data analysis, a relatively small number of samples had been tested on this platform.

In conclusion, our analysis of a large cohort of patients demonstrates that high titers aPL are persistently positive. This observation has the potential to impact clinical practice, allowing for an earlier diagnosis and risk assessment at the time of the initial screening. Conversely, a retest is necessary for low titers. The high correlation between two methodologies suggests that these findings are independent of assay platform. Finally, the low to moderate correlation between PC4d and aPL, combined with findings in previous studies (14, 22, 23, 28), might suggest a potential additive value to evaluate association with thrombosis in autoimmune diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author upon reasonable request.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants’ legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

RA wrote the manuscript and contributed to sample testing and data analysis. SS conceived the study. RB analyzed the data. TO contributed to data analysis. AK contributed to sample testing and data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

RB, TO, AK, and RA are current or former employees of Exagen Inc. and/or stockholders of Exagen Inc.

The remaining author declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.949919/full#supplementary-material

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