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ORIGINAL ARTICLE

Influence of different IgG anticardiolipin antibody cut-off values on antiphospholipid syndrome classification

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Summary. *Background:* While medium to high titers of anticardiolipin (aCL) antibodies, defined as >40 GPL units or >99th percentile, is a laboratory criteria for the 'definite' diagnosis of antiphospholipid syndrome (APS), agreement between the two cut-offs has not been validated. *Objective:* To validate the current aCL laboratory criterion by verifying the effect of the two cut-offs on APS classification. *Patients/methods:* Ninety aCL positive APS patients were selected on the basis of their GPL values above the 99th percentile (17.4 GPL), which was calculated by testing 100 age- and sex-matched healthy subjects. *Results:* A significant difference in the IgG positivity ($P < 0.0001$) was found between the APS laboratory profiles as 20 out of the 24 (83.3%) patients with single positivity (aCL alone), six out of the 23 (26.1%) with double positivity (aCL plus lupus anticoagulant or anti-$\beta_2$glycoprotein I), and none out of the 43 with triple positivity (aCL plus lupus anticoagulant and anti-$\beta_2$glycoprotein I) had titers between the 99th percentile and 40 GPL units. Moreover, the rate of aCL values between the 99th percentile and 40 GPL units was significantly higher ($P < 0.0001$) in patients with pregnancy morbidity (73.7%) as compared to those with vascular thrombosis (16.9%) and those with both conditions (16.7%). *Conclusion:* The 99th percentile cut-off level seems more sensitive than the >40 GPL value for APS classification, as it includes subjects with aCL positivity alone as well as patients with pregnancy morbidity.

Keywords: anticardiolipin antibodies, antiphospholipid syndrome, cut-off value.

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Introduction

According to the International Consensus Document that updated the classification criteria for antiphospholipid syndrome (APS) [1], a 'definite' diagnosis implies vascular thrombosis and/or pregnancy morbidity and at least one of the following antibodies: lupus anticoagulant (LA) and/or IgG/IgM anti-$\beta_2$glycoprotein I (α-β_2GPI) and/or IgG/IgM anticardiolipin (aCL) antibodies. Patients are to be allocated to classification categories on the basis of positivity to more than one test (category I) or to a single test (category II). ACL titers must be medium/high, defined as levels above 40 GPL or MPL units or higher than the 99th percentile [1] calculated in normal subjects.

Despite its popularity and several standardization workshops [2–6], aCL enzyme-linked immunosorbent assay (ELISA) still shows high intra- and inter-laboratory variability. Inter-laboratory variations in numerical results and methodologies, and the lack of consensus about semi-quantitative data continue to be reported [3,6]. As demonstrated by Escalante *et al.* [7], who quantified the diagnostic accuracy of aCL ELISA using ROC curves, aCL IgG titers are important for predicting APS-related clinical events. As aCL ELISA does not have a normal distribution [8], the Committee of the IV European Forum on APS advised calculating the cut-off value in percentiles instead of using +2, +3 or +5 SD of the mean optical density of normal samples [2]. Some authors [9–11] have proposed using a value higher than 40 GPL as the cut-off for medium/high titers, as it is clinically useful to distinguish patients at risk of thrombosis. In accordance with these indications, the Sydney Consensus Document states that levels >99th percentile or >40 GPL units are to be considered the cut-off for medium/high titers. Agreement between these two cut-off values has not yet been validated.

Given the discrepancies observed in daily laboratory activities, we were interested in comparing the influence of these cut-off values on APS classification in a large group of patients in an attempt to validate the current laboratory criterion for aCL [1].

Patients and methods

Patients and controls

One hundred and forty-one patients were evaluated, who were affected with primary APS and who all met the Sydney criteria for APS classification [1]. The clinical features for APS were vascular thrombosis and/or pregnancy morbidity. In accordance with the international criteria [1], pregnancy morbidity signified: (i) one or more unexplained fetal deaths at or beyond the 10th week of gestation; or (ii) one or more premature births before the 34th week of gestation of morphologically normal neonates as a result of severe preeclampsia or recognized symptoms of placental insufficiency; or (iii) three or more unexplained consecutive spontaneous abortions before the 10th week of gestation. Laboratory criteria included detection of at least one of the following antibodies: LA and/or IgG/IgM α-β_2GPI and/or IgG/IgM aCL antibodies at medium/high titers using the 99th percentile as the cut-off value. One hundred healthy age- and sex-matched subjects were evaluated to calculate the 99th percentile for the aCL and α-β_2GPI assays. All of the subjects participating in the study signed an informed consent statement.

aPL assays

A single operator performed all laboratory tests. IgG and IgM aCL were detected using an 'in-house' ELISA that followed the 'minimal requirements' proposed by the European Forum on antiphospholipid antibodies [2]. Polysorb plates (Nunc, Roskilde, Denmark), coated with cardiolipin (Sigma, St Louis, MO, USA) overnight at 4 °C, were incubated for 2 h at room temperature with 10% fetal calf serum/phosphate buffered saline (FCS/PBS). After washing with 10% FCS/PBS, plates were incubated with 1/50 diluted sera for 1 h. Following three washes with 10% FCS/PBS, plates were incubated with alkaline phosphatase-conjugated goat antihuman IgG and antihuman IgM (Sigma) for 1.5 h. The wells were washed as before and incubated at 37 °C with *p*-nitrophenyl phosphate (Sigma), and absorbance at 405 nm measured in a microplate reader (Versamax; Molecular Devices, Sunnyvale, CA, USA). Blank samples were included. In accordance with the recommendations of the European Forum, two well-defined dilutions of the antibodies HCAL and EY2C9 were used as external controls. Moreover, LAPL GM-200 calibrators (Louisville APL Diagnostics, Inc., Doraville, GA, USA) were used to make calibration curves [12]. PL or MPL values of LAPL GM-200 calibrators ranged from 96 to 16 units. The initial evaluation of the patients' sera was made using the same assay that was used later to calculate the data. Each sample was run twice for data calculation and the ELISA assay was repeated twice on different days.

LA was detected by a series of clotting tests following internationally accepted recommendations [13]. As previously described [14], the dilute Russell viper venom time was used as the screening test, and the kaolin clotting time test was performed [15] in plasma negative in this test.

Anti-β_2GPI antibodies were tested in accordance with the proposals of the Standardization Group of the European Forum on antiphospholipid antibodies [16]. The results were expressed as arbitrary units using an eight dilution point standard curve obtained from a pool of positive samples calibrated on Koike's monoclonal antibodies (HCAL for the IgG α-β_2GPI and EY2C9 for the IgM α-β_2GPI) [17].

Statistical analysis

Paired-samples *t*-test was used to calculate the inter- and intra-test variability. Pearson's chi-square test was employed to compare the results in the different subsets of IgG aCL-positive APS patients. The mean GPL values in the patient groups were compared using a one-way ANOVA test and post hoc multiple comparisons. The relationship between thrombosis or pregnancy morbidity with a >40 GPL cut-off value was analyzed by calculating the crude odds ratio (OR) and the 95% confidence interval (CI).

Results

APS patients were selected using the 99th percentile value (17.4 for IgG and 26.8 for IgM aCL). One hundred and seven out of the 141 patients were positive for aCL antibodies when this cut-off was used. Of these, 72 patients were positive for IgG aCL alone, 17 for IgM aCL alone, and 18 for both isotypes. The latter patients were counted in both the IgG and IgM groups (Fig. 1). The IgM aCL patients were not considered any further as the group was too small to achieve statistical significance. The study group thus included 90 APS IgG aCL-positive patients. Table 1 outlines the study groups stratified according to their antibody profiles and clinical features. Sixty-four out of 90 patients (71%) had titers above 40 GPL units, while the remaining 26 fell between the 99th percentile (17.4 GPL) and 40 GPL units. As illustrated in Fig. 2, 20 out of the 24 patients with only IgG aCL (83.3%) fell between the 99th percentile and

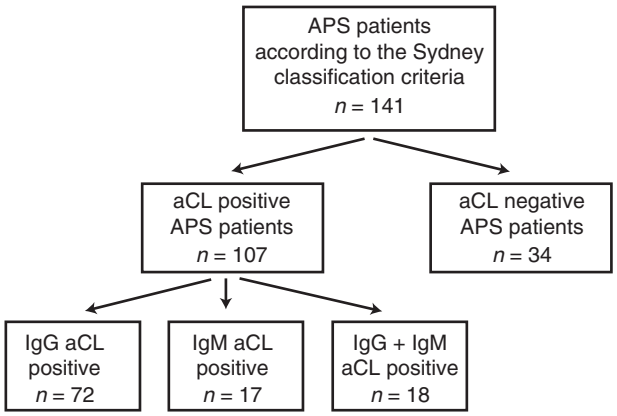

Fig. 1. Study population divided according to anticardiolipin (aCL) positivity and aCL isotypes.

Table 1 Antibody profiles and clinical characteristics of the IgG anticardiolipin (aCL)-positive antiphospholipid syndrome (APS) patients

	Pregnancy morbidity	Thrombosis	Both	Total
IgG aCL alone	13	9	2	24
IgG aCL + LA or α-β_2GPI	3	18	2	23
IgG aCL + LA + α-β_2GPI	3	32	8	43
Total	19	59	12	90

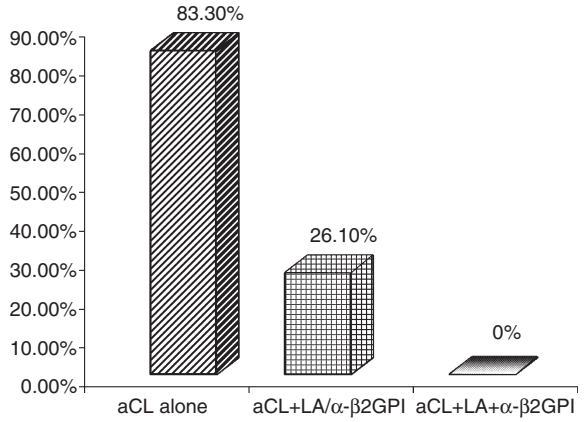

Fig. 2. Prevalence (%) of IgG anticardiolipin (aCL)-positive antiphospholipid syndrome (APS) patients with aCL level between the 99th percentile and 40 GPL units, divided according to antibody profile.

40 GPL units. When the >40 GPL cut-off value was used, these patients were no longer considered APS. Out of the 23 patients with double positivity, six (26.1%) fell between the 99th percentile and 40 GPL units, and when the >40 GPL cut-off was used their antibody category went from I to II. The titers of the 43 triple positive patients were all higher than 40 GPL units. A significant difference was observed between the three groups ($P < 0.0001$).

The mean value (± SD) of the IgG aCL levels was 34.0 (± 16.0) in patients with IgG aCL alone, 68.8 (± 29.2) in those with IgG aCL plus LA or α-β_2GPI, and 140.6 (± 93.7) in those with IgG aCL and LA plus α-β_2GPI (Fig. 3). The comparison between the mean levels in the three groups was highly significant ($P < 0.0001$).

There were 14/19 patients (73.7%) with only pregnancy morbidity, 10/59 subjects (16.9%) with only vascular thrombosis, and 2/12 patients (16.6%) with both features had values between the 99th percentile and 40 GPL units (Fig. 4). There was a significant difference between these three groups ($P < 0.0001$). The mean IgG aCL values in the three groups were 47.2 (± 46.3), 94.7 (± 61.7), and 163.5 (± 144.1), respectively, and their difference was statistically significant ($P < 0.0001$; Fig. 5).

Univariate analysis showed a significant crude OR (13.8; 95% CI, 4.2–45.5) for the association between thrombosis and values higher than 40 GPL units. However, there was a significant crude OR (0.2; 95% CI, 0.1–0.5) for the association between pregnancy morbidity and values lower than 40 GPL units.

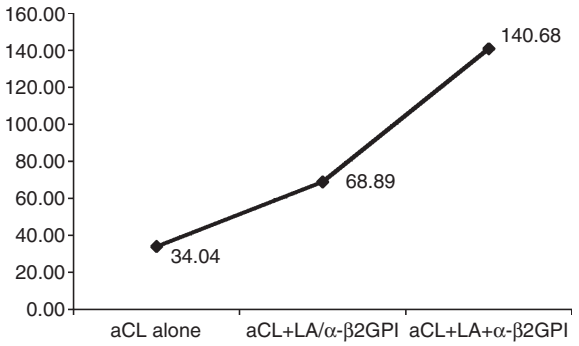

Fig. 3. Mean antibody levels of IgG anticardiolipin (aCL)-positive antiphospholipid syndrome (APS) patients divided according to antibody profile.

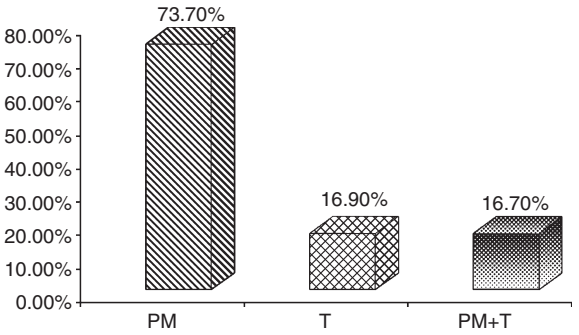

Fig. 4. Prevalence (%) of IgG anticardiolipin (aCL)-positive antiphospholipid syndrome (APS) patients with aCL level between the 99th percentile and 40 GPL units, divided according to the clinical features. PM, pregnancy morbidity; T, thrombosis; PM + T, pregnancy morbidity + thrombosis.

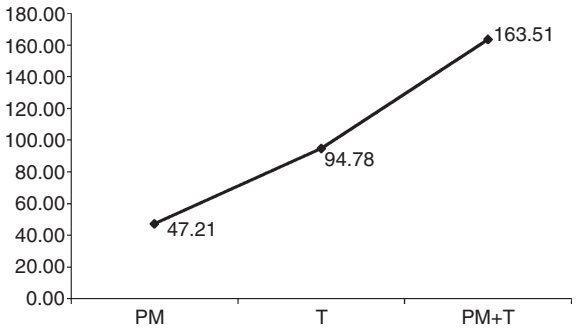

Fig. 5. Mean antibody levels of IgG anticardiolipin (aCL)-positive antiphospholipid syndrome (APS) patients divided according to the clinical features. PM, pregnancy morbidity; T, thrombosis; PM + T, pregnancy morbidity + thrombosis.

Discussion

As the International Consensus Conference proposed that aCL titers above the 99th percentile and higher than 40 GPL units both be used as the cut-offs for APS, it seemed important to verify whether there is an agreement between these values. When aCL ELISA was carried out using an eight dilution point

curve and employing international standard materials as external controls to ensure that the results were valid, no significant intra- and inter-assay variability was observed. The 99th percentile cut-off value, calculated on the basis of the results obtained in 100 age- and sex-matched healthy subjects, was first used to select IgG aCL-positive APS patients. Among these, 64 out of 90 had titers above 40 GPL, while the remaining 26 fell between the 99th percentile and 40 GPL units. In this case, the 20 who were positive to IgG aCL alone would not be considered APS if the >40 GPL cut off value was used, but would be overdiagnosed if the 99th percentile was employed.

As the double and triple positive patients normally register higher IgG aCL levels, they continued to be considered aCL positive when the >40 GPL cut-off was used in most of the former and in all of the latter cases.

From a clinical point of view, most of the patients with pregnancy morbidity were no longer aCL positive when the >40 GPL cut-off was used, while most of the subjects with thrombosis and all those with both features continue to be considered positive. Why do the pregnancy morbidity APS patients have lower mean antibody levels than vascular thrombosis APS subjects? At present, it is impossible to answer this question. On the basis of the literature available [18,19], we can only hypothesize that the mechanisms of action in placental injury [20,21] are different from those in thrombosis [22]. Thus, despite the lack of consistent evidence to this effect, it would seem that the quantity and perhaps even the binding characteristics of IgG aCL in APS patients might differ in the two groups.

On the basis of the present findings, it is apparent, in any case, that the number and type of APS patients do, in fact, vary greatly depending on the IgG aCL cut-off, which needs to be specified whenever clinical trials are undertaken.

Finally, as a significant association was found between vascular thrombosis and titers >40 GPL units and between pregnancy morbidity and titers <40 GPL units, using different cut-off values depending on the patient's clinical features seems, for the moment, to be the best solution.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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