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Establishment of ELISA-comparable moderate and high thresholds for anticardiolipin and anti-β2 glycoprotein I chemiluminescent immunoassays according to the 2023 ACR/EULAR APS classification criteria and evaluation of their diagnostic performance

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Abstract

Objectives: Recently published 2023 ACR/EULAR APS classification criteria emphasize the importance of quantifying single-, double-, and triple-antiphospholipid antibody positivity, distinguishing between IgG and IgM isotypes, and delineating moderate/high levels of anticardiolipin (aCL) and anti-β2 glycoprotein I (anti-β2GPI) antibodies. We aimed to establish clinically important moderate/high thresholds for aCL and anti-β2GPI IgG/IgM chemiluminescent immunoassays (CLIA), in particular QUANTA Flash, comparable to our in-house ELISAs used for over two decades, and to evaluate their diagnostic performance.

Methods: QUANTA Flash CLIA and in-house ELISAs were used to measure aCL and anti-β2GPI IgG/IgM. Moderate thresholds for QUANTA Flash CLIA were determined using a non-parametric approach, calculating a 99th percentile on serum samples from 139 blood donors, and by mirroring the diagnostic performance of in-house ELISA on 159 patient samples.

Results: Thresholds for QUANTA Flash CLIA achieving diagnostic performance equivalent to in-house ELISAs were 40 CU for moderate and 80 CU for high levels for aCL and anti-β2GPI IgG and IgM. The assays showed good qualitative agreement, ranging from 76.10 to 91.19 %. When considering in-house ELISA results, 14 out of 80 (17.5 %) patients did not fulfill the new ACR/EULAR laboratory classification criteria, while 27 out of 80 (33.8 %) did not when considering QUANTA Flash CLIA results.

Conclusions: We determined moderate and high thresholds for aCL and anti-β2GPI IgG and IgM detected with QUANTA Flash CLIA, aligning with long-established in-house ELISA thresholds. These thresholds are crucial for seamlessly integrating of the new 2023 ACR/EULAR classification criteria into future observational clinical studies and trials.

Keywords: antiphospholipid syndrome; anticardiolipin; anti-β2GPI; classification criteria

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Introduction

The international consensus statement on the classification criteria for antiphospholipid syndrome (APS) published in 1999 [1] and revised in 2006 [2] recognizes the association of arterial/venous thrombosis and/or recurrent miscarriages with the persistent presence of antiphospholipid antibodies (aPL). These antibodies, detectable by anticardiolipin (aCL) and anti-β2 glycoprotein I (anti-β2GPI) enzyme-linked immunosorbent assays (ELISA) or lupus anticoagulant (LAC) functional coagulation assays, constitute crucial laboratory criteria for definite APS. According to the consensus statement, a moderate or high level of aCL antibodies is defined as either

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>40 IgG and IgM phospholipid units (GPL or MPL) or >99th percentile of results obtained in healthy blood donors using standardized ELISA. Similarly, the threshold for positive anti-β2GPI antibodies should align with the 99th percentile of healthy blood donors. This approach is adopted due to the absence of standard or reference material, necessitating laboratories to establish their own normal ranges based on control and patient populations. Moderate/high levels of aPL or multiple positivity confer a heightened risk for vascular and obstetric outcomes compared to low levels or single aPL positivity [3, 4]. While there is general agreement that only moderate/high aPL levels in ELISAs are significant for diagnosing vascular APS, there is also some evidence for the relevance of low aPL level in the obstetric APS [5, 6].

The determination of thresholds to define semi-quantitative aPL ranges, i.e., low, moderate and high levels, has therefore long been considered crucial for assessing the risk of recurrent adverse events in patients. In 2023, new ACR/EULAR APS classification criteria emerged, integrating an entry criterion and additive weighted criteria across six clinical and two laboratory domains, including LAC, and ELISA for aCL and anti-β2GPI IgG/IgM [7]. These criteria emphasize the importance of quantifying single-, double-, and triple-aPL positivity, distinguishing between IgG and IgM isotypes, and delineating moderate and high levels of aCL/anti-β2GPI positivity. The new criteria specify the use of standardized ELISA methods for both aCL and anti-β2GPI with defined thresholds for moderate positivity (40–79 units) and high positivity (≥80 units). The exclusion of other solid phase aPL assay methods in new classification criteria is intriguing and justified by the absence of clinical research studies using automated test systems. The limitation of classification criteria to ELISA assays for aCL and aβ2GPI contradicts current diagnostic practice, as ELISA testing is now rarely utilized in most diagnostic laboratories [8].

Efforts to standardize the aCL test began in the mid-1980s when the importance of the isotype and positivity level was recognised, particularly the association of higher IgG levels with APS. Despite numerous efforts, including international workshops and collaborations like the European forum, the Australasian Anticardiolipin Working Group, the College of American Pathologists, and NEQAS in the United Kingdom [9], standardization of aCL and anti-β2GPI asays has remained a challenge due to various pre- and post-analytical factors, including variations in antigens/antigen epitopes and methodologies (ELISA vs. automated immunoassays). Researchers recognise that aCL and anti-β2GPI assays, both in-house and commercial, encounter common issues inherent in autoantibody assays, such as antigen source and purity, detection antibody specificity, and the heterogeneous avidity spectrum of detected antibodies, issues related to assay preparation and calibration, or the way thresholds are established. While automated platforms have substituted ELISA methods in recent years, there remains a lack of standardized tests for aCL and anti-β2GPI [10, 11]. Notably, the issue of arbitrary units used in ELISA and CLIA persists, with manufacturers labelling them as GPL/MPL or U/mL, which are not directly comparable. Recently 1st WHO International Standard for anti-β2GPI Immunoglobulin G in human serum has been introduced, to help laboratories calibrate and harmonize the methods used to measure anti-β2GPI in human serum. However, similar standards for antinuclear (ANA) testing, anti-neutrophil cytoplasmic antibody (ANCA) testing and rheumatoid factor (RF) have not succeeded in achieving uniform results or standardization [12].

Our in-house aCL and anti-β2GPI ELISAs have been routinely performed for over two decades. Crucially, these assays were initially calibrated using the first available international KAPS standards [13] and subsequently with monoclonal anti-β2GPI antibodies, HCAL (IgG) and EY2C9 (IgM), specifically distributed for this purpose [14]. The new 2023 ACR/EULAR APS classification criteria urge investigators to identify and validate moderate/high thresholds for each platform, correlating them with the established aCL/anti-β2GPI ELISA thresholds to promote methodologic transparency [7]. Our objectives were therefore to evaluate the analytical performance of the QUANTA Flash CLIA for the detection of aCL (IgG/IgM) and anti-β2GPI (IgG/IgM), to establish clinically important moderate and high thresholds comparable to our in-house ELISAs, and to evaluate the agreement between the QUANTA Flash CLIA and ELISA results in compliance with the new ACR/EULAR APS classification criteria for laboratory domain scoring.

Materials and methods

Study design

The retrospective study included consecutive patients with clinical symptoms significant for APS and control patients with various systemic autoimmune diseases referred to the Department of Rheumatology at the University Medical Centre Ljubljana from March 2019 to October 2020. The presence of aCL, anti-β2GPI, and LAC was assessed in patients with clinical symptoms significant for APS at two time points: initially, at study entry and subsequently 3–6 months thereafter. Blood samples were centrifuged at 1,800×*g* for

10 min within 2 h of collection, and serum was separated directly from the cell pellet. Samples were stored at 4 °C and analysed the following day, or aliquoted into tubes and stored at −80 °C for future analyses. All measurements were performed on the same serum samples, and diagnostic accuracy analysis was conducted using results from the initial time point. Data regarding clinical records were not accessible to the performers and assessors of each test. In accordance with the Declaration of Helsinki, informed consent was obtained from all patients and controls. The study was approved by the National Medical Ethics Committee, Ljubljana, Slovenia (KME 0120-7/2019/5) and (KME 99/04/15). The involvement of patients or the public in the design, conduct, reporting or dissemination plans of our research was not appropriate.

Measurement of antiphospholipid antibodies

In-house aCL ELISA (reference standard)

aCL IgG and IgM were measured with in-house ELISA according to the protocol first described in 1997 [15] and later repeatedly evaluated [16, 17]. Briefly, cardiolipin-coated medium-binding microtiter plates were blocked with 10 % fetal bovine serum (FBS) in phosphate-buffered saline (PBS). After washing with PBS, serum samples diluted 1:100 in 10 % FBS-PBS were applied and incubated at room temperature (RT) for 2.5 h. After incubation with secondary antibodies, anti-IgG/IgM conjugated with alkaline phosphatase, the bound antibodies were detected with *para*-nitrophenyl phosphate in diethanolamine buffer (pH 9.8) and the OD_{405} was measured kinetically with a spectrometer. The detailed principle and analytical characteristics of the in-house ELISA are listed in Figure 1.

In-house anti-β2GPI ELISA (reference standard)

Anti-β2GPI IgG and IgM were measured with in-house ELISA as previously described [18] and evaluated by the European Forum for aPL [19]. Briefly, high-binding polystyrene microtiter plates were coated with 10 µg/mL β2GPI in PBS isolated from human plasma [20] and incubated for 2 h at RT. A wash step with PBS containing 0.05 % Tween-20 (PBS-Tween) was followed by a 30 min incubation with serum samples diluted 1:100 in PBS-Tween. Antibody binding was detected in the same way as with the in-house aCL ELISA. The results were expressed in arbitrary units (AU). The detailed principle and analytical characteristics of the in-house ELISA are listed in Figure 1.

Lupus anticoagulant assay

LAC was determined according to the guidelines of the International Society on Thrombosis and Haemostasis ISTH [21] in a three-step procedure using two screening tests (diluted Russell's viper venom time (dRVVT) and activated partial thromboplastin time (aPTT)), and the following mixing and confirmation step for the positive assays in the screening procedure. The assay was performed with blood

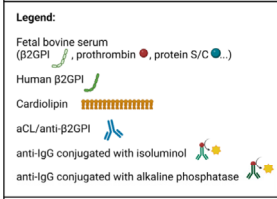
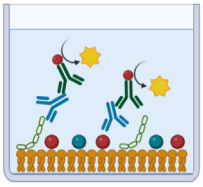
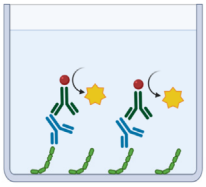
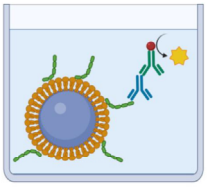
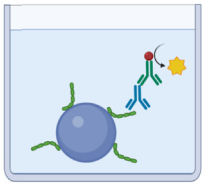
Assay	aCL (IgG/IgM) ELISA	anti-β2GPI (IgG/IgM) ELISA	aCL (IgG/IgM) CLIA (QUANTA Flash)	anti-β2GPI (IgG/IgM) CLIA (QUANTA Flash)
				
Manufacturer	In-house	In-house	Inova Diagnostics, San Diego, CA, USA	Inova Diagnostics, San Diego, CA, USA
Antigen	FBS in complex with CL on the medium-binding microtiter plate	human β2GPI on the high-binding microtiter plate	human β2GPI in complex with CL on the beads	human β2GPI on the beads
Units	AUG/AUM	AUG/AUM	CU	CU
99th percentile cut-off (IgG/IgM)	16/14	4/2	20	20
Range	1 – 60	1 – 34	IgG: 2.6 – 2024 IgM: 1.0 – 774	IgG: 6.4 – 6100 IgM:1.1 – 841
Traceability	KAPS standards HCAL/EY29C	HCAL/EY29C	HCAL/EY29C	HCAL/EY29C

Figure 1: aCL and anti-β2GPI assays characteristics. FBS, fetal bovine serum; CL, cardiolipin; HCAL anti-β2GPI IgG monoclonal antibody; EY2C9 anti-β2GPI IgM monoclonal antibody. Created with BioRender.com.

samples collected in tubes containing 0.109 M sodium citrate. Platelet-free plasma was obtained with double centrifugation at 2,500×*g* for 15 min dRVVT screening test was performed on a CS-2500 automated coagulation analyzer (Sysmex, Japan) using LA1 Screening and LA2 Confirmatory reagents (Siemens, Germany) and the aPTT was measured on a StArt semi-automated coagulation analyzer using the StaClot LA reagent kit (both Diagnostica Stago, France). dRVVT results were normalized with normal pool plasma and normalized ratio above 1.2 was considered positive for LAC. An absolute difference between the two APTTs (w/o phospholipids in hexagonal phase II) more than 8 s was considered positive for LAC.

Chemiluminescence immunoassay – QUANTA flash methods (index test)

QUANTA Flash aCL (IgG and IgM) and QUANTA Flash β2GPI (IgG and IgM) (Inova Diagnostics Inc., San Diego, CA, USA) were used according to the manufacturer's instructions developed for the BIO-FLASH® analyser (Inova Diagnostics Inc., San Diego, CA, USA). Briefly, magnetic microparticles coated with cardiolipin and purified human β2GPI are used to capture aCL, or coated with purified human β2GPI to capture anti-β2GPI from the sample. The results are expressed in chemiluminescence units (CU). According to the manufacturer, the threshold for a positive aCL and anti-β2GPI test is 20.0 CU. The detailed principle and analytical characteristics of the CLIA assay are shown in Figure 1.

Verification of the CLIA assays

The precision performance and linearity of the QUANTA Flash CLIA aCL (IgG and IgM) and anti-β2GPI (IgG and IgM) were verified in accordance with the relevant guidelines of the Clinical and Laboratory Standards Institute (CLSI), EP5-A3. To evaluate within-run and between-run imprecision, samples were tested five times daily over a 5-day period. To find significant outliers among the results, the Grubb's test was performed. For the linearity study, a positive sample and its dilutions (1:2, 1:4, 1:8, 1:16) were tested and a linear regression was calculated. Samples from healthy blood donors were used to verify the manufacturer's threshold for CLIA assays.

Establishment of QUANTA flash CLIA and ELISA thresholds for aCL and anti-β2GPI

To verify the manufacturer's QUANTA Flash CLIA thresholds in local setting, we calculated the 99th percentile of results from 139 sex-matched blood donors, fulfilling the ISTH SSC recommendation to test at least 120 samples [22]. Next, we performed Receiver Operating Characteristic (ROC) analysis of QUANTA Flash CLIA aCL (IgG and IgM) and QUANTA Flash β2GPI (IgG and IgM) results to calculate the threshold that provides similar clinical performance (diagnostic sensitivity and specificity) to the already established in-house ELISA [10, 23].

In-house ELISA thresholds, discriminating between negative, low, moderate and high levels of aCL, were established by testing 320 healthy blood donors following recommendations from international guidelines [2]. The 95th percentile of the healthy population was used to calculate the threshold for low positive aCL and anti-β2GPI, the 99th percentile for moderate positive and the threshold for high positive was twice as high as the threshold for moderate. In-house ELISA thresholds for clinically significant levels of anti-β2GPI were calculated as the 99th percentile of healthy blood donors.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 9. Diagnostic performance was determined with add-in software Analyse-it for Excel (Version 6.15.4). Spearman's rank correlation coefficient was used to analyze the quantitative correlations, and Cohen's kappa test was used to determine the qualitative agreement between the results of the different assays using the thresholds established in this study. p-Values of less than 0.05 were considered significant.

Results

Participants

This study included 80 patients with APS (30 men and 50 women, mean age 48.4 years) classified based on the revised Sapporo criteria [2]. 74 patients had primary APS, and six patients had APS secondary to another autoimmune disease. 14 patients had APS with obstetric complications (oAPS) and 66 had APS with a history of thrombosis (tAPS) (online Supplementary Table 1). The first control group of patients consisted of 28 patients with various systemic autoimmune diseases without history of thrombosis or pregnancy complications: systemic lupus erythematosus (SLE), systemic sclerosis (SSc), Sjögren's disease (SjS) and rheumatoid arthritis (RA) (4 men and 24 women, mean age 55.4 years). The second control group consisted of 51 patients with

clinical symptoms significant for APS but negative for aPL (11 men and 40 women, mean age 38.5 years). 15 patients had thrombotic clinical manifestations (non-tAPS) and 36 had obstetric clinical manifestations (non-oAPS). Data on previous venous thrombosis, arterial thrombosis and obstetric complications were available for all patients. 20 patients were included in the study during the COVID-19 pandemic (6 APS and 14 non-APS), but all patients who visited the outpatient clinic were tested for COVID-19, making it unlikely that they had acute COVID-19.

This study included two cohorts of healthy blood donors (online Supplementary Table 1). Serum samples from 320 healthy blood donors, including 229 men and 91 women, with a mean age of 43.4 years were used to determine thresholds for aCL and anti-β2GPI measured with our in-house ELISAs. Serum samples from 139 donors, consisting of 68 men and 71 women with a mean age of 42.6 years were used to validate thresholds for QUANTA Flash CLIA aCL and anti-β2GPI.

Verification of the QUANTA flash CLIA

To evaluate the measurement precision of the QUANTA Flash CLIA, we determined within-run and between-run imprecision for aCL (IgG and IgM) and anti-β2GPI (IgG and IgM) using the 5x5 model. The within-run coefficients of variation (%CV) ranged from 2.00 to 2.41 % for aCL and 2.88–3.48 % for anti-β2GPI. The between-run % CV ranged from 2.24 to 2.90 % for aCL and 3.29–4.53 % for anti-β2GPI. For the linearity study, a sample was serially diluted and the values of aCL (IgG, IgM) and anti-β2GPI (IgG, IgM) were measured at all concentration levels (online Supplementary Table 2, online Supplementary Figure 1). We have shown the linearity of the method in the measurement range for all measured values with regression slopes of 0.996 (95 % CI 0.958–1.033) and 1.003 (95 % CI 0.980–1.026) for aCL IgG and IgM, respectively. The coefficient of determination (R^2) for aCL IgG and IgM were 1.000. Anti-β2GPI IgG had a slope of 1.023 (95 % CI 0.913–1.133) with R^2 of 0.997. Anti-β2GPI IgM had a slope of 1.005 (95 % CI 0.945–1.064) with an R^2 of 0.999.

Establishment of moderate and high thresholds

Verification of the QUANTA flash CLIA thresholds

The reference range of the QUANTA Flash CLIA has been established by the manufacturer according to the 2006 revised Sapporo classification criteria, which define the clinically significant threshold for aCL and anti-β2GPI as the 99th percentile of values from the healthy population [2]. Here, the 99th percentile of values from 139 blood donors for aCL IgG corresponded to a value of 42.1 CU and 45.6 CU for aCL IgM (Table 1). While the 99th percentile for anti-β2GPI IgG and anti-β2GPI IgM was 47.4 CU and 33.5 CU, respectively. The 99th percentiles in our setting differed from those suggested by the manufacturer. We also set the threshold for low positive aCL and anti-β2GPI values based on the 95th percentile of the healthy population in accordance with previous studies [24]. These were 16.5 CU for aCL IgG, 17.5 CU for aCL IgM, 32.2 CU for anti-β2GPI IgG and 11.1 CU for anti-β2GPI IgM.

Thresholds for aCL and anti-β2GPI in-house ELISA results

The thresholds between negative and low positive aCL levels were calculated based on the 95th percentile of the healthy population and amounted to 9 AUG for aCL IgG and 6 AUM for aCL IgM (Table 2). The 99th percentile, which defines the clinically important threshold for distinguishing between low and moderate positive levels, according to the 2006 classification criteria, corresponding to 40 GPL/MPL [2], was calculated as 16 AUG for aCL IgG and 14 AUM for aCL IgM. The threshold for discriminating between moderate and high positives was twice as high as the threshold for low/moderate levels, corresponding to 80 GPL/MPL. Importantly, the monoclonal anti-β2GPI antibodies HCAL (IgG) and EY2C9 (IgM) have been used as external controls in the past to calibrate our method [14, 25].

Regarding thresholds for anti-β2GPI, the 2006 revised Sapporo international consensus statement did not distinguish

Table 1: Verification of cut-off values for QUANTA Flash aCL and anti-β2GPI.

Cut-off	aCL, CU		Anti-β2GPI, CU	
	IgG	IgM	IgG	IgM
Manufacturer cut-off (99th percentile of 252 blood donors	20.0	20.0	20.0	20.0
99th percentile of 139 blood donors	42.1	45.6	47.4	33.5
95th percentile of 139 blood donors	16.5	17.5	23.2	11.1

Results, including the specified threshold values, are considered positive.

Table 2: Thresholds for in-house ELISA aCL and anti-β2GPI.

Thresholds[a]	aCL		Anti-β2GPI[b]	
	IgG [AUG] (n=316)	IgM [AUM] (n=318)	IgG [AUG] (n=312)	IgM [AUM] (n=311)
Low positive (95th percentile)	9	6	2	1
Moderate positive (99th percentile)	16	14	4	2
High positive	32	28	8	4

[a]Results including the shown thresholds are considered positive. [b]The 99th percentile was used to define a clinically important threshold that distinguished between negative and positive anti-β2GPI levels.

between low, moderate, or high levels of anti-β2GPI; therefore, these thresholds were never used in our clinical setting, but were hovever calculated for the purpose of this study (Table 2). In rutine laboratory setting, the 99th percentile was used to define a clinically important cut-off that distinguished between negative and positive levels. The calculated threshold was 4 AU for anti-β2GPI IgG and 2 AU for anti-β2GPI IgM (Table 2).

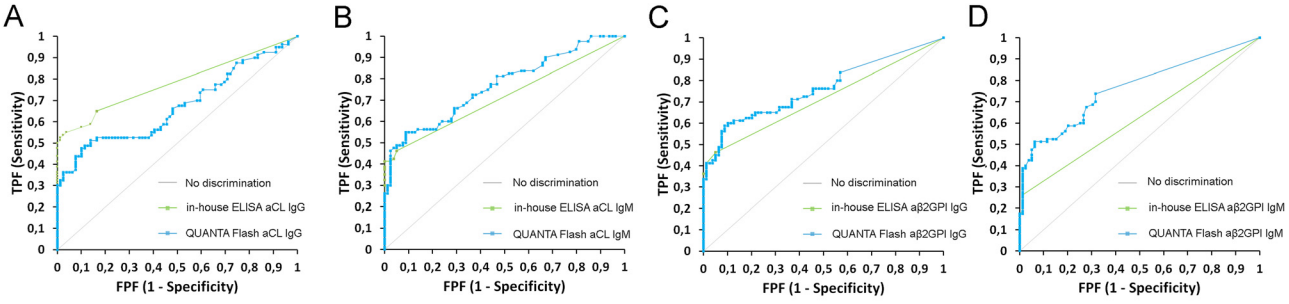
Comparison of QUANTA flash CLIA and ELISA thresholds using ROC curve analysis

In addition to the non-parametric approach described above, another way to determine threshold values of the assay is to analyze and compare its diagnostic sensitivity and specificity [10, 23] and compare them with already established methods. In this way, we calculated thresholds for aCL and anti-β2GPI on the QUANTA Flash CLIA that correspond to the thresholds of the in-house ELISA in a group of 80 patients with APS and the two control groups of patients without APS (n=79).

The areas under the ROC curve of the two assays showed no significant differences for aCL IgM and anti-β2GPI IgG. The area under the curve (AUC) values ranged from 0.626 to 0.783 for all assays. CLIA showed better clinical performance in terms of AUC compared to ELISA for anti-β2GPI IgM (0.626 for ELISA vs. 0.763 for CLIA) but worse for aCL IgG (0.783 for ELISA vs. 0.672 for CLIA) (Figure 2).

At the threshold between negative and low positive levels, the sensitivity of the aCL IgG and IgM ELISA was 53.8 % and 42.5 % with a specificity of 97.5 % and 96.2 %, respectively. A comparable clinical performance was obtained for both CLIA assays at 20 CU (Table 3), the value close to above measured 95th percentile (Table 1).

At a threshold between low and moderate positive levels, the sensitivity of the aCL IgG and IgM ELISA was 50.0 % and 30.0 %, respectively and the specificity was 100.0 % (Table 3). For the anti-β2GPI IgG and IgM ELISA, the sensitivity at the threshold between low and moderate positive values was 38.8 % and 20.0 % respectively, with a specificity of 98.7 %. A lower diagnostic sensitivity and specificity of the QUANTA Flash CLIA was achieved at 40 CU as a threshold between low and moderate positive values. Importantly, this value is close to above measured 99th percentiles (aCL IgG – 42.1 CU, aCL IgM – 45.6 CU, anti-β2GPI IgG – 47.4 CU and anti-β2GPI IgM – 33.5 CU, Table 1).



n = 159	in-house ELISA		QUANTA Flash CLIA	
	IgG	IgM	IgG	IgM
aCL	0.783 (0.719 – 0.846)	0.716 (0.656 – 0.775)	0.672 (0.587 – 0.757)	0.767 (0.694 – 0.840)
anti-β2GPI	0.715 (0.656 – 0.775)	0.626 (0.576 – 0.676)	0.764 (0.690 – 0.838)	0.763 (0.693 – 0.833)

Figure 2: ROC analysis: comparison of area under the curve (AUC) between in-house ELISA and QUANTA flash CLIA. (A) aCL IgG, (B) aCL IgM, (C) anti-B2GPI IgG, (D) anti-B2GPI IgM.

Table 3: Diagnostic sensitivity and specificity of aCL and anti-β2GPI IgG and IgM in-house ELISA and QUANTA Flash at certain threshold value.

n=159	aCL IgG		aCL IgM		Anti-β2GPI IgG		Anti-β2GPI IgM	
	In-house ELISA	QUANTA flash	In-house ELISA	QUANTA flash	In-house ELISA	QUANTA flash	In-house ELISA	QUANTA flash
Threshold for low positive	**9 AU**	**20.0 CU**	**6 AU**	**20.0 CU**	**2 AU**	**20.0 CU**	**1 AU**	**20.0 CU**
Sensitivity, %	53.8	43.8	42.5	35.0	46.3	56.3	26.3	27.5
Specificity, %	97.5	89.9	96.2	97.5	94.9	92.4	98.7	98.7
Threshold for medium positive	**16 AU**	**40.0 CU**	**14 AU**	**40.0 CU**	**4 AU**	**40.0 CU**	**2 AU**	**40.0 CU**
Sensitivity, %	50.0	33.8	30.0	25.0	38.8	42.5	20.0	18.8
Specificity, %	100.0	97.5	100.0	100.0	98.7	94.9	98.7	98.7
Threshold for high positive	**32 AU**	**80.0 CU**	**28 AU**	**80.0 CU**	**8 AU**	**80.0 CU**	**4 AU**	**80.0 CU**
Sensitivity, %	30.0	31.3	11.3	13.8	33.8	37.5	16.3	11.3
Specificity, %	100.0	98.7	100.0	100.0	100.0	98.7	100.0	100.0

Determined threshold values are presented in bold.

The sensitivity of aCL IgG and IgM in the in-house ELISA at the threshold between moderate and high was 30.0 % and 11.3 %, respectively. In this study, we found that QUANTA Flash CLIA values of 80.0 CU for both aCL IgG and aCL IgM can be considered equivalent to the moderate/high threshold of 32 AU and 28 AU of the in-house ELISA aCL IgG and IgM, respectively. For anti-β2GPI, the sensitivity determined with ELISA was 33.8 % for IgG and 16.3 % for IgM. With the QUANTA Flash anti-β2GPI tests, a comparable clinical performance was achieved at threshold values of 80.0 CU for IgG and IgM as with the in-house ELISA at threshold values of 8 AU for IgG and 4 AU for IgM.

Qualitative agreement and correlation between methods

We evaluated the qualitative agreement between QUANTA Flash CLIA and ELISA results on the entire patient population (n=159) (Table 4). A graphical presentation comparing ELISA and CLIA results is presented in online Supplementary Figure 2. The assays demonstrated good qualitative agreement, ranging from 76.10 to 91.19 %. The Cohen's kappa coefficient ranged from 0.466 to 0.596, indicating moderate agreement except for anti-β2GPI IgG where it was 0.683 indicating substantial agreement.

Our study also showed significant correlation between the quantitative results of QUANTA Flash CLIA and in-house ELISA for aCL and anti-β2GPI with Spearman coefficients ranging from 0.2714 to 0.6200 ($p<0.01$) (Table 4).

Comparative performance of ELISA and CLIA against the 2023 ACR/EULAR laboratory classification criteria

In our cohort of APS patients initially classified based on the revised Sapporo classification criteria, 50 of 80 (62.5 %) were found to meet the new ACR/EULAR laboratory classification criteria, while 11 of 80 (13.8 %) would not meet the criteria, regardless of the method used to determine aPL. The remaining 19 would fulfil the criteria according to one of the two methods, namely three according to CLIA and 16 according to ELISA (Figure 3).

In other words, it was found that 14 out of 80 (17.5 %) patients would not fulfil the new ACR/EULAR laboratory classification criteria when considering only the in-house ELISA results, due to their single IgM positivity. However, among these patients, three individuals would meet the laboratory classification criteria when considering QUANTA Flash CLIA results. This was attributed to moderately positive anti-β2GPI IgG. Upon reviewing their medical records, it was

Table 4: Qualitative agreement and correlation between QUANTA Flash CLIA and in-house ELISA.

	aCL IgG	aCL IgM	Anti-β2GPI IgG	Anti-β2GPI IgM
Cohen's kappa coefficient (95 % CI)	0.466 (0.341–0.591)	0.550 (0.426–0.673)	0.683 (0.558–0.809)	0.596 (0.415–0.777)
Total agreement	76.10 %	83.65 %	88.68 %	91.19 %
Spearman's rank correlation coefficient, p	0.2714 (p=0.001)	0.6200 (p<0.0001)	0.5173 (p<0.0001)	0.5357 (p<0.0001)

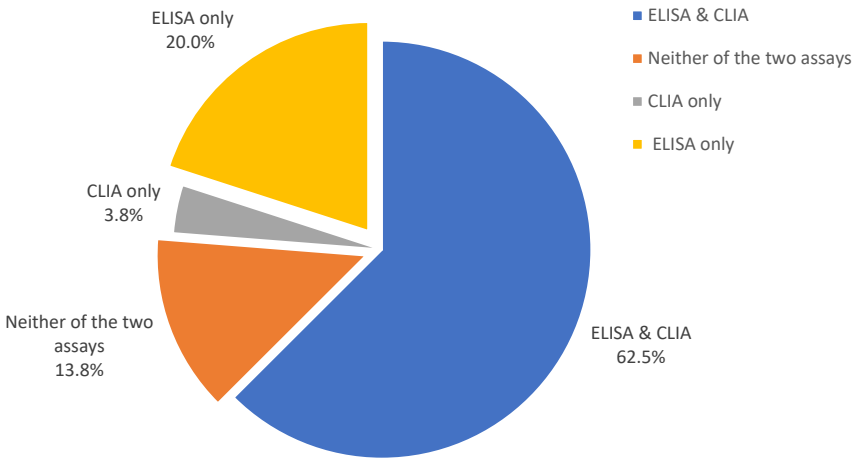


Figure 3: Percentage of APS patients originally classified using the 2006 revised Sapporo classification criteria who fulfill the new ACR/EULAR laboratory criteria, according to the method used.

confirmed that one of them would also meet the new clinical criteria, while two patients would not meet the criteria due to their high-risk VTE profile (in the case of one patient) or obstetric complications (in the case of one patient).

On the other hand, when evaluating the QUANTA Flash CLIA results, it was observed that 27 out of 80 patients (33.8 %) would not meet the new ACR/EULAR laboratory classification criteria. However, among these patients, 16 individuals would meet the laboratory criteria when considering the in-house ELISA results. Specifically, this was due to moderate positive aCL IgG in nine patients, high positive aCL IgG in four patients, and high positive anti-β2GPI IgG in three patients. In addition, review of their medical records confirmed that 14 of these patients would fulfil the new clinical criteria, while two patients would not be eligible due to either their high-risk CVD profile or obstetric complications.

In the SLE control cohort, which included 10 patients with SLE, all tested negative for aPL by ELISA and LAC. However, one patient had a moderately positive IgG anti-β2GPI result by QUANTA Flash CLIA (66.4 CU), but persistent positivity could not be confirmed.

In the non-APS control group, which consisted of 51 patients with clinical symptoms significant for APS but negative for aPL, three patients had low-positive IgG aCL (31.0–37.5 CU) and one had moderately positive IgG anti-β2GPI (56.2 CU) as measured by QUANTA Flash CLIA. The latter patient met the clinical criteria for pregnancy-related APS and would therefore meet the new ACR/EULAR laboratory criteria when considering CLIA, but persistent positivity could not be confirmed.

Discussion

The new 2023 ACR/EULAR APS classification criteria have recently been published for use in observational studies and trials [7]. New criteria offer a comprehensive framework that emphasizes precise laboratory features, including the distinction between IgG and IgM isotypes and the delineation of moderate and high levels based on standardized ELISA. Here we addressed key challenges in aligning QUANTA Flash CLIA thresholds with long-standing in-house ELISA thresholds, thereby facilitating the seamless integration of these new criteria into future trials. This is important as ACR/EULAR classification criteria, which comprise an additive, weighted system, assessing an individual's relative probability of APS, precisely define laboratory features of the APS. The novel laboratory features include the following: 1) quantifying single-, double-, and triple-aPL positivity, 2) separating aCL/anti-β2GPI IgG and IgM isotypes, and 3) defining two levels of aCL/anti-β2GPI positivity that will be interpreted as clinically relevant by most investigators. The steering committee agreed there should be two levels of aCL/anti-β2GPI positivity ("moderate" and "high") based on ELISA technique [7].

Clinical guidelines recommend a distinction between positive aCL and anti-β2GPI levels as either in the "low range" or in the "moderate-to-high range" for APS diagnosis, classification and risk profiling. Studies typically use specific thresholds in the low range (20–40 "units") and moderate-to-high range (>40 "units" or >99th percentile). It is important to note that these studies often do not distinguish between different analytical platforms such as ELISA, CLIA or multiplex flow immunoassay (MFI) [26]. In addition, researchers face the issue of arbitrary units used in ELISA and CLIA, although they are referred to GPL/MPL, U/mL or CU by manufacturers who claim to have run out of Harris standards to calibrate their products [27, 28]. Originally, Harris et al. defined a level that enables best distinction between APS and non-APS patients. This level is at least above 20 GPL (or MPL) or even more reliably above 40 GPL (or MPL) as a threshold for moderate aCL IgG/IgM positivity and 80

GPL/MPL as a threshold for the high positive aCL IgG/IgM range [13, 29]. However, Harris standards are no longer available to calibrate any commercial or in-house assay. Therefore, researchers and routine laboratories are faced with the problem of arbitrary units used in ELISA and CLIA. Automated platforms using different techniques, such as CLIA and MFI, have some advantages over ELISA, are commercially available as alternatives for ELISA, and perform well. However, they also show inter-assay variability and limited numerical agreement with ELISA. Here, we re-evaluated thresholds of our long-standing in-house ELISA with non-parametric approach determining 99th percentile of 320 blood donors for moderate threshold being 16 AUG and 14 AUM for aCL IgG and IgM, and 4 AUG and 2 AUM for anti-β2GPI. It is crucial to emphasise that our in-house ELISAs have been calibrated more than decade ago using international standard sample β2GPI dependent aCL monoclonal antibodies HCAL (IgG) and EY2C9 (IgM), and that these thresholds can be translated into 40/80 GPL/MPL [14, 30].

QUANTA Flash CLIA, a fully automated assay, takes approximately 30 min to complete a test, saving time and reducing operator handling. Automation reduces variability within and between laboratories and improve reproducibility. These are all advantages that led us to implement and validate this platform in our laboratory. Here we have confirmed the precision and linearity of the QUANTA Flash CLIA. The manufacturer states the cut-off value as 20 CU but does not specify the thresholds for moderate and high levels. In this study, we determined 40 CU for aCL and anti-β2GPI IgG and IgM antibodies as a low/moderate threshold for the QUANTA Flash CLIA, which provides equivalent clinical performance to our in-house aCL and anti-β2GPI ELISA. In addition, these thresholds were also confirmed using a non-parametric approach in which the 99th percentile is calculated. This is a previous recommendation published in the revised Sapporo classification criteria [2] and is also endorsed in a recent commentary on the 2023 ACR/EULAR APS classification criteria [28]. The 99th percentile determined by measuring aCL and anti-β2GPI in the sera of 139 blood donors were 42.1 CU and 45.5 CU for aCL IgG and IgM, respectively, and 47.4 and 33.5 CU for anti-β2GPI IgG and IgM, respectively. Importantly, we were able to demonstrate a significant correlation between the quantitative results of QUANTA Flash CLIA and the in-house ELISAs when using 40/80 CU thresholds. The assays showed good qualitative agreement, particularly for aCL IgM and anti-β2GPI IgG and IgM which ranged from 83.7 to 91.2 %, and slightly lower for aCL IgG at 76.1 %. The lower qualitative agreement between the in-house ELISA and QUANTA Flash CLIA aCL results can be explained by the use of different antigens in both systems. The source of protein cofactors, such as β2GPI and prothrombin, bound to cardiolipin as the antigen in our in-house aCL ELISA is foetal bovine serum (FBS). In contrast, the antigen in QUANTA Flash CLIA aCL is human β2GPI in complex with cardiolipin. Consequently, positive results in QUANTA Flash CLIA aCL and β2GPI are attributed to β2GPI-dependent antibodies, whereas our in-house aCL ELISA can also detect antibodies directed against other antigens such as protein C, S, prothrombin. It is important to emphasize that aCL antibodies are a heterogeneous group of antibodies that bind to cardiolipin in the presence of plasma proteins. While most are directed against β2GPI, some can also recognize other proteins. The source of antigen in methods used for aCL determination varies widely, with many commercially available methods now being β2GPI-dependent. Although initial international anti-cardiolipin standardization workshops recommended ELISA protocols using adult or fetal calf serum as a source of antigen, the comparison of results remains challenging or even unachievable [13].

Only three previous studies compared the clinical performance of a CLIA with in-house or commercial ELISA [10, 23, 26], all of which reported their own threshold values, which are hardly comparable for aCL IgG, while the comparison for aCL IgM is better (Supplementary online Table 4). A study by Meneghel et al. compared the HemosIL AcuStar CLIA with their in-house ELISAs and reported the aPL levels as U/mL using a standard curve obtained from a pool of positive samples calibrated to Koike's monoclonal antibodies (HCAL for the IgG and EY2C9 for the IgM aPL antibodies) [23]. Despite a lower comparative sensitivity, CLIA showed a higher comparative specificity for some aPL and a good level of agreement and correlation with the in-house ELISAs. Their thresholds for aCL IgG/IgM were 16.2 and 23.6 U/ml, respectively. The study by Iwaniec et al. was the first to compare QUANTA Flash CLIA with commercial QUANTA Lite ELISAs [10]. Their study showed good clinical performance and a strong correlation of the automated CLIA aPL assays with clinical symptoms of APS. They also established low/moderate antibody thresholds for the aCL antibodies using a ROC curve approach to achieve similar clinical performance (diagnostic sensitivity and specificity) for the QUANTA Flash CLIA as for ELISAs with a threshold of 40 GPL and MPL. According to their results, QUANTA Flash CLIA values of 95 CU for aCL IgG and 31 CU for aCL IgM can be considered as equivalent to the 40 GPL and MPL low/moderate thresholds. Later, a third research group performed a semi-quantitative evaluation of aCL and anti-β2GPI measured with CLIA [26]. Vandevelde et al. reported that the use of 40/80 units as moderate/high thresholds are acceptable for aCL/anti-β2GPI IgG ELISA, but not for CLIA and MFI.

Their thresholds for moderate levels of aCL IgG/IgM, corresponding to the standard value of 40 units in the aCL ELISA, were 202 CU and 45 CU, respectively. Taken together, the QUANTA Flash CLIA thresholds for moderate aCL IgG levels and the associated diagnostic sensitivities differ significantly, i.e. 40 CU in our study, 95 CU in Iwaniec's study and 202 U/mL in Vandevelde's study, with associated sensitivities ranging from 29 % in Vandevelde's study, 34 % in our study and 55 % in Iwaniec's study. The lower thresholds and lower sensitivity in our study may be partly explained by the fact that our cohort of APS patients was initially selected based on in-house aCL ELISA results. Our in-house aCL ELISA detected more subpopulations of aPL compared to other commercial aCL ELISA or QUANTA Flash CLIA aCL due to the difference in antigen, as explained previously. In addition, our cohorts of patients and controls originated from the general population that was routinely tested for aPL. Importantly, the thresholds we identified are closely comparable to the 99th percentile of healthy blood donors, whereas the differences in other studies are larger. However, the thresholds for aCL IgM were much more comparable. In this study, we found a value of 40 CU, while Iwaniec et al. reported a value of 31 CU and Vandevelde et al. a value of 45 CU, all with similar diagnostic sensitivity and specificity. A possible explanation would be that a larger proportion of aCL IgM antibodies are β2GPI-dependent and the differences in antigen have less influence on the result than with aCL IgG.

To our knowledge, the only other research group that has established thresholds for anti-β2GPI is the study by Vandevelde et al. [26]. They set the ROC sensitivity-based threshold for moderately positive levels of anti-β2GPI IgG at 1959 U/ml in the thrombotic test population and 1552 U/ml in the obstetric test population, both of which are significantly different from our thresholds. Their thresholds for anti-β2GPI IgM were 31 U/ml and 33 U/ml, which is much more comparable to our study (Supplementary online Table 5). Taken together, the results of our study and previous studies reporting moderate thresholds for aCL IgG or anti-β2GPI IgG vary considerably, rising questions regarding the possibility of fixed cut-off values that can be used as thresholds in different detection systems. While the data for aCL IgM or anti-β2GPI IgM are more consistent, hovering around 40 CU or 40 U/ml, further studies are needed to confirm and validate these results. Similar concerns regarding the use of fixed cut-off values and variability between ELISA and CLIA systems have also been reported by others [28]. Although the indication of the aPL levels in terms of low, moderate or high values in the laboratory reports is primarily a matter of classification criteria and not diagnostic criteria, our results nevertheless also have diagnostic significance, as the antibody levels is also indicative for the probability of disease and for determining the risk of relapse [31].

All studies evaluating the analytical performance of QUANTA Flash CLIA showed satisfactory precision of the tests in terms of analytical reproducibility and linearity. Most studies showed good clinical performance and a strong correlation of the automated CLIA with the clinical symptoms of APS. The publication of the new 2023 ACR/EULAR APS classification criteria allowed us to redefine our research cohort, which was initially defined with LAC and with aCL and anti-β2GPI in-house ELISAs according to the revised Sapporo classification criteria. Importantly, the Phase IV (validation) of the new APS criteria showed that the new APS criteria compared to the 2006 revised Sapporo criteria have a specificity of 99 % vs. 86 % and a sensitivity of 84 % vs. 99 %. Our results support this statement, as a significant proportion of APS patients classified according to the revised Sapporo criteria did not meet the new 2023 ACR/EULAR APS laboratory classification criteria. Specifically, 14 of 80 (17.5 %) patients did not meet the new criteria when using in-house ELISA, and 27 of 80 (33.8 %) did not meet the criteria when using QUANTA Flash CLIA. Although more patients meet laboratory criteria when ELISA is used, validation of a classification system may be challenging in the absence of a definitive gold standard and may be affected by an inherent bias towards ELISA, as the original cohort was initially defined using the in-house ELISA results. In addition, the difference in percentages could also be due to the differences in antigens used in both platforms, as explained earlier.

We acknowledge some limitations in our study. First, the diagnostic performance of the CLIA and ELISA tests was retrospectively calculated based on the previous classification criteria, as our patient cohort was originally defined according to the revised Sapporo classification criteria. Unfortunately, not all clinical data required to assess the patients' disease signs according to the new classification criteria were always available in the medical records. This limitation applies not only to our study but also to many others, as emphasised by Favaloro et al. who discuss the difference between classification and diagnostic criteria [8]. As defined by the ACR Subcommittee on Classification and Response Criteria [32], diagnosis involves the evaluation of an individual patient's signs, symptoms, and supportive tests to determine the origin or nature of the disease. Consequently, a group of signs, symptoms, and tests used in standard clinical care to guide the clinical decision-making process for specific patients constitutes diagnostic criteria. Instead, the classification criteria are uniform definitions that are mostly used to create different, comparatively homogeneous patient groups for clinical research. Secondly, our study is

limited by a relatively small sample size. This limitation did not affect our main objective, which was to establish clinically important thresholds for QUANTA Flash CLIA that are comparable to our in-house ELISAs, as all sample size recommendations were met. However, the impact of the thresholds on clinical practise will only become apparent in the long term and with a larger number of patients.

Despite these limitations, our study makes an important contribution as one of the first papers to show thresholds for moderate and high levels of aCL and anti-β2GPI IgG and IgM for QUANTA Flash CLIA. Such findings are crucial not only at the local level but also within the broader international medical community. The new classification criteria emphasize the stratification of aPL positivity, yet there is a notable lack of studies reporting thresholds for different platforms. Given the current recommendation to primarily employ ELISA until further validation on alternative platforms, our findings play a pivotal role in guiding researchers worldwide in establishing relevant thresholds and enhancing diagnostic accuracy across diverse methodologies.

In conclusion, the study aimed to align QUANTA Flash CLIA thresholds with long-standing in-house ELISA thresholds, crucial for implementing the new ACR/EULAR APS classification criteria. These criteria emphasize precise laboratory features, including the quantification of aPL positivity and the definition of moderate and high levels. Despite challenges in standardization, our study determined thresholds for moderate and high aCL and anti-β2GPI IgG/IgM levels in QUANTA Flash CLIA, crucial for clinical implementation. Notably, applying the new 2023 ACR/EULAR APS classification criteria revealed a substantial portion of patients no longer meeting updated laboratory criteria. The comparison with in-house ELISAs showed significant quantitative correlation, confirming the reliability of the method. Despite the limitations, this study contributes valuable insights for establishing relevant threshold values and aligning diagnostic methods, thus supporting future research and clinical practice.

What is already known about this subject?

- The recently published 2023 ACR/EULAR APS classification criteria recommend that the aCL and anti-β2GPI thresholds of moderate (40–79 units) and high (>80 units) should be determined based on standardized enzyme-linked immunosorbent assay (ELISA) results. In addition, researchers should focus their efforts on identifying and validating the moderate/high thresholds of their platform by correlating them with the moderate/high thresholds determined by ELISA.

What does this study add?

- Thresholds in QUANTA Flash CLIA, which reflect the long-established in-house ELISA thresholds, were firmly set at 40–79 CU for moderate and ≥80 CU for high levels of aCL and anti-β2GPI IgG and IgM.
- Our study clearly showed a significant correlation between quantitative results obtained from both QUANTA Flash CLIA and in-house ELISAs.
- Applying the new 2023 ACR/EULAR APS classification criteria to our APS cohort, previously classified according to the revised Sapporo classification criteria, revealed that a significant proportion of patients no longer met the updated laboratory criteria. This confirms that the new 2023 ACR/EULAR APS classification criteria, compared to the 2006 revised Sapporo classification criteria, demonstrate a specificity of 99 % vs. 86 %.

How this study might affect research, practice or policy

- This study provides valuable insights for establishing relevant thresholds, which are crucial for seamlessly integrating the new 2023 ACR/EULAR classification criteria into future observational clinical studies and trials.

Research ethics: The study was approved by the National Medical Ethics Committee, Ljubljana, Slovenia (KME 0120-7/2019/5) and (KME 99/04/15).
Informed consent: Informed consent was obtained from all individuals included in this study.
Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission. PŽ, MO and SČ contributed to the conception and study design. AA and EB checked medical records. PŽ, NK and MO analysed the data. PŽ and MO contributed to the interpretation of the data. PŽ and MO wrote the first version of the manuscript and SS and ŽR revised it critically. PŽ, MO, AA, ŽR and SČ read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Data availability: The raw data can be obtained on request from the corresponding author.

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