

# Optimising testing for phospholipid antibodies

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## Abstract

**Aim**—To compare anticardiolipin (ACL) and anti- $\beta$ 2 glycoprotein 1 ( $\beta$ 2gp1) enzyme linked immunosorbent assays (ELISAs) in the diagnosis of antiphospholipid syndrome (APS) and to incorporate these results into a meta-analysis of published data.

**Method**—Three representative commercial ACL ELISAs and an in house  $\beta$ 2gp1 assay were optimised and then assessed on 124 sera from normal donors, patients with infection, or patients with APS. A Medline search was screened for papers meeting defined criteria to conduct a meta-analysis. The performance of the assays used in this study was included.

**Results**—A non-quantitative ACL assay performed at least as well as the anti- $\beta$ 2gp1 assay in the diagnosis of APS. Meta-analysis confirmed that neither assay is perfect, although the anti- $\beta$ 2gp1 assay had a higher specificity and lower sensitivity than the ACL assay.

**Conclusions**—The pooled data suggest that the ACL assay is used to investigate thrombosis without overt underlying pathology and that the improved specificity of the anti- $\beta$ 2gp1 assay is exploited where infection, connective tissue disease, or atheroma are present.

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Keywords: antiphospholipid syndrome; anticardiolipin antibodies; anti- $\beta$ 2 glycoprotein 1; sensitivity

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enzyme linked immunosorbent assays (ELISAs) in which cardiolipin is the antigen.

Anticardiolipin antibodies associated with infection, originally described in syphilis, are not linked to thrombosis. These antibodies may cause confusion when investigation for APS takes place in settings where infection is common—for example, systemic lupus erythematosus (SLE)<sup>6</sup> or stroke.<sup>7</sup>

$\beta$ 2gp1 ( $\beta$ 2glycoprotein 1) is a plasma glycoprotein that can act as a physiological anticoagulant, in vitro at least, inhibiting the clotting cascade<sup>8</sup> and platelet aggregation.<sup>9</sup>  $\beta$ 2gp1 is required as a cofactor for the binding of ACL to cardiolipin in APS, but not in infection.<sup>10</sup> Irradiated ELISA plates have been recommended for use in anti- $\beta$ 2gp1 assays<sup>11</sup> and may yield specificity and sensitivity for APS as high as 95% and 83%, respectively.<sup>12</sup>

Despite claims of improved specificity, there is no evidence that anti- $\beta$ 2gp1 is superseding ACL. For example, although over 150 centres participate in the UK National External Quality Assurance Scheme for anticardiolipin antibodies, only 18 centres submit results for  $\beta$ 2gp1.<sup>13</sup> Faced with increasing requests for antiphospholipid antibodies in a population with a high incidence of infection, we aimed to compare the performance of commercial anticardiolipin and in house anti- $\beta$ 2gp1 assays.

We also aimed to see whether our results and results from other published studies were substantiated in a meta-analysis, when larger patient numbers and a broader case mix were included.

## Patients and methods

### PATIENTS

The local research ethics committee oversaw our study and samples were obtained with informed consent from consecutive patients during a two month period. So as not to prejudice our data, ACL positivity was not a prerequisite to a diagnosis of primary or secondary APS. We relied on clinical diagnostic criteria,<sup>14</sup> exclusion of postoperative state, oestrogens, protein C, protein S, antithrombin III deficiencies, and activated protein C resistance. Twenty two consecutive patients with primary APS who had thrombosis but no evidence of connective tissue disease were recruited from a haematology clinic. Thirty four consecutive patients with secondary APS who had a history of thrombosis and either SLE or rheumatoid arthritis were recruited from a rheumatology clinic.

Samples seropositive for syphilis (positive rapid plasma reagin and *Treponema pallidum* haemagglutination test; 15 donors) and human immunodeficiency virus (HIV) infection (16

Phospholipid antibodies are associated with thrombosis in patients with antiphospholipid syndrome (APS), either as a primary disorder or secondary to connective tissue diseases. If untreated, there is a high risk of recurrent thrombosis in APS, usually within a year.<sup>1</sup> Tests for APS are often used to investigate unexpected thrombosis, with the aim of preventing recurrence in otherwise healthy patients or on a background of connective tissue disease.

The lupus anticoagulant (LAC) test for APS relies on the paradoxical ability of patient plasma to inhibit clotting in vitro, in a phospholipid dependent fashion. This test is more difficult in patients receiving anticoagulants, may not always be reproducible,<sup>2</sup> and has been found to have poor sensitivity in several studies.<sup>3</sup>

To overcome some of these disadvantages, serological tests for anticardiolipin antibodies (ACL) are widely used and have been cited as an absolute prerequisite to the diagnosis of APS,<sup>4</sup> or a requirement when lupus anticoagulant is not detected.<sup>5</sup> Phospholipid antibodies are usually detected by commercially available

Table 1 Performance characteristics of optimised assays

Assay	Cut off point (SDs above mean)	Mean specificity (CI)	Mean sensitivity (CI)	Mean positive predictive value (CI)	Mean negative predictive value (CI)
Non-quantitative total ACL	3	0.98 (0.96 to 1.00)	0.55 (0.46 to 0.64)	0.92 (0.87 to 0.97)	0.79 (0.71 to 0.87)
Quantitative total ACL	3	0.88 (0.82 to 0.94)	0.59 (0.50 to 0.68)	0.72 (0.64 to 0.80)	0.80 (0.72 to 0.88)
IgG ACL	4	0.98 (0.96 to 1.00)	0.34 (0.26 to 0.42)	0.88 (0.82 to 0.94)	0.73 (0.65 to 0.81)
IgM ACL	3	0.95 (0.91 to 0.99)	0.34 (0.26 to 0.42)	0.79 (0.72 to 0.86)	0.71 (0.62 to 0.80)
Either IgG or IgM ACL	—	0.95 (0.91 to 0.99)	0.50 (0.41 to 0.59)	0.85 (0.79 to 0.91)	0.78 (0.70 to 0.86)
IgG anti-β2gp1	1	0.92 (0.87 to 0.97)	0.43 (0.34 to 0.52)	0.76 (0.68 to 0.83)	0.75 (0.66 to 0.84)
IgM anti-β2gp1	4	0.97 (0.94 to 1.00)	0.18 (0.11 to 0.25)	0.8 (0.73 to 0.87)	0.68 (0.59 to 0.77)
Either IgG or IgM anti-β2gp1 positive	—	0.89 (0.83 to 0.95)	0.48 (0.39 to 0.57)	0.70 (0.62 to 0.78)	0.76 (0.67 to 0.85)

ACL, anticardiolipin; anti-β2gp1, anti-β2 glycoprotein 1; CI, confidence interval.

donors) were obtained from consecutive patients attending a genitourinary medicine clinic who had no history of thrombosis. Fifty two normal control sera were obtained from healthy blood donors (obtained via the National Blood Authority) and were negative for HIV and syphilis.

Sera were stored at -20°C for at least two months, after which patient details were checked (MH) to confirm clinical grouping. Twelve samples from patients with APS and three samples from patients with syphilis were excluded because data required for clinical classification were incomplete.

#### ASSAYS

Three representative commercial ACL ELISAs were selected, each using cardiolipin coated plates but differing in the source of cofactor protein and conjugate specificity.

Non-quantitative total ACL (Alpha, Eastleigh, Hampshire, UK): contains purified β2gp1 in the sample diluent and uses polyvalent conjugate (anti-IgG, anti-IgA, anti-IgM).

Quantitative total ACL (Genesis, Cambridge, UK): purified β2gp1 is present on plates and uses polyvalent conjugate (anti-IgG, anti-IgA, anti-IgM).

Separate IgG and IgM ACL (Autozyme; Cambridge Life Sciences, Cambridge, UK): uses fetal calf serum as a source of β2gp1 on plates and in sample diluent and separate assays with anti-IgG and anti-IgM conjugates.

Each assay was carried out according to the manufacturer's instructions, including exact incubation times, although we based our results on optical density rather than the standards supplied with the assays.

In the anti-β2gp1 ELISA, purified β2gp1 (Crystal Chem, Chicago, Illinois, USA) at 10 µg/ml (50 µl/well in 0.05M bicarbonate buffer, pH 9.6) was incubated in γ irradiated

plates (Nunc Maxisorp: Nunc, Roskilde, Denmark) overnight at 4°C. After washing, non-specific sites were blocked with 2% porcine gelatine (150 µl/well) for one hour at 37°C. The sample (100 µl; 1/50 dilution in PBS Tween 20) was added before antibody detection, using horseradish peroxidase conjugated anti-IgG or anti-IgM in separate assays (both Dako; 1/1000 dilution). The reaction was developed with *o*-phenylenediamine (0.1 mg/ml) and stopped after five minutes with 1M sulphuric acid. Absorbency was read at 490 nm. Antigen and conjugate dilutions had been optimised previously. Operators were blind to clinical details.

#### STATISTICS

We optimised all ELISA cut offs points using the receiver operating characteristic (ROC) process.<sup>15</sup> This compares the effects of different cut offs points (each the mean optical density derived from normal sera, plus increasing units of SD) on sensitivity and specificity for the whole data set. For these calculations, sera from patients with syphilis and HIV were grouped with the normal sera and referred to as negatives, and sera from the patients with primary and secondary APS were grouped as positives.

In the ROC for a hypothetical poorly discriminating test, increasing the cut off point increases specificity and decreases sensitivity in a linear fashion (broken lines in fig 1A-F). We selected optimum cut off points for each assay by identifying the maximum perpendicular distance between the observed data and that of the hypothetical test.

Studies comparing the performance of phospholipid and β2gp1 assays for APS were sought using Medline (using the terms: "antiphospholipid syndrome", "antiphospholipid antibodies", "anticardiolipin antibodies", or "β2gp1" and "sensitivity" or "specificity"). Medline was searched for English language publications from 1966 to June 1999 and yielded 53 publications, which were then screened for inclusion, using a checklist for evaluating diagnostic tests.<sup>16</sup> We did not attempt to include unpublished data. The pooled data were derived by weighting each individual study according to its variance, with greater weight being given to larger studies.

The 95% confidence intervals (CI) for proportions were estimated using the formula:

$CI = p \pm 1.96 \sqrt{p(1-p)/n}$ , where  $p$  is the proportion of interest and  $n$  is the number of

Table 2 Distribution of positive sera in anticardiolipin (ACL) assays

Patient group	Normal	HIV	Syphilis	Primary APS	Secondary APS
Non-quantitative total ACL only positive	0	0	0	0	0
Non-quantitative and quantitative total ACL positive	0	0	0	2	3
Non-quantitative total and separate IgG/IgM ACL positive	0	0	0	2	0
All ACL assays positive	0	1	0	9	8
Quantitative total ACL only positive	3	1	5	1	1
Quantitative total and separate IgG/IgM ACL positive	0	0	0	1	1
Separate IgG/IgM ACL only positive	3	0	0	3	0
Total samples in group	52	16	12	22	22

APS, antiphospholipid syndrome; HIV, human immunodeficiency virus.

samples. McNemar's test was used to test the hypothesis that samples will produce the same results for both tests under comparison and is based on the number of discordant pairs.<sup>17</sup>

## Results

Table 1 shows the selected cut off points for each assay. The separate ELISAs detecting IgG and IgM ACL alone were of poor sensitivity

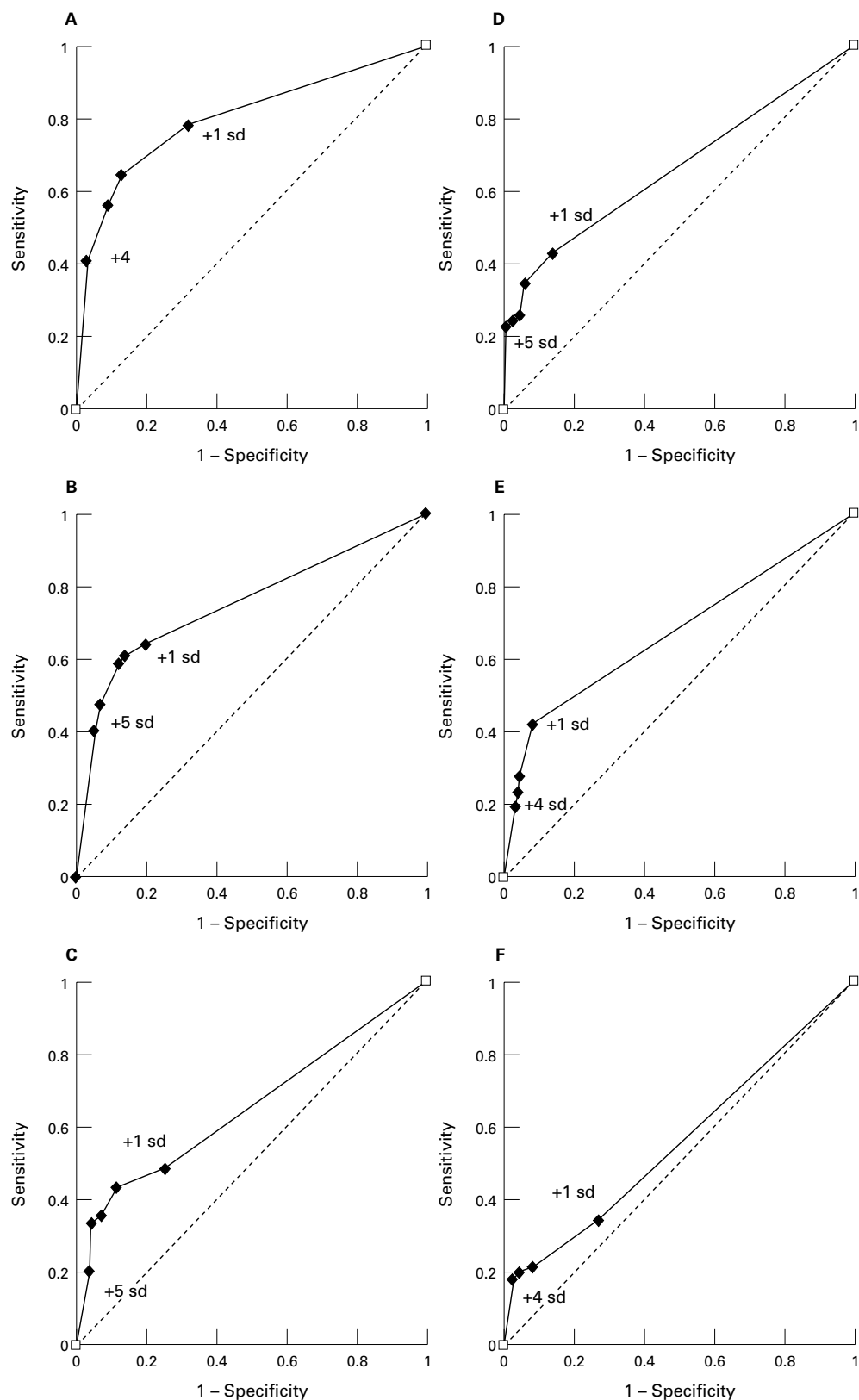


Figure 1 Receiver operating characteristic curves for anticardiolipin (ACL) and anti-β2 glycoprotein 1 (anti-β2gp1) assays. Assays are: (A) non-quantitative total ACL, (B) quantitative total ACL, (C) IgG ACL, (D) IgM ACL, (E) IgG anti-β2gp1, (F) IgM anti-β2gp1.

Table 3 Concordance between anti- $\beta$ 2 glycoprotein 1 (anti- $\beta$ 2gp1) and non-quantitative total anticardiolipin (ACL) assays by clinical group

Patient group	Normal	HIV	Syphilis	Primary APS	Secondary APS
Anti- $\beta$ 2gp1 only positive	3	1	4	1	2
Both anti- $\beta$ 2gp1 and ACL positive	0	1	0	9	9
ACL only positive	0	0	0	4	2
Total samples in group	52	16	12	22	22

APS, antiphospholipid syndrome; HIV, human immunodeficiency virus.

(fig 1) and the results of these assays combined were used for analysis. Table 2 shows the distribution of positive results for the three ACL assays in each disease group. There was no significant difference in the sensitivities of the ACL assays. The assays differed greatly in their false positive rates (table 1), and the specificity of the non-quantitative ACL assay was significantly better than that for the quantitative total ACL (McNemar's test;  $p < 0.001$ ) and the combined IgG/IgM ACL assay ( $p < 0.05$ ). The non-quantitative total ACL assay had an interassay coefficient of variation of 13%, derived from the optical density of a positive control used in each assay run.

We combined the results of the IgG and IgM anti- $\beta$ 2gp1 ELISAs and compared these with the non-quantitative total ACL assay. Table 3 shows the distribution of positive results by disease group and highlights the false positives seen with anti- $\beta$ 2gp1, which are responsible for the significantly lower specificity than non-quantitative total ACL (McNemar's test;  $p < 0.05$ ). There was no difference in the sensitivity of the anti- $\beta$ 2gp1 and the non-quantitative ACL assays.

If both anti- $\beta$ 2gp1 and non-quantitative total ACL are positive, specificity is improved slightly to 0.99 (95% CI, 0.97 to 1.00), but sensitivity falls to 0.41 (95% CI, 0.32 to 0.49). The requirement that sera were positive for either anti- $\beta$ 2gp1 or non-quantitative total ACL improved sensitivity to 0.61 (95% CI, 0.53 to 0.70) but specificity fell to 0.88 (95% CI, 0.82 to 0.93).

Medline identified 53 studies that assessed the performance of ACL and anti- $\beta$ 2gp1 in diagnosing APS: 15 of these studies compared both assays. For us to make direct comparisons between ACL and anti- $\beta$ 2gp1, six studies that used positive ACL tests as a prerequisite for APS diagnosis were excluded. Further studies were excluded because clinical details were inadequate (one study), there were no details of assay reproducibility (two studies), or the approach to defining ELISA cut off points was not given (two studies).

Table 4 Concordance between anti- $\beta$ 2 glycoprotein 1 (anti- $\beta$ 2gp) and non-quantitative total anticardiolipin (ACL) assays by clinical group for pooled data

Patient group	Normal	Atheroma	Infection	Connective tissue disease no thrombosis	Total no APS	Primary APS	Secondary APS	Total APS
ACL positive alone	0	38	74	59	171	16	21	37
ACL/anti- $\beta$ 2gp1 positive	0	2	11	33	49	67	50	117
Anti- $\beta$ 2gp1 positive alone	3	0	7	3	13	3	4	7
Total samples in group	100	109	239	213	661	139	145	284

The infection column refers to infection with human immunodeficiency virus, syphilis, hepatitis C, rickettsial infection. APS, antiphospholipid syndrome.

Four papers were selected for meta-analysis.<sup>3 12 18 19</sup> Of these, three studies<sup>12 18 19</sup> used purified cardiolipin as antigen. The fourth study<sup>3</sup> used a mixture of phospholipids.

Table 4 shows the distribution of positive results for ACL and anti- $\beta$ 2gp1 for each clinical group used in the meta-analysis.

Because each study included variable case mixes, we did not compare positive predictive value as a performance indicator. Sera from healthy controls and patients with infection, atheroma, or connective tissue disease without thrombosis were combined as negatives and sera from patients with primary or secondary APS were combined as positives. Figure 2A and B shows the sensitivity and specificity for ACL and anti- $\beta$ 2gp1 for each report and for the pooled data. The pooled data, based on 952 patients, show the  $\beta$ 2gp1 assay had better specificity than ACL (0.97 v 0.89), but worse sensitivity (0.75 v 0.97) (95% CI for these proportions all  $< 0.001$ ). Although methodological differences make comparisons difficult, we noted that assays separately detecting IgM as well as IgG for ACL<sup>3</sup> or anti- $\beta$ 2gp1 (this study and Day and colleagues<sup>3</sup>) did not have improved sensitivity (not shown).

In studies where irradiated plates were used (this study and Guerrin *et al*,<sup>12</sup> Sanmarco and colleagues<sup>18</sup>) the overall sensitivity (0.58; 95% CI, 0.54 to 0.62) and specificity (0.95; 95% CI, 0.94 to 0.97) were higher than for studies where non-irradiated plates were used<sup>3 19</sup> (sensitivity, 0.35; 95% CI, 0.30 to 0.40; specificity, 0.78; 95% CI, 0.73 to 0.82). Direct comparisons between the use of irradiated and non-irradiated plates may not be valid because of the different case mix composition of the studies.

## Discussion

We found that performing separate IgG and IgM ACL ELISAs and the quantitation of results did not improve performance. These findings may translate directly into laboratory economies. The non-quantitative total ACL kit was selected because of its improved specificity at our selected cut off point, which differed from that recommended by the manufacturer. Our data show that a well optimised ACL assay performs at least as well as the anti- $\beta$ 2gp1 assay in the diagnosis of APS.

The pooled data show that the ACL assay has slightly higher sensitivity and the anti- $\beta$ 2gp1 assay has higher specificity. The individual studies show variable results, which we attribute to three factors:

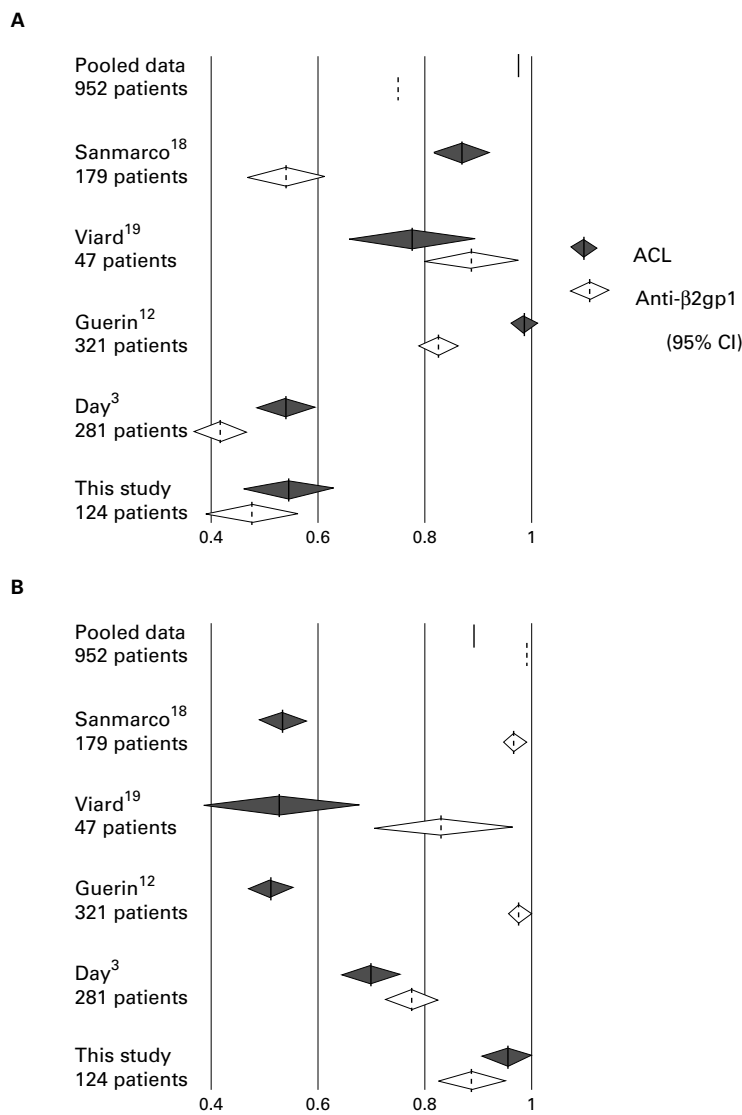


Figure 2 Sensitivities (A) and specificities (B) for anticardiolipin (ACL) and anti-β2 glycoprotein 1 (anti-β2gp1) assays in individual reports and derived from pooled data.

(1) The lack of specificity of the ACL assay in some studies may reflect the case mix of patients included and the unreliability of ACL in infection.

(2) The meta-analysis suggests that plate irradiation improves the sensitivity of anti-β2gp1 assays. Our anti-β2gp1 ELISA had comparable specificity to those included in the meta-analysis, despite the false positives noted in syphilis sera, which has been mentioned by other authors.<sup>20 21</sup> The sensitivity of our anti-β2gp1 assay was low compared with that of the pooled data, despite optimisation of antigen concentrations. Higher concentrations of β2gp1 do not necessarily improve sensitivity and have negative effects on specificity.<sup>20</sup> The use of irradiated ELISA plates improves the detection of anti-β2gp1 by inducing conformational changes in the antigen,<sup>22</sup> improved antigen binding,<sup>23</sup> or a combination of both.<sup>11</sup>

(3) The studies discussed here may highlight genuine biological differences between patient groups. In patients with APS, human major

histocompatibility complex (HLA) polymorphisms affect the frequency of patients positive for ACL.<sup>24</sup> Similarly, positivity for anti-β2gp1 is affected by racial factors, HLA,<sup>24</sup> and polymorphisms in the β2gp1 gene itself.<sup>25</sup> We are unable to draw conclusions on this possibility from the data shown here.

An earlier meta-analysis comparing these two assays recommended the routine use of anti-β2gp1 in the diagnosis of APS.<sup>4</sup> This analysis included studies with small numbers of patients and no normal controls, factors likely to exaggerate the performance of a test. Combinations of ACL and anti-β2gp1 have been proposed as a means of improving performance in diagnosing APS, although we have confirmed earlier work<sup>7</sup> showing that such combinations increase sensitivity at the cost of decreased specificity.

What is the best way to use these assays? We suggest that in clinical practice the choice of test depends on the clinical setting. For example, in an otherwise healthy patient who has had a thrombosis but has no evidence of atheroma, infection, or connective tissue disease, the higher sensitivity of a well optimised ACL assay may be preferable in screening for primary APS. In a series of 50 patients with the worst manifestation of APS—catastrophic antiphospholipid syndrome—most patients had no underlying connective tissue disease and 96% had ACL antibodies.<sup>26</sup> In the setting of atheroma, connective tissue disease, or infection, the improved specificity of anti-β2gp1 is required to confirm APS reliably. This suggestion is supported by the data in table 4. Inappropriate use of either of these tests might lead to the wrong decisions being made on anticoagulation. Finally, further studies are needed to evaluate ACL and anti-β2gp1 assays in a range of ethnic groups.

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