Table 1 Evidence table of included studies

Author, year	*Study design*	*Population*	*Women (pregnancies)*	*aPL tests*	*Intervention/ comparison*	*Pregnancy complications assessed*
Kaaja et al., 1993[14]	RCT	SLE aPL (+)[a]	6 (6)	IgG aCL LA	ASA (50 mg/day) (F 2, P 2) vs. placebo (F 4, P 4)	Spontaneous abortion Pre-eclampsia Birth weight
Julkunenet al., 1994[15]	Retrospective	SLE aPL (+)	8 (16)	LA	Medical treatment (F 4, P 4)[b] vs. non-treatment (F 4, P 12)	Fetal death Prematurity IUGR
Cowchock and Reece, 1997[16]	RCT	Low-risk pregnant women with aPL (+)[c]	19 (19)	IgG/M aCL LA	ASA (81 mg/day) (F 11, P 11) vs. usual care (F 8, P 8)[d]	Fetal death, Fetal distress at term Birth weight
Kahwa et al., 2006[17]	RCT	Primiparae aCL (+)[e]	48 (48)	IgG/M/A aCL >1 occasion IgG/M/A anti-β2GPI only in aCL (+)	ASA (60 mg/day) (F 28, P 28) vs. placebo (F 20, P 20)	Spontaneous abortion Stillbirth, Pre-term delivery Low birth weight Pre-eclampsia, Eclampsia
Del Ross et al., 2013[18]	Retrospective	Asymptomatic aPL carriers[f]	40 + X (65)[g]	IgG/M aCL, IgG/M anti-β2GPI LA confirmed 6 or 12 weeks apart	ASA (P –47) vs. non-treatment (P 18)	Spontaneous abortion Delivery ≤ 34 weeks

aPL: antiphospholipid antibodies; RCT: randomized controlled trial; F: number of women; P: number of pregnancies, (+): positive; aCL: anticardiolipin antibodies; LA: lupus anticoagulant, anti-β2GPI: anti-β2glycoprotein I antibodies; ASA: acetylsalicylic acid; IUGR: intrauterine growth retardation; SLE: systemic lupus erythematosus.

[a]Three women with antiphospholipid antibodies and systemic lupus erythematosus had a history of one miscarriage during the first trimester of gestation.

[b]Medical treatment: prednisone and aspirin, prednisone and heparin, prednisone and aspirin or prednisone alone.

[c]Women were identified in the course of a multicentre trial.[20] Pregnant woman at low risk were defined as those who had zero to two spontaneous abortions.

[d]One patient in the aspirin group and two patients in the usual care group had a history of two or fewer spontaneous abortions.

[e]Of the pregnant women who participated in the Jamaica Low Dose Aspirin Trial and consented to phlebotomy,[21] 901 women were evaluated and a history of previous pregnancies was identified in 45 women (91% had only one pregnancy), in which 24 had spontaneous abortions and 21 had elective abortions.

[f]Defined as any titre of aPL and no previous pregnancy or no pregnancy loss. Some women might have non-specified autoimmune disease.

[g]Sixty-five pregnancies occurred in aPL carriers: 40 occurred in woman who did not have any previous pregnancy and 25 occurred in a non-defined number of women.

statistically significant (RR 2.15; 95% CI 0.63 to 7.33, 132 women, I^2=0%) (Figure 3(b)). No statistical significance was observed to reduce preterm birth (RR 1.71; 95% CI 0.32 to 8.98, 132 women, I^2=0%) (Figure 3(c)) or low birth weight (RR 0.98; 95% CI 0.07 to 13.54, 132 women, I^2=33%) (Figure 3(d), Table 3), and no statistically significant interaction was noted between subgroups (study design).

Discussion

Based on the available literature, there is no evidence to show the benefit of prophylactic treatment with aspirin to prevent pregnancy complications in aPL carriers in the absence of other risk factors; however, this evidence is not yet fully confirmed.

According to the literature search, five published articles addressed, in part, our research question. The selected articles examined asymptomatic patients with aPL: two studies were performed in women with SLE,[14,15] and three in asymptomatic women with different backgrounds.[16–18] The pregnancy outcomes assessed in these studies did not favour the use of aspirin as prophylactic treatment.

Pregnant women might have aPL and these antibodies could be persistently positive before pregnancy or appear for the first time during pregnancy. Physicians encountering asymptomatic primigravida with aPL but no history of thrombosis have to consider the beneficial effect of

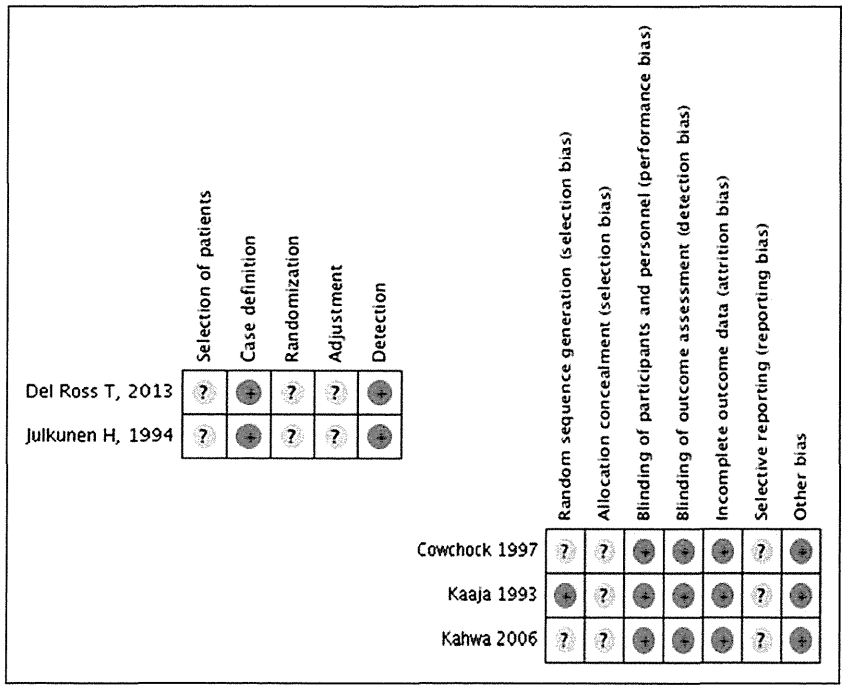

Figure 2 Risk of bias summary. Investigators' judgements of risk of bias items for each included study.

Table 2 Pregnancy complications in included studies

Author, year	*Results/conclusion*
Kaaja et al., 1993[14]	No pregnancy complications reported in women treated with ASA or placebo. No differences in birth weights observed between infants born to mothers treated with ASA and those receiving placebo.
Julkunen et al., 1994[15]	In the treatment group, one (25%) pregnancy ended in prematurity and three pregnancies were full term without IUGR. Fisher test, $P = 0.00714$. 100% of untreated pregnancies had pregnancy complications (nine abortions, two preterm births (one resulted in neonatal death) and one IUGR).
Cowchock and Reece, 1997[16]	No statistically significant differences were found in the obstetric outcome between both groups ($P < 0.05$). Prevalence of any complication in the ASA group was 18% (one fetal death and one fetal distress at term) compared with 13% in the control group (one low birth weight delivery) (OR 1.55, 95% CI 0.1–20.85).
Kahwa et al., 2006[17]	No differences in pregnancy outcome was observed between ASA and placebo treated primiparae. Prevalence of any pregnancy complication in the ASA group was 14% (one stillbirth, two low birth weight deliveries and one preterm birth) compared with 5% in the control group (one preterm birth) (OR 3.1, 95% CI 0.3–30.73).
Del Ross et al., 2013[18]	No differences in pregnancy outcome was observed between ASA and placebo groups. Prevalence of evaluated pregnancy complications in the ASA group was 13% (two abortions and four deliveries ≤34 weeks) compared with 6% in the untreated group (one abortion) (OR 2.5, 95% CI 0.3–22.3).

ASA: acetylsalicylic acid; IUGR: intrauterine growth retardation; OR: odds ratio, CI: confidence interval.

Table 3 Outcome of pregnancies in published studies comparing aspirin and placebo in asymptomatic pregnant women with antiphospholipid antibodies

Outcome	*Studies*	*Pregnancies*	*Effect estimate Risk ratio (95% CI)*	*Heterogeneity I^2 (%)*
Complicated pregnancy	3	132	2.15 (0.63–7.33)	0
Spontaneous abortion and/or fetal death	3	132	1.14 (0.18–7.31)	0
Preterm birth	3	132	1.71 (0.32–8.98)	0
Low birth weight	3	67	0.98 (0.07–13.54)	33

CI: confidence interval.

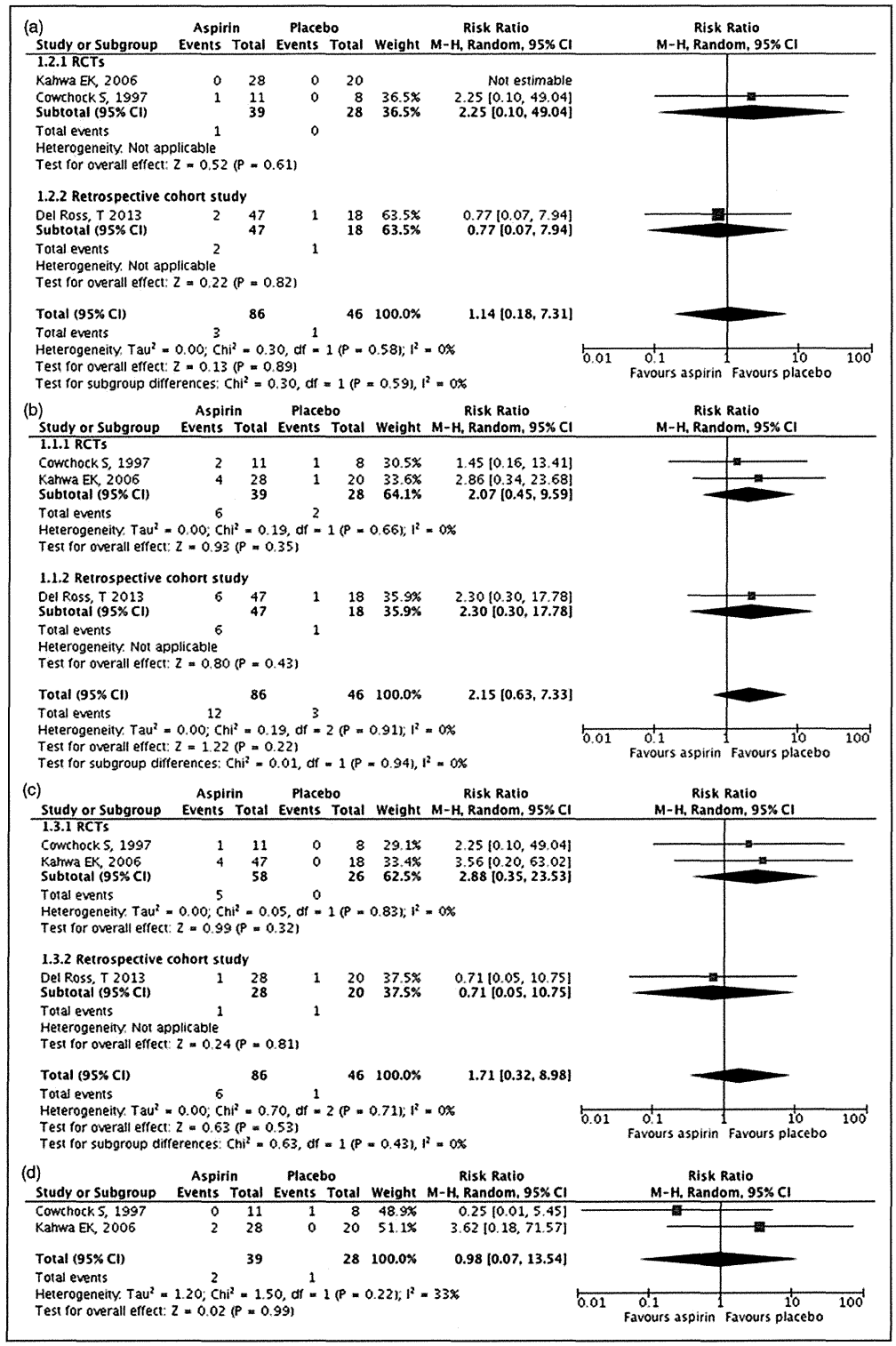

Figure 3 (a) Forest plot of spontaneous abortion and/or fetal death. Comparison of aspirin versus placebo in asymptomatic women with antiphospholipid antibodies in 132 pregnancies. (b) Forest plot of pregnancy complications. Comparison of aspirin versus placebo in asymptomatic women with antiphospholipid antibodies in 132 pregnancies. (c) Forest plot of preterm delivery. Comparison of aspirin versus placebo in asymptomatic women with antiphospholipid antibodies in 132 pregnancies. (d) Forest plot of low birth weight delivery. Comparison of aspirin versus placebo in asymptomatic women with antiphospholipid antibodies in 67 pregnancies.

prophylactic treatment to prevent obstetric complications.

In this review, our clinical question was initially centred on asymptomatic pregnant women with aPL and no history of previous pregnancy. However, most clinicians do not routinely investigate the presence of aPL in otherwise asymptomatic pregnant women, and rarely before the first pregnancy. We consider this group of asymptomatic aPL carriers especially vulnerable because the risk of unfavourable obstetric outcome is not known. Unfortunately, in asymptomatic women with no underlying autoimmune disease, aPL testing is usually conducted after the first pregnancy failure, but rarely as of the first pregnancy. Therefore, studies with asymptomatic primigravida who are aPL carriers are limited. We extended our selection criteria to include asymptomatic aPL-positive pregnant women with a history of successful pregnancies. A major problem when trying to identify from the literature an optimal prophylactic therapy for women with aPL during pregnancy was the difficulty of stratifying women by the presence or absence of obstetric history in most studies. In some instances, past obstetric history is referred to as 'no abortion' history,[16] and women for whom the only clinical manifestations are one or two early miscarriages (primiparae) are regarded as having the same condition as primigravida women.

Another difficulty of this review is that participants' characteristics varied between studies. Two studies included only women with SLE,[14,15] and three studies enrolled a heterogeneous population of aPL-positive pregnant women,[16,17] containing patients with non-specific autoimmune disease.[18] Nevertheless, this mixed population may reflect the variety of patients that clinicians manage in daily clinical practice, thus we accepted these studies as relevant enough for the analysis. In patients with underlying autoimmune disease, especially patients with SLE, aPL are routinely tested before pregnancy. Among asymptomatic aPL-positive SLE patients, primary prophylaxis with aspirin and hydroxychloroquine appeared to reduce the frequency of thrombotic events.[19]

Four out of the five studies included in this review could not find significant benefits for primary prophylaxis with aspirin.[14,16 18] On the other hand, the study reported by Julkunen et al.[15] showed that medical treatment during pregnancy seems to have a beneficial role in the obstetric outcome of aPL-positive patients with SLE. In the latter study, all treated women received prednisone, either alone or in combination with aspirin or heparin, while in the other four studies treatment consisted of aspirin alone. Discrepancies in the results might therefore be related to the different prophylactic treatment regimens administered.

In conclusion, this systematic review could find no evidence to show that the use of aspirin is superior to placebo or usual care to prevent unfavourable obstetric outcomes in otherwise healthy women with aPL during the first pregnancy. Pregnant women with aPL should be informed of the potential risk during pregnancy and advised on the different treatments available, with the final decision on treatment made by the patient in conjunction with the physician and obstetrician.

Large RCTs or prospective observational studies are needed to explore the real benefit of prophylactic treatments for pregnancy complications in asymptomatic aPL carriers. As clinicians often manage asymptomatic aPL carriers with SLE, and the condition could pose an additional risk for obstetric complications, there is a particular need for future studies to distinguish women suffering from SLE.

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Conflict of interest statement

Atsuko Murashima has taken part in speakers' bureaus for Mitsubishi Tanabe Pharma Co., Takeda Pharmaceutical Co. Ltd. and Astellas Pharma Inc. Tatsuya Atsumi has received grant/research support from Chugai Pharmaceutical Co. Ltd., Eisai Co. Ltd., Bristol-Myers Squibb Co., Astellas Pharma Inc., Daiichi Sankyo Co. Ltd. and Mitsubishi Tanabe Pharma Co., and has taken part in speakers' bureaus for Astellas Pharma Inc. and Mitsubishi Tanabe Pharma Co. The other authors have no conflicts of interest to declare.

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PAPER

Determination of clinically significant tests for antiphospholipid antibodies and cutoff levels for obstetric antiphospholipid syndrome

T Kitaori[1], M Sugiura-Ogasawara[1], K Oku[2], W Papisch[3], T Ebara[4], Y Ozaki[1], K Katano[1] and T Atsumi[2]
[1]Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan; [2]Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [3]Thermo Fisher Scientific, Freiburg, Germany; and [4]Occupational and Environmental Health, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan

Objective: The objective of this paper is to determine which kinds of assays for antiphospholipid antibodies (aPL) should be tested for clinical practice for patients with recurrent pregnancy loss (RPL). **Materials and methods:** We studied 560 patients with a history of RPL prospectively. We determined the obstetric significance of 11 commercially available tested assays for lupus anticoagulant (LA)-aPTT StaClot, phosphatidylserine-dependent antiprothrombin (aPS/PT) IgG, IgM, classical cardiolipin (CL) IgG, IgM, CL IgG, IgM, IgA, and β2glycoprotein I (β2GPI) IgG, IgM, IgA Phadia. Obstetric significance was defined as the potential for anticoagulant therapy to improve the subsequent live birth rate, or a difference in the live birth rate between positive and negative untreated cases. **Results:** The LA-aPTT StaClot assay and aPS/PT IgG assay, but not CL IgG, were found to have obstetric significance. Our conventional tests covered positive cases with the aPS/PT IgM and classical CL IgG assays. The results of the LA-aPTT StaClot, LA-aPTT and LA-RVVT assays showed different distributions, although strong or moderate correlation was observed. **Conclusion:** LA-aPTT StaClot and aPS/PT IgG might be suitable for use in routine practice for patients with RPL. Each test for aPL should be ascertained for obstetric significance, because similar assays may have different outcomes. *Lupus* (2015) **24,** 1505–1519.

Key words: Antiphospholipid antibodies; lupus anticoagulant; phosphatidylserine-dependent antiprothrombin antibody; recurrent pregnancy loss

Introduction

Established causes of recurrent pregnancy loss (RPL) include presence of antiphospholipid antibodies (aPL), uterine anomalies and abnormal chromosomes, particularly translocations, in either partner.[1–5] Antiphospholipid syndrome (APS) is the most important treatable etiology. The reported live birth rate in women with APS treated with low-dose aspirin plus heparin is 70–80%.[6–10]

According to the international criteria, patients with a positive assay result for lupus anticoagulant (LA) by activated partial thromboplastin time (aPTT) and dilute Russel viper venom time (RVVT), anticardiolipin antibodies (aCL) or anti β2glycoprotein I (β2GPI) antibodies sustained for 12 weeks can be diagnosed as having APS.[11] Thus, we currently measure LA by 5× diluted aPTT established in our laboratory (LA-aPTT), commercially available LA-RVVT and β2GPI-dependent aCL (β2GPI aCL) as our standard aPL tests in daily clinical practice. The calculated prevalence of APS using these assays was only 4.5% in our previous study carried out in 1676 patients with RPL.[3]

The aforementioned prevalence estimated in our study was low as compared to the reported rate by a systematic review of 10–15%.[9,10] A meta-analysis concluded that treatment with unfractionated heparin plus aspirin conferred a significant benefit on the live birth rate. On the other hand, Laskin et al. concluded that there was no difference in the live birth rates between treatment with low-molecular-weight heparin plus aspirin (77.8%, 35/45) or aspirin alone (79.1%, 34/43) based on the detection of aPL, inherited thrombophilia and antinuclear antibodies.[12] The live birth rate in patients treated with aspirin alone was high as compared with the rates reported from Rai's or Kutteh's study.[7,8]

Correspondence to: Tamao Kitaori, Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medical Sciences, Mizuho-ku, Nagoya, 4678601, Japan.
Email: tama-og@med.nagoya-cu.ac.jp
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Since antinuclear antibodies have been considered to play no role in RPL,[13] this major discrepancy of the results among studies is speculated to be related to the methods used for detecting aPL. Each study group used different cutoff values of aCL immunoglobulin (Ig)G and IgM or different principles of assays for LA.[10] The poorly standardized aPL tests and widely varying populations investigated in each study make it difficult to interpret the results of meta-analysis through systematic reviews.

It is well known that the true antigens of aPL are not phospholipid, but phospholipid-binding plasma proteins such as β2GPI, prothrombin, kininogen, protein C and protein S.[14 16] Thus, there are a number of assays for aPL and aPL-related autoantibodies, and clinicians should be aware of how many and which tests should be performed for obtaining better outcomes of pregnancy in patients with RPL.

We found that β2GPI aCL was predictive of intrauterine fetal death (IUFD), intrauterine growth restriction and preeclampsia in 1125 normal pregnant women.[17] However, it is unclear whether β2GPI aCL causes recurrent early miscarriage, because sera were taken at eight to 10 weeks' gestation in the previous study. In a large proportion of cases of RPL, the miscarriage occurs early. We have established a method for the LA-aPTT assay and demonstrated that treatment could improve the live birth rate in patients with a history of RPL.[18] The later procedure is important before clinical use.

Many tests have been available for use in clinical practice. Few facilities measure aPL in their own laboratories in Japan, with most facilities sending their samples to some central laboratories. The obstetric significance of each assay is proven either "when treatment based on the results of the test improves the live birth rate" or "when the live birth rate in RPL patients with a positive test result is poorer than that in RPL patients with a negative test result."

Therefore, "clinically significant positive" or "threshold for treatment" may be discrepant from a "statistically significant result in the healthy population."

We therefore conducted the present study to determine "the obstetric significance" of 11 commercially available tests in patients with RPL.

Patients and methods

Patients

We studied 560 patients with a history of RPL (defined as two or more consecutive pregnancy losses). The mean (SD) age and number of previous pregnancy losses were 33.8 (4.4) and 2.69 (0.98), respectively.

Conventional examinations were completed in all the patients including hysterosalpingography, chromosomal analysis of both partners, our standard tests for aPL, including our in-house LA-aPTT, commercially available LA-RVVT and β2 GPI aCL (conventional aPL), and blood tests for hypothyroidism and diabetes mellitus, before a subsequent pregnancy.[18] Plasma for the test assay was taken at the same time as that for the conventional aPL assay and frozen at −70°C until use.

Patients with identifiable causes, such as uterine or chromosomal abnormalities in either partner, were excluded.

Study design

Subsequent pregnancies were established between April 2005 and May 2013 in Nagoya City University Hospital. APS was diagnosed according to the classification criteria for definite APS.[11] Patients with two spontaneous abortions were included although the APS criteria recommend three or more consecutive recurrent miscarriages. Patients with APS were treated with low-dose aspirin plus heparin as soon as possible after pregnancy was ascertained. Occasional aPL-positive cases were included as a separate group, based on our report that the live birth rate in these patients improved by treatment with low-dose aspirin alone.[19] Some patients with unexplained RPL were treated with low-dose aspirin and/or heparin based on the patients' will even after she has been informed that aspirin or heparin is reported, in general, to have no effect on the live birth rate in patients with unexplained recurrent miscarriage.[20]

Gestational age was calculated from the basal body temperature charts. Treatment was decided before the subsequent pregnancy and was started as soon as possible after the urine-derived human chorionic gonadotropin (u-hCG) test turned positive. Ultrasonography was performed once a week from four weeks to eight weeks of gestation. Dilation and curettage was performed on all patients diagnosed as having miscarriage. Part of the villi was cultured, and the cells were harvested after six to 22 days of cultivation for chromosomal analysis by G-banding.

We examined 11 assays for aPL, including LA-aPTT by StaClot, phosphatidylserine-dependent antiprothrombin (aPS/PT) assay for IgG, IgM,[21] classical aCL IgG, IgM assays by Harris's methods,[22] and aCL IgG, IgM, IgA and

anti-β2GPI IgG, IgM, IgA measured commercially by Phadia K. K. after the pregnancy outcome, to avoid selection bias.

Analysis

Primary analysis

We conducted multivariable logistic regression analysis after adjusting for age and number of previous miscarriages to compare the live birth rate between patients with positive test results treated and not treated with anticoagulant(s) and between patients with positive and negative test results not receiving any medication.

We also compared the live birth rate among three groups after excluding miscarriage caused by abnormal embryonic karyotype. First, we examined the 99th percentile value in healthy control women as the cutoff value. Then other cutoff values were also examined.

Furthermore, the obstetric significance of all assays was examined after excluding patients with at least one that tested positive on our conventional aPL.

Secondary analysis

We examined the frequency, sensitivity and specificity of the 11 assays for detecting APS diagnosed based on the conventional tests for aPL. The area under the curve (AUC) of receiver-operating characteristic (ROC) was analyzed in positive patients with and without medication. We examined the correlation coefficient (CC) among 11 tests and three conventional aPL. We examined the distribution of the results of the 11 tests for aPL and the conventional test results for aPL.

The analysis was carried out using the SPSS for Windows, version 22.0.

The study was conducted with the approval of the Research Ethics Committee at Nagoya City University Medical School.

Laboratory analysis

Conventional assays for the measurement of aPL

β2GPI-dependent aCL IgG assay. β2GPI aCL was measured using the Yamasa kit (Yamasa Corp., Choshi, Japan).[14,18,23] Test results for β2GPI-dependent and -independent aCL were considered positive when the antibody level was above the 99th percentile for 283 normal non-pregnant control sera. This was more than 1.9 units/ml for β2GPI-dependent aCL and more than 6.3 units/ml for β2GPI-independent aCL.[23] In addition, in order to avoid false-positives due to nonspecific binding, a β2GPI-dependent assay had to show a higher value than the β2GPI-independent assay performed in parallel, to be considered positive.

Assay for LA by the diluted aPTT method

LA was detected using the fivefold diluted aPTT method, as previously described, with brain cephalin (Platerin-Aauto; Kyowa Medex Co., Ltd, Japan) as the phospholipid reagent.[18] The 1:1 mixing test was performed at the same time. The clotting time was measured using a KC4 DELTA (Trinity Biotech Plc, Ireland). LA was considered positive when prolonged clotting times (>7.37 seconds) failed to correct after mixing 1:1 with standard plasma. It has been demonstrated for LA-aPTT that treatment based on the result of this test could improve the live birth rate in patients with a history of RPL.

Assay for LA with reference to the diluted RVVT

LA-RVVT was measured by an LA-RVVT kit (Gradipore Ltd, Pyrmont, Australia). LA-RVVT was considered positive when T1/T2 was over 1.3, the 99th percentile value in 98 healthy controls.

Test assays for the measurement of aPL

Assay for LA by aPTT using the StaClot kit

For measurement of the aPTT, a sensitive reagent with a low phospholipid concentration (PTT-LA test; Diagnostica Stago) was used for the screening and mixing tests, and the results were confirmed with the use of the StaClot LA kit (Diagnostica Stago). The cutoff level for a positive LA result was 1.59, over the previously established 99th percentile value in 40 healthy individuals. Cutoff values of 1.0, the 98th percentile value, and 6.3, the value recommended by Roche, were also examined.

Assay for aPS/PT IgG and IgM

The aPS/PT enzyme-linked immunosorbent assay (ELISA) procedure has been described in detail previously.[21] Briefly, phosphatidylserine was immobilized on plain polystyrene plates. After blocking, 10 μg/ml of purified prothrombin (Diagnostica Stago, Asnieres, France) was added in the presence of 5 mmol/l calcium chloride ($CaCl_2$). IgG/M bound to phosphatidylserine-prothrombin complex was detected by the standard ELISA procedure. Cutoff levels were set at 1.2 and 5.2 (99th percentile), established with sera obtained from 132 healthy volunteers. In addition, to avoid false positives due to nonspecific binding, the prothrombin-dependent assay had to show a higher value

than the prothrombin-independent assay performed in parallel, or vice versa, to be considered positive.

Classical assay for aCL IgG and IgM established by Harris

aCL IgG and IgM were assayed according to a standard aCL ELISA.[22] The cutoff levels were previously established at 19.2 and 23.4, based on the 99th percentile value in 132 healthy controls.

Assay for aCL IgG, IgM and IgA by Phadia K. K.

aCL IgG, IgM and IgA were measured by Phadia K.K. using the EliA Cardiolipin IgG, IgM or IgA commercial kit (Phadia GmbH, now part of Thermo Fisher Scientific). The results of aCL IgG, IgM and IgA were considered to be weakly positive by Phadia when the antibody levels were above 10 (97.7th percentile 400 normal controls), 10 (92.8th percentile) and 14 (98.2th percentile), respectively. The 99th percentile cutoff points were 23.8, 29.9 and 17.1, respectively. This procedure was performed by Phadia K. K.

Assay for anti-β2GPI IgG, IgM and IgA by Phadia K. K.

β2GPI IgG, IgM and IgA were measured by Phadia K.K. using the EliA β2-Glycoprotein I IgG, IgM or IgA kit. The results of these assays were considered to be weakly positive when the antibody titers were over 7 (92.0th percentile for 400 normal controls), 7 (99.1th percentile) and 7 (98.2th percentile), respectively. The 99th percentile cutoff points were 17.7, 5.7 and 8.7, respectively. This procedure was performed by Phadia K. K.

Results

The subsequent live birth rate was 68.8% (385/560). The frequency at which aPL were detected by the conventional tests β2GPI aCL, LA-aPTT and LA-RVVT assays were 4.6%, 6.8% and 3.4% (Table 1). The test results remained positive for more than 12 weeks in 88.5% (23/26) of patients with β2GPI aCL, 68.4% (26/38) of patients with LA-aPTT and 89.5% (17/19) of patients with LA-RVVT. Thus, 6.8% (38/560) of the participants were diagnosed as having APS according to the international criteria by the conventional tests for aPL (Figure 1). Twelve patients tested positive by all of the three assays.

The frequency of aPL detected by the LA-aPTT StaClot, aPS/PT IgG and IgM, classical aCL IgG and IgM, aCL IgG, IgM and IgA and anti- β2GPI IgG, IgM and IgA Phadia assays using 99th percentile value in healthy controls as the cutoff values were 6.1%, 4.5%, 0.7%, 2.1%, 0%, 5.9%, 1.4%, 2.1%, 2.0%, 2.9% and 3.6% (Table 1). The specificity of all the assays for APS was greater than 95%.

CC

The CCs of the results among all assays are shown in Table 2. Strong correlations were observed between β2GPI aCL and β2GPI IgG, between β2GPI IgG and classical CL IgG, between LA-aPTT and LA-aPTT StaClot, between LA-aPTT StaClot and LA-RVVT, and between CL IgA and β2GPI IgA. Moderate correlations were observed between β2GPI aCL and classical CL IgG, between CL IgG and β2GPI IgG, and between LA-aPTT and LA-RVVT.

Distribution of the results of the assays

The distribution in each of the assays and the three conventional aPL tests using the 99th percentile value in healthy controls as cutoff and also for other cutoff values are shown in Figure 1, because Phadia recommends lower cutoff values of 10 (97.7 percentile of controls), 10 (92.8), 14 (99.7), 7 (92.4), 7 (99.1) and 7 (98.2) for CL IgG, IgM and IgA, and β2GPI IgG, IgM and IgA, and StaClot recommends use of a cutoff value of 6.3.

Obstetric significance of the test assays

In regard to LA-aPTT StaClot, almost all cases were included in the results of the conventional aPL tests, when the cutoff of 6.3 recommended by StaClot was used (Figure 1). LA-aPTT StaClot, LA-aPTT and LA-RVVT were positive in different patients, although strong correlation was observed. There were many single positive cases identified when the 98th or 99th percentile values were used as cutoff.

Treatment tended to improve the live birth rate when administered based on the test result obtained using the 99th percentile value as the cutoff value, after excluding cases with abnormal embryonic karyotype ($p = 0.14$, Table 3). This tendency increased when the 98th percentile value was used as the cutoff ($p = 0.04$). LA-aPTT StaClot was found to have clinical significance in patients with RPL. Thus, a cutoff value of the 98th percentile was more appropriate for LA-aPTT StaClot. Patients with positive results on LA-aPTT StaClot were distinct from patients with positive results for LA-aPTT (Figure 1). Thus, both tests should be selected for clinical practice.

Table 1 The frequency, the sensitivity and specificity for APS, and the area under the curve of the ROC for each assay

Assays cutoff (percentile of healthy controls)		*Frequency %*	*Sensitivity %*	*Specificity %*	*AUC of ROC (95% CI) for live birth rate*	*AUC of ROC after excluding patients with + abnormal EK*[a]
Conventional aPL	β2GPI aCL >1.9 (99)	4.6	**60.5**	**99.4**	**0.56 (0.31–0.82)**	**0.58 (0.30–0.87)**
	LA-aPTT >7.4 (99)	6.8	**63.2**	**97.7**	**0.55 (0.33–0.77)**	**0.58 (0.31–0.86)**
	LA-RVVT >1.3 (99)	3.4	**42.1**	**99.6**	**0.67 (0.27–1.00)**	**0.75 (0.30–1.00)**
LA-aPTT StaClot >1.0 (98)		8.6	47.4	94.0	**0.66 (0.49–0.82)**	**0.71 (0.54–0.88)**
>1.59 (99)		6.1	42.1	96.4	**0.64 (0.44–0.85)**	**0.69 (0.45–0.94)**
aPS/PT IgG >1.2 (99)		4.5	23.7	96.8	**0.62 (0.39–0.86)**	**0.70 (0.46–0.94)**
aPS/PT IgM >5.2 (99)		0.7	10.5	100	–	–
Classical CL IgG >19.2 (99)		2.1	28.9	99.8	**0.70 (0.37–1.00)**	**0.83 (0.49–1.00)**
Classical CL IgM >23.4 (99)		0	0	100	–	–
CL IgG >10 (97.7)		14.1	44.7	88.1	**0.42 (0.29–0.56)**	**0.41 (0.25–0.57)**
>23.8 (99)		5.9	26.3	95.6	**0.52 (0.31–0.74)**	**0.52 (0.29–0.75)**
CL IgM >10 (92.8)		9.8	26.3	91.4	**0.56 (0.40–0.72)**	**0.53 (0.33–0.74)**
>29.9 (99)		1.4	10.5	99.2	**0.71 (0.27–1.00)**	**0.71 (0.27–1.00)**
CL IgA >14 (98.2)		2.9	18.4	98.3	**0.50 (0.19–0.82)**	**0.50 (0.19–0.82)**
>17.1 (99)		2.1	18.4	99.0	**0.69 (0.34–1.00)**	**0.69 (0.34–1.00)**
β2GPI IgG >7 (92.0)		4.1	36.8	98.3	**0.53 (0.27–0.78)**	**0.54 (0.28–0.81)**
>17.7 (99)		2.0	21.1	99.4	**0.52 (0.16–0.88)**	**0.54 (0.16–0.93)**
β2GPI IgM >7 (99.1)		2.3	21.1	99.0	**0.58 (0.23–0.94)**	**0.83 (0.58–1.00)**
>5.7 (99)		2.9	21.1	98.5	**0.58 (0.25–0.92)**	**0.83 (0.61–1.00)**
β2GPI IgA >7 (98.2)		3.8	31.6	98.3	**0.71 (0.42–0.99)**	**0.57 (0.20–0.95)**
>8.7 (99)		3.6	31.6	98.5	**0.73 (0.45–1.00)**	**0.60 (0.22–0.99)**

[a]EK: embryonic (fetal) karyotype. APS: antiphospholipid syndrome; ROC: receiver-operating characteristic; aPL: antiphospholipid antibodies; β2GPI: β2glycoprotein I; aCL: anticardiolipin antibodies; LA-aPTT: lupus anticoagulant-activated partial thromboplastin time; LA-RVVT: lupus anticoagulant-Russel viper venom time; aPS/PT: phosphatidylserine-dependent antiprothrombin; Ig: immunoglobulin; CL: cardiolipin; AUC: area under the curve; CI: confidence interval.

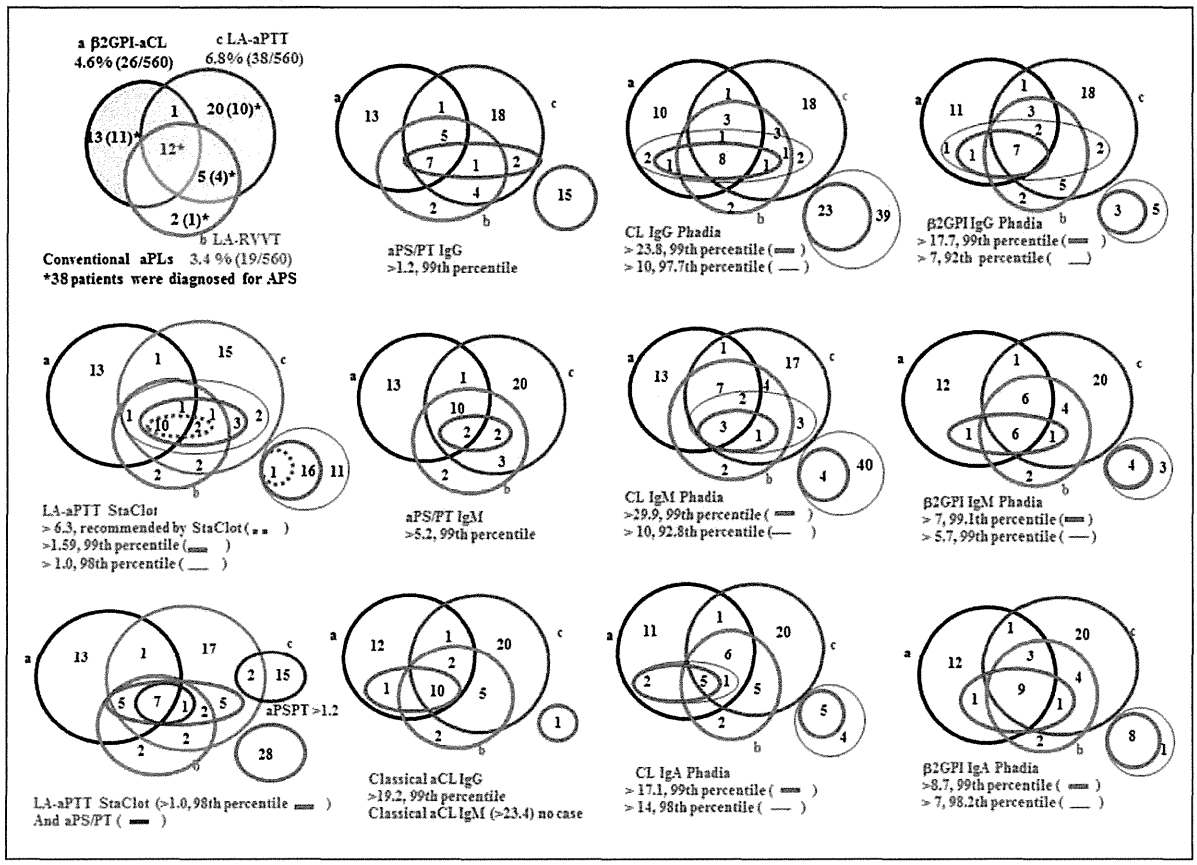


Figure 1 The distribution in each of the assays (red) and the three conventional aPL tests, β2glycoprotein I-dependent anticardiolipin antibody (β2GPI aCL, (a) black), lupus anticoagulant-RVVT (LA-RVVT, (b) green) and LA-aPTT in-house ((c) blue) using the 99th percentile value in healthy controls as cutoff and also other cutoff values as shown in Figure 1. The distribution in tested assays with 99th percentile values is shown in bold red and that in tested assays with other cutoff values is shown in light red. The distribution in both LA-aPTT StaClot (red) differed from that in aPS/PT IgG (purple).
aPL: antiphospholipid antibodies; RVVT: Russel viper venom time; aPTT: activated partial thromboplastin time; aPS/PT: phosphatidylserine-dependent antiprothrombin; IgG: immunoglobulin G.

In regard to the aPS/PT IgG, the live birth rate of patients with a positive result who received no medication was inferior to that of patients with a negative result, after excluding patients with miscarriage caused by abnormal embryonic karyotype ($p = 0.02$). There were 15 single-positive cases, although 10 patients had both aPS/PT IgG and LA-aPTT (Figure 1). Thus, aPS/PT IgG should be considered to be used in clinical practice.

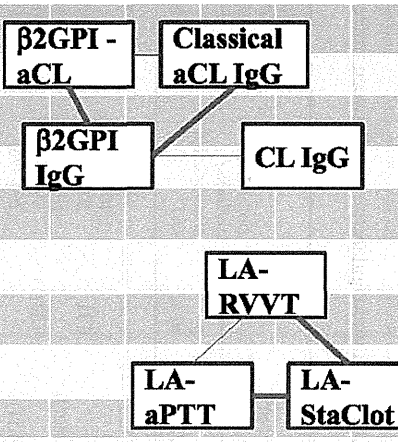
In regard to aPS/PT IgM, the specificity for APS was 100% and the test must have clinical significance. However, aPS/PT IgM assay is not necessary for clinical practice, because all four cases were included in the patients with positive results on LA-aPTT and/or LA-RVVT.

Almost all cases with classical aCL IgG were included in the cases testing positive for conventional aPL and the specificity was 99.8%. Classical aCL IgG must have clinical significance, although its obstetric significance was not ascertained statistically (Table 3). There was no patient with classical aCL IgM. Classical aCL IgM assay is not necessary in the testing of women with RPL, because none of the 560 patients showed a positive result.

In regard to aCL IgG determined using cutoff values of both 10 and 23.8, there were many single-positive cases (Figure 1). Treatment could not improve the live birth rate in any of the positive patients (Table 3). There were also 44 single-positive cases of aCL IgM determined using a cutoff of 10. Treatment could not improve the live birth rate significantly in these cases either. Thus aCL IgG was ascertained to have no obstetric significance, although the significance of aCL IgM could not be ascertained using the cutoff of 29.9 (99th percentile) because of the small sample size.

Table 2 Correlation coefficients among the results for each antiphospholipid antibody

	β2GPI aCL	LA-aPTT	LA-RVVT	StaClot	aPS/PT IgG	aPS/PT IgM	Classical CL IgG	Classical CL IgM	CL IgG	CL IgM	CL IgA	β2GPI IgG	β2GPI IgM	β2GPI IgA
β2GPI aCL	1													
LA-APTT	0.61	1												
LA-RVVT	0.62	0.71	1											
StaClot	0.58	0.81	0.80	1										
aPS/PT IgG	0.38	0.42	0.41	0.52	1									
aPS/PT IgM	0.19	0.42	0.53	0.61	0.14	1								
Classical CL IgG	0.75	0.43	0.36	0.35	0.24	0.16	1							
Classical CL IgM	0.39	0.19	0.23	0.20	0.09	0.10	0.26	1						
CL IgG	0.68	0.48	0.45	0.40	0.25	0.11	0.63	0.29	1					
CL IgM	0.56	0.41	0.52	0.45	0.27	0.17	-	0.44	0.44	1				
CL IgA	0.39	0.47	0.31	0.41	0.10	-	0.37	0.15	0.32	0.21	1			
β2GPI IgG	0.84	0.58	0.50	0.52	0.36	0.21	0.82	0.24	0.73	0.25	0.33	1		
β2GPI IgM	0.54	0.46	0.56	0.49	0.28	0.33	0.20	0.36	0.39	0.70	0.19	0.36	1	
β2GPI IgA	0.24	0.41	0.27	0.39	0.17	0.12	0.20	-	0.15	0.11	0.82	0.22	0.24	1



All correlation were significant.
Moderate or strong correlations were shown in red.

We could not determine the obstetric significance of CL IgA, β2GPI IgG, IgM and IgA because of the small number of single-positive cases. However, they might not be necessary as the tests of first choice, because there were only a few single-positive cases among the 560 patients.

A similar tendency was observed after excluding 53 cases that tested positive with our conventional aPL (Table 4). However, the difference ceased being significant because of the small sample size.

The AUCs of the ROC for LA-aPTT and aPS/PT were 0.71 and 0.70 with no significance, respectively (Table 1). The AUCs were very small even though the assays had obstetric significance.

Discussion

The present study demonstrated the obstetric significance of LA-aPTT StaClot when the 98th percentile value in healthy controls was used as the cutoff, and aPS/PT IgG. The number of controls used for establishing the LA-aPTT StatClot assay was relatively small, and represents a major limitation. However, cutoff (threshold) setting for daily clinical practice is possible after the obstetric significance of each assay is ascertained, that is, treatment based on a positive result could improve the live birth rate.

The results of similar assays, LA-aPTT and LA-aPTT StaClot, showed different distributions, although a strong correlation was observed (Figure 1). The reason could be the differences in the reagents used in the two assays. Our previous cohort study indicated that anticoagulant therapy improved the live birth rate from 46.2% to 80.4% in LA-aPTT-positive patients.[18] Thus, both assays should be used in the management of RPL patients in daily clinical practice.

The association between aPS/PT and RPL is well-known.[24] However, there has been only one prospective study in which the pregnancy outcome

Table 3 Comparison of the live birth rate between patients with a positive test results treated and not treated with anticoagulant(s), and between untreated patients with positive and negative test results each test

		Live birth rate % (n)	*Crude analysis*		*Multivariable logistic regression*		*Live birth rate excluding abnormal EK % (n)*	*Multivariable logistic regression*	
			OR (95% CI)	p *value*	*OR (95% CI)*	p *value*		*OR (95% CI)*	p *value*
StaClot >1.59	Positive no treatment	58.8% (10/17)	Reference		Reference		71.4% (10/14)	Reference	
	Positive treatment	82.4% (14/17)	3.27 (0.68–15.82)	0.14	3.65 (0.74–18.04)	0.11	93.3% (14/15)	5.97 (0.57–62.66)	0.14
	Negative	70.7% (261/368)	1.71 (0.63–4.60)	0.29	1.76 (0.64–4.81)	0.27	79.5% (260/326)	1.61 (0.49–5.37)	0.44
StaClot >1.0	Positive no treatment	59.3% (16/27)	Reference		Reference		66.7% (16/24)	Reference	
	Positive treatment	85.7% (18/21)	4.13 (0.97–17.47)	0.05	4.93 (1.14–21.26)	0.03	94.7% (18/19)	10.26 (1.14–92.42)	0.04
	Negative	70.9% (254/357)	1.70 (0.76–3.78)	0.20	1.86 (0.82–4.19)	0.14	80.1% (254/316)	2.20 (0.89–5.44)	0.09
aPS/PT IgG >1.2	Positive no treatment	50% (5/10)	Reference		Reference		50.0% (5/10)	Reference	
	Positive treatment	73.3% (11/15)	2.75 (0.51–14.86)	0.24	3.20 (0.56–18.31)	0.19	84.6% (11/13)	5.65 (0.76–41.79)	0.09
	Negative	71.2% (264/371)	2.44 (0.69–8.62)	0.16	2.8 (0.76–10.29)	0.12	80.7% (264/327)	4.74 (1.28–17.52)	0.02
aPS/PT IgG >1.0	Positive no treatment	54.5% (6/11)	Reference		Reference		54.5% (6/11)	Reference	
	Positive treatment	72.2% (13/18)	2.17 (0.45–10.44)	0.34	2.41 (0.48–12.13)	0.29	81.3% (13/16)	3.67 (0.63–21.39)	0.15
	Negative	71.1% (263/370)	2.05 (0.61–6.86)	0.25	2.25 (0.65–7.75)	0.20	80.7% (263/326)	3.77 (1.09–13.09)	0.04
aPS/PT IgM >5.2	Positive no treatment	0% (0/0)	Reference		Reference		0% (0/0)	Reference	
	Positive treatment	100% (4/4)	–	–	–	–	100% (4/4)	–	–
	Negative	70.6% (269/381)	–	–	–	–	79.8% (269/337)	–	–
Classical CL IgG >19.2	Positive no treatment	0% (0/2)	–		Reference		0% (0/2)	Reference	
	Positive treatment	77.8% (7/10)	–	–	–	–	87.5% (7/8)	–	–
	Negative	70.8% (267/377)	–	–	–	–	80.2% (267/333)	–	–

(contined)

Table 3 Contined

		Live birth rate % (n)	*Crude analysis*		*Multivariable logistic regression*		*Live birth rate excluding abnormal EK % (n)*	*Multivariable logistic regression*	
			OR (95% CI)	p *value*	*OR (95% CI)*	p *value*		*OR (95% CI)*	p *value*
CL IgG >23.8	Positive No treatment	64.3% (9/14)	Reference		Reference		69.2% (9/13)	Reference	
	Positive treatment	68.4% (13/19)	1.20 (0.28–5.18)	0.80	1.41 (0.32–6.33)	0.65	72.2% (13/18)	1.40 (0.28–7.08)	0.69
	Negative	70.6% (274/388)	1.34 (0.44–4.07)	0.61	1.43 (0.46–4.46)	0.54	80.4% (274/341)	1.92 (0.56–6.58)	0.30
CL IgG >10	Positive no treatment	74.4% (32/43)	Reference		Reference		82.1% (32/39)	Reference	
	Positive treatment	61.1% (22/36)	0.54 (0.21–1.41)	0.21	0.68 (0.25–1.81)	0.43	68.8% (22/32)	0.60 (0.19–1.87)	0.37
	negative	69.9% (251/359)	0.80 (0.39–1.64)	0.54	0.86 (0.41–1.78)	0.68	79.7% (251/315)	0.92 (0.38–2.19)	0.84
CL IgM >29.9	Positive No treatment	80% (4/5)	Reference		Reference		80% (4/5)	Reference	
	Positive treatment	100% (3/3)	–	–	–	–	100% (3/3)	–	–
	Negative	70.3% (279/397)	0.59 (0.07–5.35)	0.64	0.59 (0.07–5.35)	0.63	80.7% (279/349)	1.02 (0.11–9.54)	0.98
CL IgM >10	Positive no treatment	65.9% (27/41)	Reference		Reference		79.4% (27/34)	Reference	
	Positive treatment	78.6% (11/14)	1.90 (0.46–7.95)	0.38	2.00 (0.47–8.49)	0.35	84.6% (11/13)	1.41 (0.25–7.99)	0.70
	Negative	70.9% (256/361)	1.26 (0.64–2.51)	0.50	1.23 (0.62–2.47)	0.55	80.0% (256/320)	1.03 (0.42–2.48)	0.96
CL IgA >17.1	Positive no treatment	33.3% (1/3)	Reference		Reference		33.3% (1/3)	Reference	
	Positive treatment	77.8% (7/9)	7.00 (0.40–123.35)	0.18	13.18 (0.67–258.07)	0.09	77.8% (7/9)	12.06 (0.61–239.6)	0.10
	Negative	70.6% (281/398)	4.80 (0.43–53.49)	0.20	8.40 (0.68–103.54)	0.10	80.3% (281/350)	13.58 (1.08–170.94)	0.04
CL IgA >14	Positive no treatment	50.0% (2/4)	Reference		Reference		50.0% (2/4)	Reference	
	Positive treatment	75.0% (9/12)	–	–	1.12 (0.37–3.37)	0.85	75.0% (9/12)	1.91 (0.62–5.82)	0.26
	Negative	70.5% (280/397)	–	–	–	–	80.2% (267/333)	–	–

(continued)

Table 3 Contined

		Live birth rate % (n)	*Crude analysis*		*Multivariable logistic regression*		*Live birth rate excluding abnormal EK % (n)*	*Multivariable logistic regression*	
			OR (95% CI)	p *value*	*OR (95% CI)*	p *value*		*OR (95% CI)*	p *value*
β2GPI IgG >17.7	Positive no treatment	50.0% (1/2)	Reference		Reference		50.0% (1/2)	Reference	
	Positive treatment	55.6% (5/9)	1.25 (0.06–26.87)	0.89	1.94 (0.08–45.49)	0.68	62.5% (5/8)	3.10 (0.12–81.52)	0.50
	Negative	70.5% (282/400)	2.39 (0.15–38.53)	0.54	4.08 (0.23–72.08)	0.34	80.1% (282/352)	8.12 (0.44–150.50)	0.16
β2GPI IgG >7	Positive No treatment	60.0% (3/5)	Reference		Reference		60.0% (3/5)	Reference	
	Positive treatment	66.7% (12/18)	1.33 (0.17–10.25)	0.78	1.78 (0.22–14.23)	0.59	70.6% (12/17)	2.15 (0.26–18.09)	0.48
	Negative	70.5% (280/397)	1.60 (0.26–9.67)	0.61	2.11 (0.34–13.23)	0.43	80.2% (280/349)	3.68 (0.57–23.66)	0.17
β2GPI IgM >5.7	Positive no treatment	66.7% (4/6)	Reference		Reference		66.7% (4/6)	Reference	
	Positive treatment	80.0% (8/10)	2.00 (0.20–19.91)	0.55	1.77 (0.17–18.69)	0.64	100.0% (8/8)	–	0.63
	Negative	70.4% (278/395)	1.19 (0.22–6.58)	0.84	1.01 (0.17–5.83)	0.99	80.1% (278/347)	1.70 (0.29–9.97)	0.56
β2GPI IgM >7	Positive no treatment	60.0% (3/5)	Reference		Reference		60.0% (3/5)	Reference	
	Positive treatment	75.0% (6/8)	2.00 (0.18–22.06)	0.57	1.96 (0.17–22.72)	0.59	100.0% (6/6)	–	0.65
	Negative	70.5% (279/396)	1.59 (0.26–9.64)	0.61	1.63 (0.26–10.25)	0.60	80.2% (279/348)	2.77 (0.44–17.68)	0.28
β2GPI IgA >8.7	Positive no treatment	40.0% (2/5)	Reference		Reference		66.7% (2/3)	Reference	
	Positive treatment	86.7% (13/15)	9.75(0.95–99.96)	0.06	10.65 (0.98–115.21)	0.05	86.7% (13/15)	3.39 (0.18–62.46)	0.41
	Negative	70.7% (280/396)	3.62 (0.59–21.95)	0.16	3.86 (0.60–25.09)	0.16	80.0% (280/350)	2.11 (0.17–26.23)	0.56
β2GPI IgA >7	Positive no treatment	50.0% (3/6)	Reference		Reference		75.0% (3/4)	Reference	
	Positive treatment	86.7% (13/15)	6.50 (0.73–57.83)	0.09	6.94 (0.75–64.08)	0.09	86.7% (13/15)	2.19 (0.14–34.49)	0.56
	Negative	70.6% (279/395)	2.41(0.48–12.09)	0.29	2.50 (0.48–13.14)	0.28	79.9% (279/349)	1.36 (0.13–13.93)	0.80

aPL: antiphospholipid antibodies; β2GPI: β2glycoprotein I; aCL: anticardiolipin antibodies; LA-aPTT: lupus anticoagulant-activated partial thromboplastin time; LA-RVVT: lupus anticoagulant-Russel viper venom time; aPS/PT: phosphatidylserine-dependent antiprothrombin; Ig: immunoglobulin; CL: cardiolipin; EK: embryonic (fetal) karyotype; OR: odds ratio; CI: confidence interval.

Table 4 Comparison of the live birth rate between patients with a positive test results treated and not treated with anticoagulant(s), and between untreated patients with positive and negative test results each test after excluding 53 patients test with our conventional aPL

		Live birth rate % (n)	*Crude analysis*		*Multivariable logistic regression*		*Live birth rate excluding abnormal EK % (n)*	*Multivariable logistic regression*	
			OR (95% CI)	p *value*	*OR (95% CI)*	p *value*		*OR (95% CI)*	p *value*
StaClot >1.59	Positive no treatment	71.4% (10/14)	Reference		Reference		83.3% (10/12)	Reference	
	Positive treatment	100% (3/3)	–		–		100% (3/3)	–	
	Negative	70.9% (261/368)	0.98 (0.30–3.18)	0.99	0.97 (0.30–3.21)	0.97	79.8% (261/327)	0.75 (0.16–3.55)	0.75
StaClot >1.0	Positive no treatment	66.7% (16/24)	Reference		Reference		72.7% (16/22)	Reference	
	Positive treatment	100% (4/4)	–		–		100% (4/4)	–	
	Negative	70.9% (254/357)	1.23 (0.51–2.97)	0.64	1.32 (0.54–3.21)	0.55	80.1% (254/316)	1.57 (0.58–4.23)	0.37
aPS/PT IgG >1.2	Positive No treatment	55.6% (5/9)	Reference		Reference		55.6% (5/9)	Reference	
	Positive treatment	50% (3/6)	0.8 (0.10-6.35)	0.83	1.14 (0.14-9.52)	0.90	60.0% (3/5)	1.65 (0.17-16.10)	0.67
	Negative	71.4% (264/370)	1.99 (0.53–7.56)	0.31	2.04 (0.52–8.02)	0.31	81.0% (264/326)	3.45 (0.88–13.55)	0.076
aPS/PT IgG >1.0	Positive No treatment	60.0% (6/10)	Reference		Reference		60.0% (6/10)	Reference	
	Positive treatment	57.1% (4/7)	0.89 (0.13–6.31)	0.91	1.28 (0.17–9.45)	0.81	66.7% (4/6)	1.84 (0.21–16.04)	0.58
	Negative	71.5% (263/368)	1.67 (0.46–6.04)	0.43	1.68 (0.45–6.23)	0.44	80.9% (263/325)	2.81 (0.75–10.45)	0.124
aPS/PT IgM >5.2	Positive no treatment	0% (0/0)	Reference		Reference		0% (0/0)	Reference	
	Positive treatment	0% (0/0)	–	–	–	–	0% (0/0)	–	–
	Negative	71.2% (269/378)	–	–	–	–	80.3% (269/335)	–	–
Classical CL IgG >19.2	Positive No treatment	0% (0/1)	Reference		Reference		0% (0/1)	Reference	
	Positive treatment	0% (0/0)	–	–	–	–	0% (0/0)	–	–
	Negative	71.2% (267/375)	–	–	–	–	80.4% (267/332)	–	–

(contined)

Table 4 Contined

		Live birth rate % (n)	*Crude analysis*		*Multivariable logistic regression*		*Live birth rate excluding abnormal EK %* (n)	*Multivariable logistic regression*	
			OR (95% CI)	p *value*	*OR (95% CI)*	p *value*		*OR (95% CI)*	p *value*
CL IgG >23.8	Positive no treatment	69.2% (9/13)	Reference		Reference		75.0% (9/12)	Reference	
	Positive treatment	60.0% (6/10)	0.67 (0.12–3.76)	0.65	1.02 (0.17–6.17)	0.98	60.0% (6/10)	0.83 (0.12–5.59)	0.85
	Negative	71.0% (274/386)	1.09 (0.33–3.60)	0.89	1.09 (0.32–3.69)	0.89	80.6% (274/340)	1.35 (0.35–5.22)	0.67
CL IgG >10	Positive no treatment	76.2% (32/42)	Reference		Reference		84.2% (32/38)	Reference	
	Positive treatment	45.0% (9/20)	0.26 (0.08–0.79)	0.018	0.37 (0.12–1.21)	0.10	50.0% (9/18)	0.28 (0.08–1.07)	0.06
	Negative	70.3% (251/357)	0.74 (0.35–1.56)	0.43	0.78 (0.37–1.66)	0.52	79.9% (251/314)	0.78 (0.31–1.97)	0.56
CL IgM >29.9	Positive no treatment	100% (4/4)	Reference		Reference		100% (4/4)	Reference	
	Positive treatment	0% (0/0)	-		–		0% (0/0)	-	
	Negative	70.6% (279/395)	–		–		80.2% (279/348)	-	
CL IgM >10	Positive no treatment	67.5% (27/40)	Reference		Reference		81.8% (27/33)	Reference	
	Positive treatment	80.0% (4/5)	1.93 (0.20–19.0)	0.58	2.04 (0.20–20.4)	0.55	80.0% (4/5)	0.95 (0.09–10.23)	0.97
	Negative	71.3% (256/359)	1.20 (0.59–2.41)	0.62	1.14 (0.56–2.33)	0.71	80.3% (256/319)	0.87 (0.34–2.22)	0.77
CL IgA >17.1	Positive no treatment	50.0% (1/2)	Reference		Reference		50.0% (1/2)	Reference	
	Positive treatment	66.7% (2/3)	2.00 (0.05–78.25)	0.71	3.60 (0.08–169.48)	0.52	66.7% (2/3)	3.16 (0.07–153.05)	0.56
	Negative	71.0% (281/396)	2.44 (0.15–39.40)	0.53	3.34 (0.17–66.54)	0.43	80.5% (281/349)	4.98 (0.24–102.40)	0.30
CL IgA >14	Positive no treatment	0% (0/0)	Reference		Reference		0% (0/0)	Reference	
	Positive treatment	66.7% (6/9)	1.22 (0.30–4.95)	0.78	1.05 (0.25–4.52)	0.94	66.7% (6/9)	1.67 (0.38–7.36)	0.50
	Negative	70.9% (280/395)	-		–		80.5% (280/348)	–	
β2GPI IgG >17.7	Positive no treatment	100% (1/1)	Reference		Reference		100% (1/1)	Reference	
	Positive treatment	0% (0/2)	0 (0–)	1.00	0 (0–)	1.00	0% (0/2)	0 (0–)	1.00
	Negative	70.9% (282/398)	0 (0–)	1.00	0 (0–)	1.00	79.7% (282/354)	0 (0–)	1.00
β2GPI IgG >7	Positive no treatment	75.0% (3/4)	Reference		Reference		75.0% (3/4)	Reference	
	Positive treatment	50.0% (2/4)	0.33 (0.17–6.65)	0.47	0.43 (0.02-8.98)	0.59	50.0% (2/4)	0.47 (0.02–9.97)	0.63
	Negative	70.9% (280/395)	0.81 (0.08–7.88)	0.86	0.93 (0.09-9.17)	0.95	80.5% (280/348)	1.55 (0.16–15.58)	0.71
β2GPI IgM >5.7	Positive no treatment	80.0% (4/5)	Reference		Reference		80.0% (4/5)	Reference	
	Positive treatment	66.7% (2/3	0.50 (0.02–12.90)	0.68	0.41 (0.02–11.01)	0.59	100.0% (2/2)	-	
	Negative	70.7% (278/393)	0.60 (0.07–5.47)	0.65	0.41 (0.04–3.83)	0.43	80.3% (278/346)	0.65 (0.07–6.30)	0.71
β2GPI IgM >7	Positive no treatment	75.0% (3/4)	Reference		Reference		75.0% (3/4)	Reference	
	Positive treatment	0% (0/1)	0 (0-)	1.0	0 (0–)	1.0	0% (0/0)	1.12 (0.11–11.31)	0.92
	Negative	70.8% (279/394)	0.81 (0.08–7.86)	0.86	0.68 (0.07–6.77)	0.74	80.4% (279/347)	–	
β2GPI IgA >8.7	Positive no treatment	50.0% (2/4)	Reference		Reference		100% (2/2)	Reference	
	Positive treatment	75.0% (3/4)	3.00 (0.15–59.89)	0.47	3.91 (0.18–85.15)	0.39	75.0% (3/4)	0 (0–)	
	Negative	71.1% (280/394)	2.46 (0.34–17.65)	0.37	2.15 (0.29–15.86)	0.45	80.2% (280/349	0 (0–)	
β2GPI IgA >7	Positive no treatment	50.0% (3/6)	Reference		Reference		100% (3/3)	Reference	
	Positive treatment	86.7% (13/15)	2.00 (0.11–35.81)	0.64	2.66 (0.14–51.46)	0.52	75.0% (3/4)	0 (0–)	
	Negative	70.6% (279/395)	1.63 (0.27–9.89)	0.59	1.47 (0.24–9.05)	0.68	80.2% (279/348)	0 (0–)	

aPL: antiphospholipid antibodies; β2GPI: β2glycoprotein I; aCL: anticardiolipin antibodies; LA-aPTT: lupus anticoagulant-activated partial thromboplastin time; LA-RVVT: lupus anticoagulant-Russel viper venom time; aPS/PT: phosphatidylserine-dependent antiprothrombin; Ig: immunoglobulin; CL: cardiolipin; EK: embryonic (fetal) karyotype; OR: odds ratio; CI: confidence interval.

was examined and that was by us. Our previous study determined that the prevalence of aPS/PT IgG was 1% and a positive case was included in LA-aPTT-positive cases.[25] Thus, we concluded aPS/PT IgG was not useful in RPL practice.

The present study showed a higher prevalence of aPS/PT (4.5%). The current assay system is not different from that in the reference, but some years ago we adjusted the cutoff of aPS/PT for daily clinical practice using the 99th percentile of a large number of a healthy sample. Previous borderline negative samples would have become positive, explaining in part the difference of prevalence between previous and current studies.

The usefulness of the β2GPI aCL, aCL IgG, antiphosphatidylethanolamine (aPE) IgG and LA-aPTT StaClot or LA-RVVT assays to predict intrauterine fetal death, intrauterine growth restriction and preeclampsia in healthy pregnant women has been demonstrated.[17,26,27] However, it is unclear whether these assays cause recurrent early miscarriage, because sera were taken at eight to 10 weeks' gestation in the previous study. In a large proportion of cases of RPL, the miscarriage occurs early. In contrast, our previous cohort study showed that the distribution of aPE IgG was distinct from that of conventional aPL and that treatment could not improve the live birth rate in the patients with a single positive test result.[28] The assay for aPE IgG, which is commercially available in Japan, and the prevalence of aPE IgG and IgM, which was relatively high, being about 20%, have no obstetric significance.[29] However, the obstetric significance of the aPE assay established by Sanmarco et al. is unclear because similar assays with a strong correlation were found to show different distributions in the present study.[30]

A strong correlation was observed among the results of the assays for β2GPI aCL, classical CL IgG and β2GPI IgG. CL IgG correlated only moderately with β2GPI IgG, and the distribution of CL IgG differed from that of classical CL IgG (Figure 1). No obstetric significance of CL IgG was found in the present study, although they are included in the classification criteria. This was in line with the previous study.[29,30] The PROMISS study concluded that LA, but not classical aCL, predicted adverse pregnancy outcomes.[31] Harris et al. confirmed that classical CL IgG and IgM were rarely associated with adverse pregnancy outcomes.[32]

In all β2GPI-dependent aCL assays, we defined APS-related-aCL-positive by confirming that the aCL really directed to β2GPI bound to CL. Actually, only one case was excluded due to nonspecific binding to CL in the absence of β2GPI. Therefore, our results would be comparable with the others.

The obstetric significance of CL IgA and β2GPI IgG, IgM and IgA could also not be ascertained in the present study, because only a few cases had each of these antibodies in the absence of others. These assays might be unnecessary in clinical practice in the management of RPL, because the positive cases can be covered by the conventional tests for aPL.

The clinical efficacy of each assay was determined by the sensitivity, specificity, odds ratio, and AUC for thrombotic manifestations of APS.[33] The AUC of LA-aPTT StaClot was only 0.71 with no significance, even though it was found to be significant. The AUCs might be meaningless in this heterogeneous group because the prevalence of aPL was too low to draw an adequate ROC curve.

The present study was not a randomized controlled trial (RCT) because an RCT for testing the obstetrical significance of 11 assays would have been impossible. We could not examine the persistence of the results of all 11 assays, which was one of the limitations of the present study. We did not distinguish between patients treated with aspirin and heparin and those treated with aspirin alone, because of the small sample size. We did not consider the titer in any of the assays. The titer tended to be high in patients with positive results of tests for two or more conventional aPL. Further studies to determine the relevant titers and the antiphospholipid score for obstetric APS are needed.

It is important to ascertain the obstetric significance of each assay before clinical use, because similar assays with a strong correlation were found to show different distributions. The LA-aPTT StaClot is a mixing and phospholipid-neutralizing test, which is included in the classification criteria.[11] LA-aPTT StaClot may be suitable for use in routine practice for patients with RPL. As the obstetric significance of aPS/PT IgG was demonstrated for the first time in the present study, further study is needed to confirm our findings.

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Mayumi Sugiura-Ogasawara contributed to the research design, performed the analysis and drafted the article.

Yasuhiko Ozaki, Kinue Katano contributed to the acquisition and analysis of the data.

Wolfgang Papisch contributed to the measurements of the CL IgG, IgM and IgA and β2GPI IgG, IgM and IgA.

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