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ORIGINAL ARTICLE

The absence of standardization in antiphospholipid antibody testing may favor the use of 99th percentile cutoffs in antiphospholipid syndrome classification

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Abstract

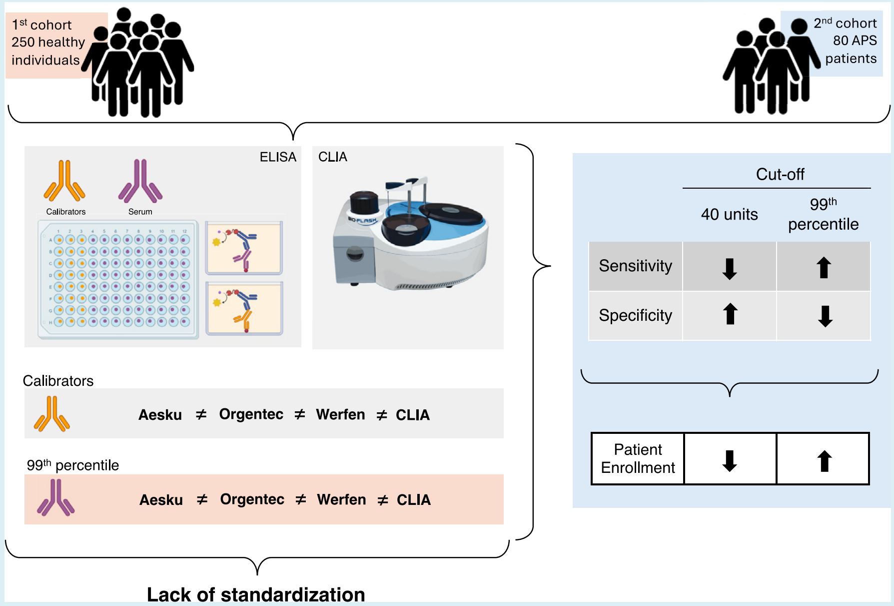
Background: Classification criteria for antiphospholipid syndrome (APS) issued by the American College of Rheumatology/European Alliance of Associations for Rheumatology necessitate a positivity for any of the 3 molecular targets: lupus anticoagulant, anticardiolipin (aCL) immunoglobulin G, or anti-β2 glycoprotein I (aβ2GPI) immunoglobulin G, with the latter 2 requiring concentrations > 40 units. This specification implies having standardized and comparable calibration strategies to achieve proper patient classification. In the past, calibrator tests suffered from poor standardization; thus, the 99th percentile was established as the cutoff point.

Objectives: We aimed to find a balance between sensitivity and specificity in the laboratory criteria for patient enrollment in APS studies by harmonizing the 99th percentile and 40-unit threshold.

Methods: In a cohort of 250 healthy individuals, we tested aCL and aβ2GPI concentrations by 4 different methods: 3 colorimetric, standardized ELISA platforms and 1 chemiluminescence assay, to define the 99th percentile. We tested cross-reactivity of standardized calibrators between kits and how to implement better accuracy for patient enrollment in a cohort of 80 APS patients.

Results: We found that the 99th percentile was substantially <40-unit cutoff and observed considerable interkit variability in the determined cutoffs, which originated from the inadequate standardization of kit calibrators. In a second cohort of 80 APS patients, we estimated the accuracy of these different methods by comparing the 99th percentile and 40-unit cutoffs. For certain ELISA kits, using a fixed cutoff of 40 units instead of the 99th percentile decreased their sensitivity without increasing specificity, which affected patient classification and thus the number of patients eligible for APS studies. Testing with 2 ELISA platforms at the 99th percentile cutoff would improve patient eligibility.

Conclusion: Our survey suggests that in the absence of standardized calibrators for testing aCL or aβ2GPI, a cutoff point at the 99th percentile of 2 different ELISA kits should be adopted.



KEYWORDS
anti-β2 glycoprotein I, anticardiolipin, antiphospholipid syndrome, chemiluminescent assay, ELISA, reference values

Essentials

- APS classification relies on aPL tests.
- Two hundred fifty controls defined aPL thresholds in 4 methods, and accuracy was tested in 80 APS patients.
- The 40-unit threshold missed APS patients compared with the 99th percentile method.
- Using 2 tests and 99th percentile cutoffs may improve the accuracy of APS study enrollment.

1 | INTRODUCTION

The new American College of Rheumatology/Europena Alliance of Associations for Rheumatology (ACR/EULAR) classification criteria for antiphospholipid syndrome (APS) [1,2] establish the use of standardized ELISA techniques to quantify the concentrations of 2 key antiphospholipid antibodies (aPL), anticardiolipin (aCL), and anti-β2 glycoprotein I (aβ2GPI) immunoglobulin (Ig) G isotypes. IgM isotypes for aCL and aβ2GPI are also considered, but with such a low weight that their positivity is insufficient to fulfill the laboratory classification criteria. Determining aCL or aβ2GPI positivity involves measuring aPL concentrations above a cutoff of 40 units (U), according to domain 8 of the ACR/EULAR classification criteria for APS [1]. Patients with positive aCL or aβ2GPI are further stratified based on their antibody concentrations: moderate positivity is defined as values between 40 and 80 U, while high positivity corresponds to values > 80 U. This stratification assigns a differential weight in scoring, reflecting the clinical significance of antibody quantification.

Setting up a proper cutoff value for aCL and aβ2GPI antibody positivity has been an ongoing endeavor since Harris et al. [3] described an immunoassay for assessing aCL concentrations in 1983. Early efforts focused on standardizing a solid-phase immunoassay protocol, culminating in multicenter studies that developed aCL calibrators purified from human sera and featured as polyclonal antibodies [4,5]. These international standardization workshops introduced recognized GPL for aCL IgG units (mg of aCL IgG/mL), and MPL for aCL IgM units (mg of aCL IgM/mL), laying the foundation for uniform antibody measurement. Interestingly, it was later discovered that aCL antibodies primarily recognize not cardiolipin itself but a plasma protein cofactor in a greater manner [6,7], identified as β2-glycoprotein I (β2GPI) [8,9], which is essential for antibody binding. This finding was further supported by studies using monoclonal aCL antibodies, either isolated from APS patients [10], such as EY2C9, or bioengineered as chimeric mouse-human antibodies [11], such as HCAL. These antibodies also demonstrated a dependence on β2GPI presence for cardiolipin recognition [10,11], and even numerical equivalence of HCAL concentration to GPL was described [11]. In turn, aβ2GPI antibodies recognize cryptic epitopes on β2GPI protein when bound to anionic phospholipids [12,13]. Antigenic regions were described in the C-terminal domains 4 and 5 of β2GPI [14,15]; however, no association between positivity to IgG aβ2GPI domains 4/5 and thromboembolism in APS patients was found [16], which was also confirmed in COVID-19 patients [17]. Studies on epitope specificity have demonstrated that aβ2GPI antibodies against the N-terminal cryptic domain 1 are pathogenic [18–21]. To validate equal aPL determinations and promote consistency across laboratories, task forces were created to ensure the availability of properly prepared and validated reference materials, including polyclonal and monoclonal antibodies, for aCL and aβ2GPI assays. These efforts also aimed to maintain standardized reporting in GPL/MPL for aCL and internationally agreed-upon U for aβ2GPI measurements [22–25], facilitating more reliable and comparable results. Currently, many commercially available standardized ELISAs for aCL and aβ2GPI antibodies use HCAL and EY2C9 as calibrators [11]. Nevertheless, solid-phase assays for aCL and aβ2GPI display considerable interassay variability, posing a challenge to the consistency and reliability of laboratory criteria for APS classification [26].

Other methodologies, such as fully automated chemiluminescence-based assays, have been implemented and have shown excellent interlaboratory agreement [27], with performance comparable to standardized commercial ELISAs [28]. However, antibody titers varied considerably between the different systems [29,30], which led to the new ACR/EULAR APS classification criteria excluding these more recently introduced methods.

The use of the 99th percentile as a cutoff value for the classification of aCL and aβ2GPI positivity has been previously recommended by the Sydney classification criteria [31] and the International Society on Thrombosis and Haemostasis (ISTH) [23]. The former also considered the cutoff of 40 GPL for aCL [31]. A disagreement between the cutoffs of the 99th percentile and 40 GPL was observed, with the 99th percentile cutoff being more sensitive [32]. In addition to the lack of standardized calibrators, solid-phase assays for aCL and aβ2GPI show interassay differences [33–35]. In this context, we hypothesized the sensitivity-specificity accuracy of the 40-U cutoff would depend on the type of assay and manufacturer used for the detection of aPL. To this end, we analyzed aCL and aβ2GPI concentrations in 250 healthy controls using 3 standardized, commercially available solid-phase ELISA kits and 1 automated chemiluminescence assay (CLIA). Our analysis showed that the different ELISA kits had different cutoff values (set at the 99th percentile), all of which were far below the universally applied 40-U cutoff. The 99th percentile for aCL with CLIA was the only one close to the 40-U cutoff. The implementation of a fixed cutoff value of 40 U for all methods still resulted in discrepancies in aPL positivity between tests, and the exclusion of patients whose aCL or aβ2GPI concentrations were >99th percentile from enrollment in research studies. This exclusion is particularly concerning, given the evidence supporting the relevance of low/medium aPL values in obstetric APS (OAPS) [32,36]. We posit that the use of a rigid 40-U cutoff risks excluding patients with clinically significant symptomatology, particularly women with obstetric manifestations of APS, from being appropriately classified and included in research studies.

2 | METHODS

2.1 | Study design

Two hundred fifty healthy blood donors from the Catalan Blood and Tissue Bank (Banc de Sang I Teixits, Generalitat de Catalunya) were selected, matched in age and sex (62% women, 38% men; mean ± SD, 42.4 ± 11.7 years old) with our cohort of APS patients. The sample size of 250 individuals was determined based on recommendations for defining reference intervals in immunoassays, aiming for statistical robustness to reliably calculate the 99th percentile while minimizing variability [37,38]. A cohort of 80 APS subjects was selected from participants enrolled in APS studies at Vall d'Hebron Barcelona Hospital and who were classified as APS patients according to the previous Sydney classification criteria [31]. All of them fulfilled clinical criteria according to the ACR/EULAR APS guidelines [1]. The medical history and laboratory data of these APS patients were summarized in a codified and anonymized database stored in our institutional repository (Hospital Universitari Vall d'Hebron Institut de Recerca). The study was originally approved by the Independent Ethics Committee of the Vall d'Hebron Barcelona Hospital (PR[AG]83/2020), and all participants signed an informed consent form. The study content, in which patients and participants took part, strictly complied with the World Medical Association's Helsinki Declaration guidelines.

2.2 | Sample collection

Blood samples were collected by venipuncture and processed for serum isolation within 2 hours of collection. After 15-minute centrifugation at 1500 × *g*, the sera were isolated from gel clotting tubes (Vacutainer, BD), aliquoted, and stored at −80 °C until analyzed.

2.3 | Laboratory clinics

At the time of enrollment in APS studies, according to Sydney classification criteria, lupus anticoagulant (LA), aCL IgG/IgM, and aβ2GPI IgG/IgM were the aPL to be tested. LA was measured at the Clinical Hematology Laboratory of Vall d'Hebron Barcelona Hospital using standardized protocols for diluted Russell's viper venom time and silica clotting time, and performed according to the published ISTH guideline [39]. Briefly, the screening test was the ratio of clotting time of patient plasma divided by clotting time of pooled normal plasma (PNP) of 40 healthy donors; mixing test was the ratio of clotting time of the 1:1 mixture of patient plasma and PNP divided by clotting time of PNP of 40 healthy donors; eventually, the confirmatory test was the ratio of clotting time of patient plasma performed in the presence of high content phospholipids divided by clotting time of PNP of 40 healthy donors. IgG and IgM isotypes for aCL and aβ2GPI were measured at the Clinical Immunology Laboratory of Vall d'Hebron

Barcelona Hospital using commercial CLIAs (QUANTA Flash, Werfen) with the BIO-FLASH system (Werfen). Subjects were considered to fulfill the laboratory criteria for APS if: (i) the normalized screening and mixing tests in the LA assay were >1.2, and phospholipid dependence was confirmed when the normalized screening test divided by the normalized confirmatory test was ≥1.2, and/or (ii) their aCL IgG or IgM concentrations were ≥40 GPL or 40 MPL, respectively, or ≥99th percentile cutoff value, and/or (iii) their aβ2GPI IgG or IgM concentrations reached ≥99th percentile cutoff value (20 U/mL) in 2 blood samples collected at least 12 weeks apart and for each type of aPL. Depending on their aPL positivity, subjects were classified into laboratory categories as follows: category I, repeatedly positive for the 3 aPL or 2 aPL; category II, repeatedly positive only for LA (IIa), aCL IgG or IgM (IIb), or aβ2GPI IgG or IgM (IIc).

2.4 | ELISA

IgG aCL and IgG aβ2GPI were quantified through 3 different commercial ELISA kits referred to as Werfen (Werfen), Orgentec (Orgentec Diagnostika GmbH), and Aesku (Aesku Diagnostics Inc). The specific characteristics of each are summarized below.

2.4.1 | QUANTA Lite ACA HRP IgG III ELISA (Werfen, #708625)

Polystyrene microwells are coated with a purified cardiolipin phospholipid as an antigen and bovine β2GPI protein as cofactor. The calibrator for aCL IgG is human serum antibodies to cardiolipin that are originally traceable to HCAL monoclonal antibodies and used at 4.2, 9.8, 18.8, 37.5, 75, and 150 standard U (GPL) [11].

2.4.2 | QUANTA Lite β2GPI IgG ELISA (Werfen, #708665)

Purified β2GPI protein from human serum is used as an antigen and is bound to the wells of a polystyrene microwell plate. The calibrators are purified aβ2GPI IgG polyclonal antibodies with 9.4, 18.8, 37.5, 75, and 150 standard IgG β2GPI U. The standard used to construct the 5-point curve refers to calibrators for IgG aβ2GPI, available from the Rheumatology Laboratory at Seton Hall University, St Joseph's Hospital and Medical Center [40].

2.4.3 | aCL IgG/IgM (Orgentec Diagnostika GmbH, #ORG515)

Highly purified cardiolipin is coated onto microwell plates saturated with β2GPI. The calibrators for aCL IgG are polyclonal antibodies used at 7.5, 15, 30, 60, and 120 standard U (GPL) and calibrated against the reference sera from E.N. Harris (Louisville), as well as the specific reference materials International Reference Preparation 97/656 and HCAL for IgG and EY2C9 for IgM [11].

2.4.4 | aβ2GPI IgG/IgM (Orgentec Diagnostika GmbH, #ORG521)

Highly purified β2GPI protein is bound to microwell plates. Calibrators are polyclonal antibodies at 6.3, 12.5, 25, 50, and 100 U/mL. Calibration is related to the reference sera from E.N. Harris (Louisville), as well as International Reference Preparation 97/656 and HCAL for IgG and EY2C9 for IgM.

2.4.5 | AESKULISA Cardiolipin-GM (AESKU Diagnostics, #3204)

The assay uses highly purified cardiolipin plus native human β2GPI for the quantitative detection of IgG and IgM antibodies against cardiolipin in human serum. Monoclonal antibodies are used in the AESKULISA Cardiolipin-GM, which is calibrated against reference sera from E.N. Harris (Louisville). The results are expressed in GPL/mL. In addition, the calibrators in AESKULISA Cardiolipin-GM are standardized using the Sapporo standards HCAL for IgG and EY2C2 for IgM.

2.4.6 | AESKULISA β2-Glyco-GM (AESKU Diagnostics, #3206)

The assay uses native β2GPI, highly purified from human plasma, for the quantitative detection of IgG and IgM antibodies against β2GPI in human serum. Calibrators are monoclonal antibodies that are standardized using the Sapporo standards HCAL for IgG and EY2C9 for IgM. Calibrators are used at 3, 10, 30, 100, and 300 U/mL.

The ELISAs were performed according to the manufacturer's instructions. Briefly, 1:100 diluted sera from healthy controls and patients were added in parallel to the calibrators in separate wells. Calibrators and samples were added in duplicate and triplicate, respectively. Extensive washes allowed for retaining only the serum components were able to bind to the cognate immobilized antigen. These antigen-bound aCL or aβ2GPI IgG antibodies were specifically recognized by a secondary anti-human IgG antibody coupled to the horseradish peroxidase enzyme. Extensive washes were performed again, and the remaining enzyme activity was measured by adding the chromogenic 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. The intensity of the color of the resulting horseradish peroxidase activity product was measured in a spectrophotometer (Varioskan LUX multimode microplate reader, Thermo Fisher Scientific) at 450 nm. A reference wavelength of 630 nm was used. The intensity readout was expressed in absorbance U, which was converted to GPL or U/mL using the curve of standardized ELISA calibrators. It is worth noting that the final readouts indicating aCL or aβ2GPI IgG antibody concentrations are not arbitrary U but standardized U (GPL or U/mL, respectively).

All the test results passed the quality control criteria to be considered valid. Specifically, the absorbance at 450 nm of the positive control, supplied by the kit's manufacturer, was lower than that of the highest concentration calibrator and more than double of the negative control. Additionally, the absorbance at 450 nm of the negative control did not exceed 0.2, and the concentration of the positive control fell within the range stated by the manufacturers.

To further evaluate performance, calibrators for each ELISA kit were tested across the 3 ELISA platforms, either directly or diluted in the sample dilution buffer specific to each platform. Serum samples positive for aPL from 4 APS patients, diluted 1:100 in the different diluents, were also tested using the Werfen ELISA platform.

2.5 | Coefficients of variation

Coefficients of variation (CVs) were calculated to evaluate the precision and reproducibility of the data. CVs were assessed for all calibrators running across 7 different testing days using multiple reagent lots and involving various experimenters.

2.6 | CLIAs

aCL and aβ2GPI IgG were measured in healthy controls and patients using the QUANTA Flash Cardiolipin IgG (Werfen) and QUANTA Flash β2GPI IgG (Werfen), respectively. Data acquisition was performed on a BIO-FLASH chemiluminescent analyzer (Werfen). The procedure was carried out following the manufacturer's instructions.

2.7 | Outlier detection

For the group of healthy individuals, we first considered excluding those individuals whose aCL or aβ2GPI concentrations were above the cutoff point as established by the manufacturer's kit in all 3 ELISAs. Outliers for aCL or aβ2GPI were considered by applying the X algorithm according to Chantarangkul et al. [41], where the difference between the maximum value and the second maximum value was greater than one-third of the range (maximum value-minimum value) of observations for aPL, including the extreme observations that were deemed as outliers. The X algorithm is a modification of Dixon's test for n samples >30, and by definition, is Reed's test. We also used Tukey's fences test to detect several outliers at once by considering 3 times the IQR. For this test, the Box–Cox transformation was applied to normalize the data using the MASS package (Brian D. Ripley and William N. Venables) in R (version 4.3.1). For both, we selected the outlier test that eliminated the fewest outliers possible to render the highest 99th percentile. It is worth noting that values checked as outliers in a specific assay were within the normal range in the other assays for that individual.

2.8 | Statistical analysis

Data were analyzed using R software (created by R. Ihaka and R. Gentleman, University of Auckland; version 4.3.1) and packages dplyr, and tidyverse (developed by H. Wickham [42]). The median and IQR are given as median (IQR). Categorical variables are presented as absolute numbers and relative frequencies. The normal distribution of data was tested with the Kolmogorov–Smirnov normality test. The normal distribution of data was rejected when P value was $\leq$ .05. Numerical variables were compared between different groups using the nonparametric Kruskal–Wallis test, followed by the Bonferroni multiple comparison test. Two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were used to identify differences between the different aPL assay kits by sex. Cohen's κ coefficient was calculated in R using the dlstats, effectsize, BayesFactor, and epiR libraries, and the epi.kappa function. Correlations between ELISA platforms and between the different ELISA and CLIA methods were estimated using Spearman statistics. Accuracy tests of sensitivity and specificity were performed with the caret library in the R software. Werfen data were considered the reference for these tests. The specific tests used in each experiment and P values are stated in the figure legends. A 2-sided α concentration of < .05 was considered statistically significant.

3 | RESULTS

3.1 | aCL and aβ2GPI 99th percentile in different commercial platforms

The cutoff of 40 U as a laboratory criterion for determining positivity for aCL or aβ2GPI prompted us to assess the similarity and homogeneity of aPL concentrations obtained from 3 different commercial ELISAs and 1 CLIA method. Initially, this evaluation was performed on healthy individuals, recruited as blood donors from the Generalitat de Catalunya Blood and Tissue Bank. The use of healthy donors allowed us to define the 99th percentile for each aPL and method, and subsequently compare it with the cutoff point of 40 U. A total of 250 individuals were recruited, and their aCL and aβ2GPI concentrations were tested in parallel using 4 different kits: 3 based on ELISA method and 1 on CLIA method. The latter is not recommended to be used for testing aPL positivity [1] because it overestimates the antibody presence compared with ELISA methods [30,43]. Nevertheless, we found that the median values of aCL IgG determined by the CLIA method were similar to those determined by 2 ELISA kits (Figure 1A). Specifically, for Aesku ELISA, the median (IQR) value was 4.66 (3.65) GPL; for Werfen ELISA, it was 4.16 (3.07) GPL; and for the CLIA method, it was 4.70 (5.22) GPL. Of note, the Orgentec ELISA yielded significantly lower aCL IgG values than the other platforms (median [IQR], 0.79 [0.9] GPL; $P < .0001$; Figure 1A). On the other hand, in the case of aβ2GPI, the median concentrations differed significantly among all kits and methods (Figure 1B). Again, Orgentec was a commercial

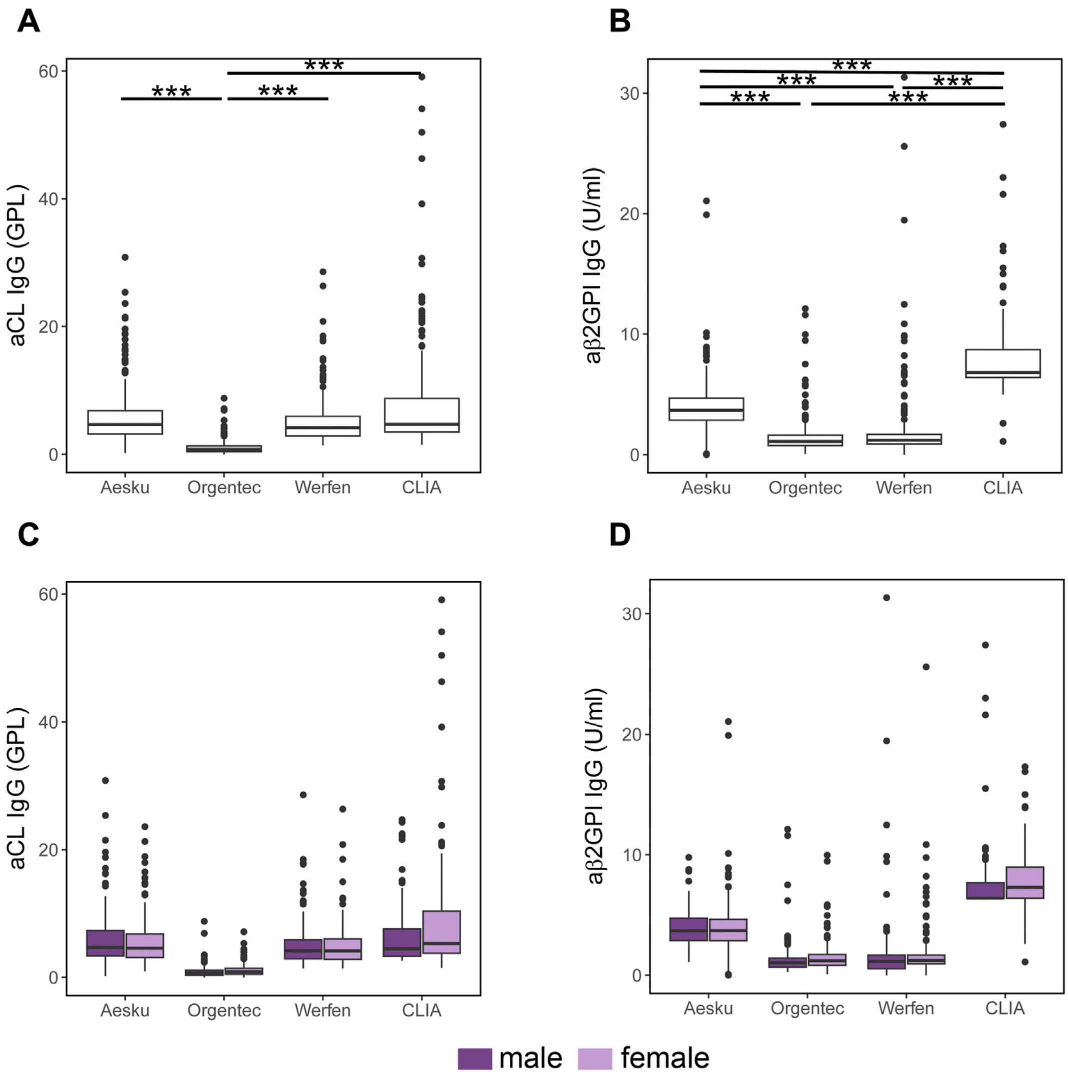


FIGURE 1 Anticardiolipin (aCL) and anti-β2 glycoprotein I (aβ2GPI) concentrations differ in quantitation by commercial platforms. Concentrations of (A, C) aCL IgG units (GPL) and (B, D) aβ2GPI in U/mL were assessed in 250 healthy individuals by Aesku, Orgentec, and Werfen ELISAs, and chemiluminescent assay (CLIA) methods. Values were assessed based on standardized calibrators for each ELISA and CLIA. (C and D) aCL and aβ2GPI concentrations differentiated by sex. Statistical analysis included the (A–D) Kolmogorov–Smirnov test for normal distribution, (A, B) the Kruskal–Wallis test followed by the Bonferroni multiple comparison test, and (C, D) 2-way ANOVA and Tukey's multiple comparisons test. P values for normality tests in all cases were $P \leq .001$, rejecting a normal distribution of the data; $^{***}P < .0001$; nonsignificant P values were observed between women and men in each method. The box plot depicts the median with the 25th and 75th percentiles, while the dots represent individuals outside the 25th and 75th percentile values. Samples were tested in triplicate. IgG, immunoglobulin G.

ELISA that measured lower aβ2GPI concentrations (median [IQR]1.1 [0.85] U/mL; $P < .0001$) compared with Aesku ELISA (3.69 [1.81] U/mL) and CLIA method (6.8 [2.3] U/mL), but similar to Werfen ELISA (1.2 [0.81] U/mL).

This variability in aPL quantitation by each method and kit resulted in differences between the corresponding 99th percentile cutoff values (Table 1), which were far from the 40-U cutoff established for determining aCL or aβ2GPI positivity. Importantly, the CLIA method estimated median concentrations of aCL similar to the ELISAs, but its 99th percentile was the highest and was closest to the cutoff of 40 U. In contrast, the median values for aβ2GPI were significantly higher for the CLIA method than for the ELISAs, but the 99th percentile was well below the cutoff point of 40 U and close to the 99th percentile of the Werfen ELISA for aβ2GPI (Table 1). These results suggest that the aCL and aβ2GPI concentrations for an individual may depend on the commercial ELISA used, and that the CLIA method should not be completely discarded to establish aPL positivity. As specificity was sought, the increased 99th percentile corrected with the CV for each assay was determined. High positivity was considered twice the corrected 99th percentile (Table 1).

We also analyzed the aCL and aβ2GPI concentrations in healthy individuals separately by sex to determine whether there were differences in the 99th percentile between women and men (Figure 1C, D, and Table 1). We observed no differences in the median values of

TABLE 1 The 99th percentiles of anticardiolipin and anti-β2 glycoprotein I for each ELISA and chemiluminescent assay methodologies, segregated by sex. Values were obtained from 250 healthy blood donors.

aPL	Method	99th	99th women	99th men	CV (%)	99th CV	2× 99th CV
aCL IgG	Aesku ELISA	22.80	20.27	25.7	16.5	26.6	53.1
	Orgentec ELISA	6.29	4.95	7.01	23.6	7.8	15.5
	Werfen ELISA	19.93	19.81	19.08	11.4	22.2	44.4
	CLIA	48.80	52.47	24.32	8	58.6	117.1
aβ2GPI IgG	Aesku ELISA	9.99	15.6	8.86	15.3	11.5	23.0
	Orgentec ELISA	9.78	7.88	11.63	16.7	11.4	22.8
	Werfen ELISA	16.87	10.37	20.41	12.7	19.0	38.0
	CLIA	20.01	16.08	23.31	8	24.0	48.0

2× 99th CV, twice the 99th corrected percentile; 99th CV, 99th percentile corrected by CV; aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; aPL, antiphospholipid; CLIA, chemiluminescent assay; CV, coefficient of variation; IgG, immunoglobulin G.

aCL or aβ2GPI concentrations between women and men within each platform. aPL concentrations measured with Orgentec ELISA were lower for both sexes in comparison with those measured with the other methods. In men, similar aCL concentrations were measured with the Aesku ELISA, Werfen ELISA, and CLIA; however, in women, higher concentrations of aCL were measured with CLIA than with the Aesku or Werfen ELISAs. This suggests that the higher overall 99th percentile for aCL measured by CLIA was due to the higher values detected in women. Notable differences in the 99th percentiles between women and men were also observed for aβ2GPI measured with Werfen and Aesku ELISAs (Table 1). Nevertheless, the Aesku ELISA showed higher 99th percentile in women, whereas the Werfen ELISA showed a higher 99th percentile in men. These data provide further evidence of inconsistencies in the determination of aCL and aβ2GPI concentrations between the different commercial methods.

3.2 | Standardized calibrators differ between platforms

We further explored whether these disagreements were due to a lack of standardization in the calibrators for aCL or aβ2GPI. Calibrators for each ELISA were tested on alternative ELISA platforms (Figure 2A). Only calibrators from Werfen were detected in both the Aesku and Orgentec ELISAs, although with different sensibility (Figure 2A left). Calibrators from Aesku and Orgentec were only detected on their own ELISA platform (Supplementary Figure S1), but not on the other ELISA platforms (Figure 2A, center and right), despite some of these calibrators containing 300 U of aCL or 300 U of aβ2GPI.

To rule out the influence of sample diluent, we first diluted the most concentrated calibrator 10 times with the corresponding sample diluent of each ELISA platform to achieve an aPL concentration of 30 GPL for aCL or 30 U/mL for aβ2GPI from Aesku, and 8 GPL for aCL or 10 U/mL for aβ2GPI from Orgentec. We could not detect a single aCL or aβ2GPI U, although the 10-fold diluted calibrators were supposed to contain enough U to be detected within the standard curve of any ELISA platform (Supplementary Figure S1). Second, we diluted 4 serum samples with the 3 different sample diluents of each ELISA manufacturer and tested them using the Werfen ELISA platform (Figure 2B). Serum samples were detected at comparable concentrations regardless of the sample diluents used. This indicated that sample diluents were not affecting the detection of the calibrators in other ELISAs, suggesting that a lack of homogeneity in the standardization of calibrators could explain the differences in aPL concentrations between the methods and manufacturers.

3.3 | Consistency of aPL positivity between platforms in a cohort of APS patients

Given the lack of homogeneity in the quantification of aCL and aβ2GPI, we investigated how the cutoff point of 40 U for aCL and aβ2GPI affected patient selection in a randomized sample of 80 individuals from our cohort of APS patients (Table 2). This cohort of APS patients was created according to the Sydney classification criteria, in which the laboratory criteria for aCL and aβ2GPI positivity for both IgG and IgM isotypes allowed the 99th percentile as the cutoff point for aPL positivity.

We tested aCL and aβ2GPI IgG concentrations in the 80 APS patients with the 3 ELISAs and the CLIA method. Paired ELISAs showed an acceptable degree of correlation among the manufacturers for aPL, aβ2GPI IgG (Aesku vs Werfen R = .61; Orgentec vs Werfen R = .79; Aesku vs Orgentec R = .59), and aCL IgG (Aesku vs Werfen R = .89; Orgentec vs Werfen R = .79; Aesku vs Orgentec R = .70; Figure 3A, B). However, some patients tested positive on only 1 platform, even with the 40-U cutoff. Agreement between paired tests was calculated using Cohen's κ coefficient, considering both the 40-U and the 99th percentile cutoffs for aCL and aβ2GPI positivity (Figure 3C). For aβ2GPI testing, the agreement was similar between the cutoff points of 40 U and the 99th percentile of each paired ELISA. In contrast, for aCL assays, higher agreement was observed with the 40-U cutoff in 2 out of the 3 pairings (Figure 3C). Regardless of the cutoff value, substantial variability was evident in the agreement between different ELISA platforms, highlighting the inconsistency in

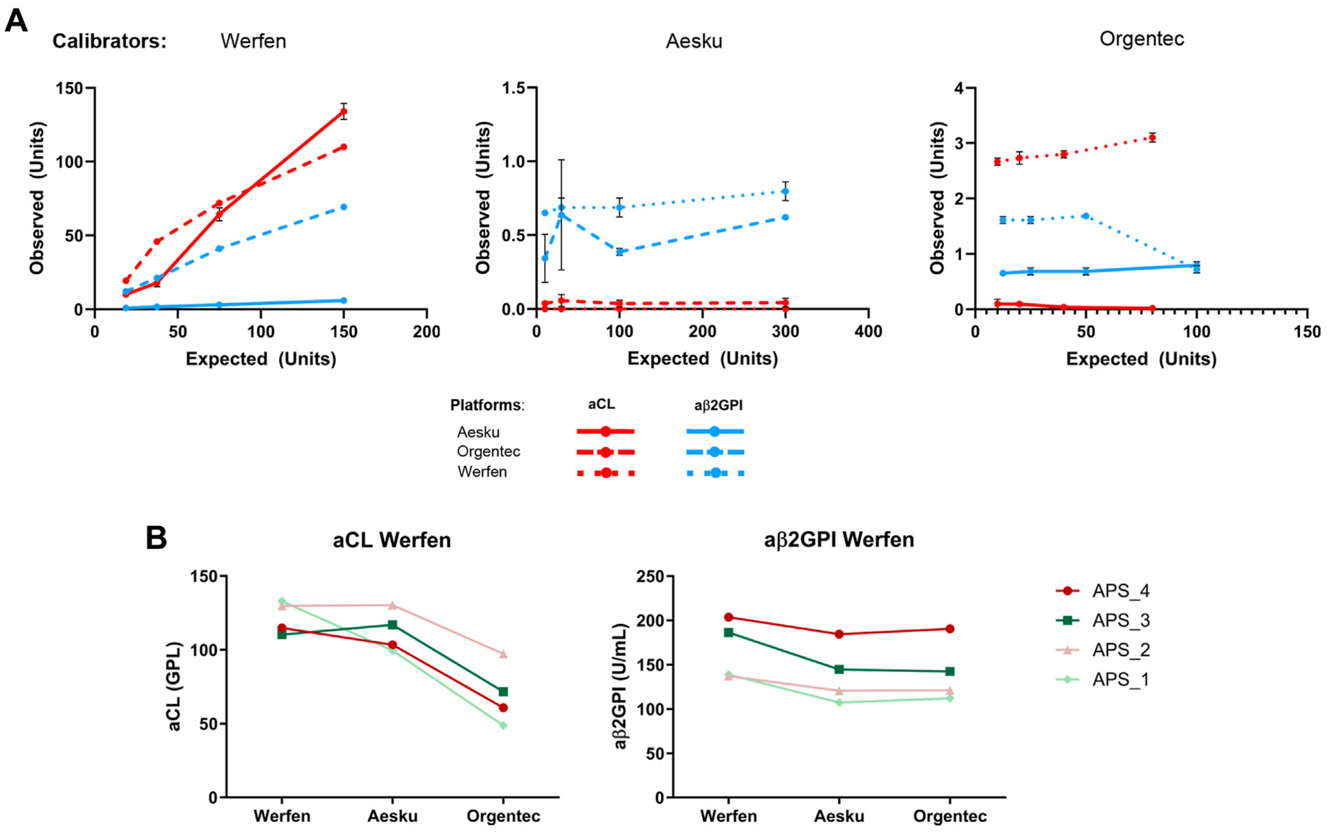


FIGURE 2 Variability in anticardiolipin (aCL) and anti-β2 glycoprotein I (aβ2GPI) assessment. (A) Calibrators for aCL and aβ2GPI from ELISA manufacturers (Werfen, left; Aesku, center; Orgentec, right) were measured on the other ELISA platforms (Aesku, solid line; Orgentec, dashed line; Werfen, dotted line) for aCL (red) or aβ2GPI (blue). The results for aCL (red) and aβ2GPI (blue) are shown. The x-axes represent the expected units (U) as indicated by each calibrator manufacturer, while y-axes display the values measured on the other ELISA platforms. (B) Sera from 4 antiphospholipid syndrome (APS) patients, diluted in sample diluent provided by each ELISA manufacturer (x-axes), were analyzed for aCL and aβ2GPI using the Werfen ELISA platform. Calibrators and samples were measured in triplicate. GPL, aCL IgG units.

results across kits. In addition, agreement between ELISA and CLIA methods was higher with the 99th percentile cutoff compared with the 40-U cutoff in all pairings (Figure 3D, Supplementary Figure S2). There was no agreement between CLIA and Orgentec ELISA for aCL with the cutoff value of 40 U (κ P = .065). Furthermore, our results showed that agreement between CLIA and ELISA methods was consistently lower than that between different ELISA manufacturers for a given aPL and cutoff value. Altogether, these data point to substantial discrepancies in agreement between kits, suggesting that the choice of 1 ELISA platform may result in false-positive (FP) patients being enrolled in study cohorts, even when the cutoff value is increased from the 99th percentile to 40 U.

Additionally, we analyzed whether women or OAPS patients had aPL concentrations within the range of the 99th percentile and the 40-U threshold for each method (Supplementary Table). However, our analysis showed that this was not the case, as neither group was significantly represented in this region.

We then decided to assess the sensitivity and specificity of the ELISA and CLIA methods by considering Werfen ELISA as the reference and the cutoff points of 40 U and the 99th percentile. Although the ELISA method presented the highest specificity for both aCL and aβ2GPI with a cutoff point of 40 U, its sensitivity was the highest at the cutoff value of the 99th percentile (Figure 4A). Specificity was lower when testing with the CLIA method, and there were no differences between the 2 cutoff values (Figure 4A). The number of true positive (TP) patients was higher for aβ2GPI tested with both ELISA and CLIA at the cutoff point of 99th percentile (Figure 4B). Thus, in our cohort of 80 patients, we screened those who were positive for aβ2GPI or aCL in at least 2 ELISA platforms at the 99th percentile cutoff or the 40-U cutoff (Figure 5). Regarding aβ2GPI, a noticeable number of patients who were positive at the 99th percentile cutoff on 2 different ELISA platforms would be lost if the cutoff were shifted to 40 U (Figure 5A). Additionally, nearly 50% of patients who were positive on all 3 ELISAs with a cutoff of the 99th percentile would not meet the positivity criteria if testing were limited to a single ELISA platform with the cutoff of 40 U (Figure 5B). For aCL, reliance on a single ELISA with a cutoff of 40 U risked including FP (Figure 5B). Overall, these results suggest that it would be beneficial for the enrollment of patients with APS in research studies to test aCL and aβ2GPI using more than 1 ELISA platform rather than increasing the cutoff value.

TABLE 2 Demographic and clinical data of a randomized sample of 80 patients from our antiphospholipid syndrome cohort.

Variable	*n* (%)	aPL positivity	*n* (%)
Sex			
Female	59 (73.75)	LA	48 (60)
Male	21 (26.25)	aβ2GPI IgG	37 (46.25)
Age (y), mean (SD)	45 (10)	aβ2GPI IgM	27 (33.25)
Ethnicity		aCL IgG	41 (51.25)
Caucasian	73 (91.25)	aCL IgM	27 (33.75)
Asian	3 (3.75)	**Laboratory cat**[a]	
Arabic	2 (2.5)	Cat I	49 (61.25)
Latin	1 (1.25)	Cat IIa	17 (21.25)
Mixed background	1 (1.25)	Cat IIb	8 (10)
Disease		Cat IIc	6 (7.5)
OAPS	43 (53.75)		
TAPS	37 (46.25)		
Female TAPS	16 (43.24)		
Male TAPS	21 (56.76)		

aβ2GPI, anti-β2 glycoprotein I; aCL, anticardiolipin; aPL, antiphospholipid; cat, category; Ig, immunoglobulin; LA, lupus anticoagulant; OAPS, obstetric antiphospholipid syndrome; TAPS, thrombotic antiphospholipid syndrome.

[a]Cat I, repeatedly positive for the 3 aPL (LA, aCL [IgG or IgM], and aβ2GPI [IgG or IgM]), or any 2 of them; Cat IIa, persistently positive only for LA; Cat IIb, consistently positive only for aCL (IgG or IgM); Cat IIc, repeatedly positive only for aβ2GPI (IgG or IgM).

4 | DISCUSSION

Our results show substantial variability in the quantification of aCL and aβ2GPI concentrations across different ELISA and CLIA methods. This divergence stems from a lack of standardized calibrators used by different commercial ELISA manufacturers, leading to discrepancies in antibody measurements for the same serum sample, depending on the ELISA platform used. Such inconsistencies are a significant challenge in determining whether serum from an individual contains enough aPL concentrations to be classified as aPL-positive or aPL-negative. To address this issue, the updated ACR-EULAR guidelines for APS classification have established a cutoff of 40 U for both aCL and aβ2GPI. However, this cutoff is considerably >99th percentile values we determined using 3 different commercial ELISAs. Raising the cutoff point to 40 U is intended to increase specificity in APS classification. Our data support this approach, since the 99th percentile cutoff showed lower agreement for aCL positivity between paired ELISAs compared with the cutoff of 40 U. In addition, specificity is globally better with the 40-U cutoff. However, for aβ2GPI positivity, the variability in the concentration of agreement between test pairs was not corrected by increasing the cutoff point from the 99th percentile to 40 U. Furthermore, the desire to reduce the likelihood of FPs being included in APS studies also means that many patients with aPL concentrations >99th percentile, who could be considered to have positive autoantibody concentrations, are being excluded from studies. In our cohort, 50% of patients with aβ2GPI concentrations >99th percentile of the 3 ELISAs from different manufacturers would be excluded from such studies if we considered only a test with the 40-U cutoff. Our finding is of value since the ACR/EULAR APS classification criteria do not reference any study to justify aligning the aβ2GPI cutoffs for medium and high positivity with those of aCL.

Using the 99th percentile as a diagnostic cutoff offers several benefits, particularly in enhancing specificity while maintaining clinical relevance. By selecting this high percentile, clinicians and researchers significantly minimize the likelihood of FPs, as it excludes the majority of the general population who do not exhibit clinically significant concentrations of aCL or aβ2GPI. This approach is particularly valuable in conditions with a long-tailed aPL distribution, ensuring that only individuals with exceptionally elevated concentrations are flagged. Additionally, the 99th percentile cutoff aligns with established clinical guidelines and evidence-based practices [39], making it a widely accepted and reliable criterion for categorizing abnormal results. This standardization also allows for better comparability across studies and clinical settings, allowing laboratories to establish and verify cutoffs specific to their aPL testing platforms. We found that the 99th percentile differed depending on the ELISA platform used. In some cases, the cutoffs were up to 4 times lower than those proposed by the ACR/EULAR APS guidelines. Additionally, adopting this strategy would allow the exploration of potential differences in aCL and aβ2GPI cutoffs between men and women, which could influence patient recruitment, particularly in OAPS. Interestingly, our results revealed significant sex-based differences in aCL concentrations when using the CLIA method at the 99th percentile; however, these differences were not confirmed with the ELISAs. As autoimmune diseases mainly affect women, among the 250 healthy individuals used to determine the 99th percentiles, 62% (155/250) were women and 38% (98/250) were men. The 99th percentile by sex was correctly assessed for women, although not for men. In addition, our findings suggest that the choice between the 99th percentile and the 40-U threshold does not disproportionately impact the classification of women or OAPS patients.

The 99th percentile cutoff, even when more adapted to each laboratory and assay, is not exempt from controversy and remains a matter of debate [44]. To identify outliers in data that are not normally distributed, we applied the X algorithm described in Chantarangkul et al. [41], which discarded fewer outliers than Tukey's fences test (3 times IQR). Our approach to outlier detection prioritized those statistical tests that considered a lower number of outliers to obtain a higher 99th percentile cutoff. Furthermore, as we observed CV values of up to 20% for some assays due to batch-to-batch variations, the 99th percentile cutoffs could be increased by this CV value. This would have the additional effect of reducing FPs arising from the statistically inevitable 1% of the normal population who would generate a result >99th percentile, thereby increasing confidence in obtaining a homogeneous population of definite APS patients. As previously proposed by Vanoverschelde et al. [44], standardized statistical criteria for calculating the 99th percentile cutoffs

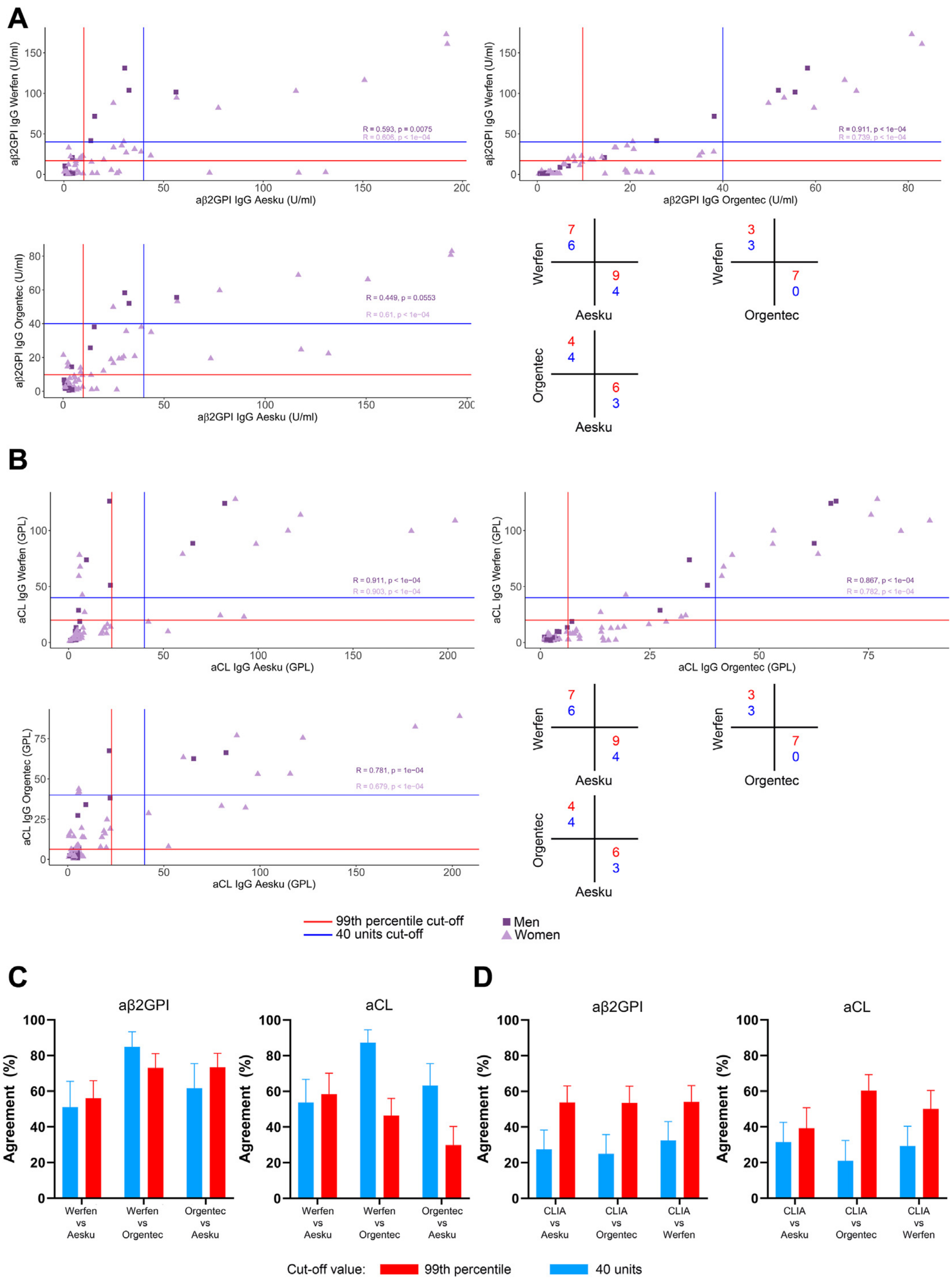


FIGURE 3 Agreements in testing anti-β2 glycoprotein I (aβ2GPI) or anticardiolipin (aCL) positivity across different methods and kit manufacturers. Variability in aβ2GPI and aCL positivity between platforms highlights the limitations of nonstandardized calibrators and the inconsistency introduced by varying cutoffs. Correlation plots between pairs of ELISA platforms for (A) aβ2GPI immunoglobulin (Ig) G or (B) aCL IgG antibodies. For each ELISA platform and antiphospholipid antibodies, the cutoff points are depicted as red lines for the 99th percentile and blue lines for the 40-unit (U) cutoff, the latter recommended by the American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) antiphospholipid syndrome guidelines. The number at the bottom right of panels A and B represents the number of patients who tested positive in only 1 of the ELISA pairs; red numbers correspond to the cutoff of the 99th percentile, and blue numbers represent the cutoff of 40 U. Antiphospholipid antibody values from women are represented by light purple triangles and those of men by dark purple squares. Spearman's correlation coefficient is indicated for women and men separately. Cohen's κ coefficients indicate the degree of agreement between (C) 3 pairs of ELISA manufacturers (Aesku, Werfen, and Orgentec), and between (D) CLIA and ELISA methods for either aβ2GPI or aCL positivity using the cutoff value of 40 U (blue) or the 99th percentile (red). Cohen's κ coefficient was calculated in R with the dlstats, effectsize, BayesFactor, and epiR libraries, and using the epi.kappa function. Bars depict estimates ± SE means Cohen's κ coefficient and its standard error. All pairs show $P < .05$, except for #$P > .05$. Samples were tested in triplicate. GPL, aCL IgG units.

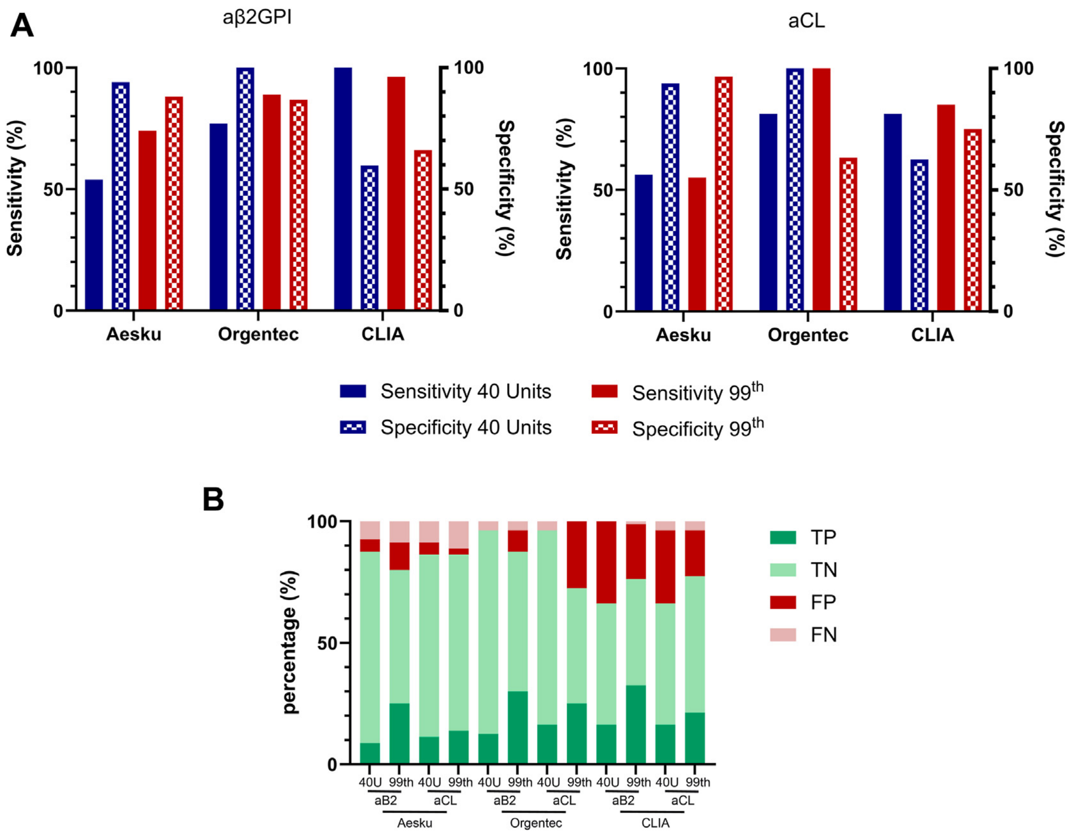


FIGURE 4 Sensitivity and specificity of ELISA and chemiluminescent assay (CLIA) methods for antiphospholipid syndrome (APS) patient classification. (A) Sensitivity and specificity of anti-β2 glycoprotein I (aβ2GPI/aB2) and anticardiolipin (aCL) detection across Aesku, Orgentec, and CLIA platforms, and (B) the distribution of true positive (TP), true negative (TN), false positive (FP), and false negative (FN) patients were assessed using the Werfen ELISA as reference. In (A), 2 cutoff points were compared between the 99th percentile (red) and 40 units (40U; blue, as per American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) APS guidelines). The ELISA platforms showed higher specificity at the 40U cutoff but better sensitivity at the 99th percentile. CLIA had lower specificity at both cutoffs, with minimal variation between them. The 40U cutoff excluded a significant number of TPs and increased FNs, highlighting the importance of cutoff selection in APS research and diagnostics.

are needed. In addition to determining whether to exclude outliers and selecting the appropriate statistical method to detect them, the CV could be included as an additional factor.

The fact that the CLIA and ELISA methods showed similar 99th percentiles and median values of aCL and aβ2GPI measurements in a healthy population, but the CLIA method estimated higher concentrations of aPL compared with the ELISA methods in APS patients, is a subject for further investigation. The CLIA method quantifies aPL concentrations by measuring relative luminescence U, which are subsequently reported as chemiluminescence U (CU) using calibrators. Routinely, these CU are translated to GPL/MPL based on the assumption by manufacturers that 1 CU equals 1 GPL/MPL. This explains why our data, as well as findings from other laboratories [45,46], show comparable cutoff values for CLIA and ELISA. By contrast, higher aCL and aβ2GPI concentrations detected by CLIA suggest that it is more sensitive than ELISA in measuring aPL concentrations [30,43]. Because of this apparent overassessment of aPL by the CLIA method, the ACR/EULAR guidelines for APS classification ruled out its use as a method for aPL determination. However, the exclusion of a method with higher sensitivity, greater reliability, and faster than ELISA methods poses a controversy.

For research purposes, classification criteria must prioritize specificity to ensure that only APS patients are enrolled, excluding individuals without the condition. On the contrary, diagnostic criteria should prioritize sensitivity, as clinicians cannot risk excluding potential patients without appropriate therapeutic management. This represents a significant challenge for clinicians specializing in APS. CLIA remains a valuable tool for diagnosing APS due to its superior sensitivity. Meanwhile, for APS classification, efforts should be made to develop protocols to harmonize aPL concentrations rather than resort to discarding the technology. One study aimed to harmonize moderate to high aCL IgG concentrations between ELISA and CLIA methods reported that ELISA thresholds of 40 U and 80 U corresponded to 31 CU and 95 CU in CLIA, respectively [47]. Whereas the study by Vandevelde et al. [30] found that an aCL IgG ELISA range of 40 U to 79 U corresponded to CLIA values between 200 U and 400 U. A recent study has proposed additional techniques that aim to harmonize the U used across both methods [46]. We observed that harmonization is highly platform-dependent. Vandevelde et al. [48] improved harmonization in the interpretation of aCL and aβ2GPI positivity across 4 analytical platforms by introducing the likelihood

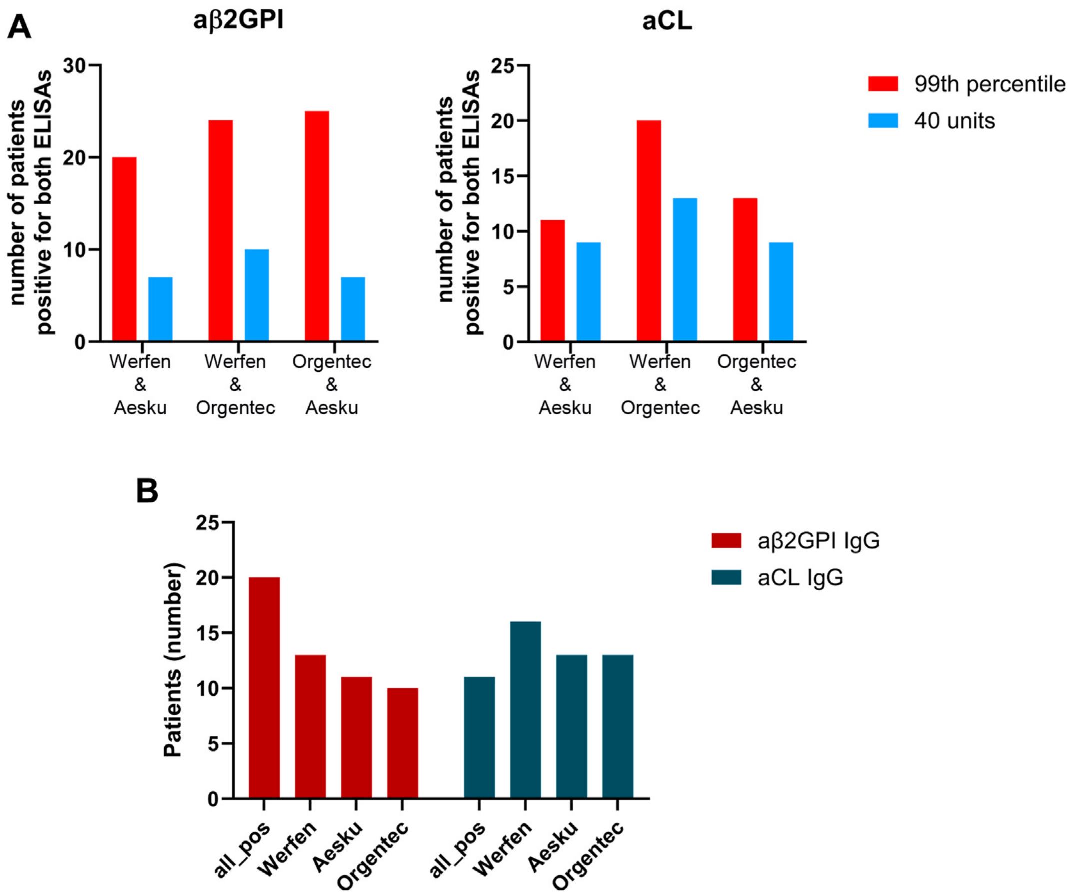


FIGURE 5 Impact of cutoff values on patient enrollment in antiphospholipid syndrome studies. (A) The number of patients testing positive for anti-β2 glycoprotein (aβ2GPI) immunoglobulin (Ig) G (left) or anticardiolipin (aCL) IgG (right) in the 2 tested ELISA platforms is shown for the 99th percentile cutoff (red) and the 40-unit cutoff (blue). The data emphasize the loss of patients classified as positive when shifting from the 99th percentile to the 40-unit cutoff. (B) The number of patients testing positive on all 3 ELISA platforms at the 99th percentile cutoff (all_pos) is compared with those testing positive with the 40-unit cutoff on a single ELISA platform. The results of aβ2GPI illustrate how the 40-unit cutoff excludes a significant proportion of patients who would otherwise fulfill the criteria for positivity using the 99th percentile.

ratios, and their applicability should be verified by independent studies [48]. Additionally, some ELISA platforms underassessed aPL concentrations. For instance, from the same cohort of 80 patients, Aesku and Werfen ELISAs identified 9 patients with aCL ≥ 80 U, while Orgentec ELISA identified only 2. Similar discrepancies were observed for aβ2GPI (10 with Werfen, 6 with Aesku, and 2 with Orgentec). Consequently, moving forward, it will be important not only to discard methodologies that overestimate aPL concentrations but also to carefully evaluate those that potentially underestimate them, thereby preventing misclassification of patients. Specifically, the ACR/EULAR APS guidelines establish a weight score based on whether aCL and/or aβ2GPI concentrations are > 80 U (highly positive). Our data backs this weight score using a value twice the 99th percentile instead of 80 U.

We hypothesized that this variability in the quantification of aPL was due to a lack of standardized calibrators in the assessment kits. This issue became apparent when we quantified the calibrators of various ELISA kits on different platforms. Only the aCL calibrators from one kit, and to a lesser extent, the aβ2GPI calibrators, were correctly quantified by all 3 ELISA platforms. In contrast, the calibrators of other kits were only measured on their respective platforms. This indicates that the calibrators are not standardized across the platforms, and caution must be taken when comparing data across these platforms. Whether these differences were due to the use of polyclonal or monoclonal antibodies as calibrators needs to be explored. Calibrators for aCL assays are polyclonal antibodies, except for Aesku, which are monoclonal antibodies. A study suggested an extreme heterogeneity of aPL antibodies, even in a single patient [49]. It would mean that the use of polyclonal calibrators could improve aPL detection. However, in our data, one of the ELISAs with polyclonal calibrators showed the lowest detection of aPL concentrations. Another aspect would be the antigen state, as the effect of β2GPI reduction on the protein structure could affect antibody binding [50].

Given the lack of standardization in laboratory methods, using the 99th percentile helps maintain a reasonable balance between sensitivity and specificity. This ensures that testing remains practical for widespread clinical application without compromising diagnostic accuracy. However, under the new ACR/EULAR APS guidelines, not only do we risk excluding people from research with *a priori* positive aPL concentrations according to the 99th percentile, but we also do not know how the borderline range of aPL affects aPL-related pathologies.

The decision on whether the 99th percentile cutoff or the unified 40-U cutoff is better depends on the specific ELISA platform. We checked classification accuracy using both cutoff points by comparing Aesku and Orgentec ELISAs, and by comparing the CLIA method with the Werfen ELISA as the reference. The Aesku ELISA had similar accuracy for aCL assessment, irrespective of the chosen cutoff point, whereas aβ2GPI assessment showed a better specificity-sensitivity balance with the 99th percentile cutoff. In contrast, the Orgentec ELISA consistently showed higher specificity but lower sensitivity using the 40-U cutoff for aPL. CLIA estimated similar values for both cutoffs; however, unlike the other 2 ELISAs, it exhibited higher sensitivity than specificity across all evaluations. This would mean that using 40 U as the cutoff value does not universally provide higher specificity than the 99th percentile of all ELISA platforms; in some cases, specificity is similar. Conversely, sensitivity is consistently better with the 99th percentile cutoff compared with the 40-U cutoff, with the latter reducing the number of patients eligible for enrollment in research studies. In addition, the specificity-sensitivity balance is higher when a patient tests positive in 2 ELISAs with a 99th percentile cutoff compared with a single ELISA with a 40-U cutoff. For research studies, it would imply that patients must test positive in 2 ELISAs with a 99th percentile cutoff. Testing with 2 ELISAs will not eliminate issues such as interlaboratory differences, assay sensitivity, or the impact of outliers. Additionally, the use of different assay platforms could yield divergent results, mainly in terms of weighing aPL positivity as moderate or high, though being positive in both.

In summary, these observations raise concerns about the potential exclusion of APS patients from research studies due to the absence of standardized calibrators, which creates inconsistencies in determining aPL positivity across different methods. This lack of alignment also led us to question whether increasing the cutoff value to 40 U truly enhances the accuracy of aPL positivity assessment compared with the 99th percentile. As long as the kit calibrators are not properly standardized, raising the cutoff from the 99th percentile to 40 U prevents the inclusion of FPs at the expense of TPs. We have listed a number of discrepancies that point to the lack of standardized methods for aCL and aβ2GPI assessment. Rather than increasing the cutoff value, which is difficult to set up without a standardized method, it will be more valuable to reference positivity by aPL to relative values, and also to promote good laboratory practices. Specifically, laboratories should adopt the 99th percentile as the preferred cutoff to enhance consistency and reliability.

Finally, regarding the use of commercialized ELISA kits, several important questions remain unanswered. To ensure specificity and reduce variability, would it be more appropriate to recommend that patients test positive on at least 2 ELISA platforms from different manufacturers before being classified as positive for aCL and aβ2GPI? If testing on 2 ELISA platforms, would a positive result on only 1 be sufficient for diagnosing APS? Addressing these questions is crucial for improving APS classification and diagnosis.

We propose that the 99th percentile cutoff, assessed individually for each technique, is more appropriate than a generalized value applied across all methods. Adopting the 99th percentile cutoff would allow for the inclusion of advanced technologies, such as CLIA, which often demonstrate higher sensitivity. Additionally, the 99th percentile offers a structured framework for differentiating between negative and positive results: the moderate positive concentration may be defined by a fold increase of the 99th percentile, while the high positive threshold may be set at twice the moderate positive value.

It is important to emphasize that this is a conceptual proposal and not a definitive conclusion. To strengthen the validity of this approach, future studies should include comparisons using a cutoff of 80 U to better understand its impact on patient stratification. Such additional analyses could provide valuable insights for refining the guidelines and enhancing the consistency and reliability of APS classification across different platforms.

5 | CONCLUSIONS

As long as there are no standardized calibrators, adopting a universal 40-U cutoff instead of a 99th percentile cutoff prevents the inclusion of FP APS patients in research studies at the expense of losing TP patients. Being able to certify that eligible APS individuals are truly TP APS patients, and thus achieving high specificity for enrollment, could be accomplished by testing aPL concentrations with at least 2 different ELISAs.

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AUTHOR CONTRIBUTIONS

Conceptualization: A.A.-L. and F.A.M.-M. Methodology and data curation: A.A.-L., J.M.-S., M.O., N.P., and F.A.M.-M. Writing original draft: F.A.M.-M. Writing–Review and Editing: A.A.-L., J.M.-S., M.O., C.A., and F.A.M.-M. Approval of the final version: all authors. Funding: F.A.M.-M. and J.A.-R.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

DATA AVAILABILITY

All data generated and analyzed during this study are stored in the Vall d'Hebron Institut de Recerca repository, and access will be granted by the corresponding authors upon request.

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SUPPLEMENTARY MATERIAL

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