

New Technologies, Diagnostic Tools and Drugs

Laboratory detection of the antiphospholipid syndrome via calibrated automated thrombography

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Summary

Lupus anticoagulants (LAC) consist of antiphospholipid antibodies, detected via their anticoagulant properties *in vitro*. Strong LAC relate to thromboembolic events, a hallmark of the antiphospholipid syndrome. We have analyzed whether detection of this syndrome would benefit from thrombin generation measurements. Therefore, calibrated automated thrombography was done in normal plasma (n=30) and LAC patient plasma (n=48 non-anticoagulated, n=12 on oral anticoagulants), diluted 1:1 with a normal plasma pool. The anti- β_2 -glycoprotein I monoclonal antibody 23H9, with known LAC properties, delayed the lag time and reduced the peak height during thrombin generation induction in normal plasma dose-dependently (0–150 μ g/ml). At variance, LAC patient 1:1 plasma mixtures manifested variable lag time prolongations and/or peak height reductions. Coupling these two most informative thrombin gen-

eration parameters in a peak height/lag time ratio, and upon normalization versus the normal plasma pool, this ratio distributed normally and was reduced in the plasma mixtures, for 59/60 known LAC plasmas. The normalized peak height/lag time ratio correlated well with the normalized dilute prothrombin time, diluted Russell's viper venom time and silica clotting time, measured in 1:1 plasma mixtures (correlation coefficients 0.59–0.72). The anticoagulant effects of activated protein C (0–7.5 nM) or 23H9 (0–150 μ g/ml), spiked in the 1:1 LAC plasma mixtures were reduced for the majority of patients, compatible with functional competition between patient LAC and activated protein C and LAC and 23H9, respectively. Hence, the normalized thrombin generation-derived peak height/lag time ratio identifies LAC in plasma with high sensitivity in a single assay, irrespective of the patient's treatment with oral anticoagulants.

Keywords

Thrombosis, α_2 -glycoprotein I, lupus anticoagulants, prothrombin, antibodies

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Introduction

The antiphospholipid syndrome (APS) is characterized by the occurrence of thrombosis in patients with so-called antiphospholipid antibodies. These antibodies are directed to plasma proteins with affinity for anionic phospholipids. Those antibodies with anticoagulant properties *in vitro*, are referred to as lupus anticoagulants (LAC) (1, 2).

Antiphospholipid antibodies develop both to proteins with or without known function in coagulation. Prothrombin (a representative of the former) and β_2 glycoprotein I (β_2 GPI, a representative of the latter) appear to be the major, but not exclusive antigen targets, implicated in the pathophysiology of APS (3). Anti- β_2 -GPI antiphospholipid antibodies promote binding of stable bivalent antibody- β_2 -GPI complexes to phospholipids, re-

sponsible for the anticoagulant effect *in vitro* (4). Anti- β_2 -GPI antibodies are thrombogenic in animal models (5) and high-avidity anti- β_2 -GPI antibodies highly correlate with thrombosis in men (6). Whereas anti- β_2 -GPI antibodies are associated with thromboembolic complications (1–3), the role of anti-prothrombin antibodies in thrombosis is more controversial (7). Yet, anti-prothrombin and anti- β_2 -GPI antibodies co-exist in APS and circulating prothrombin-anti-prothrombin complexes were demonstrated in LAC patients (2, 8).

Several mechanisms may explain the thrombogenic effects of antiphospholipid antibodies. Dimerized β_2 -GPI supports platelet adhesion and aggregation under venous conditions, phenomena depending on its interaction with platelet receptors GPIIb/IIIa and apolipoprotein E receptor 2' (9). Recently, antiphospholipid antibodies were found to interfere with the anticoagulant activity of

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annexin A5 (10) and of activated protein C (APC) (3). Thus, antibody- β_2 -GPI complexes may compete with components of the APC complex for the limited number of binding sites on the phospholipid surface, or, alternatively, disrupt the APC complex (3). Antibody- β_2 -GPI complexes can also disturb other phosphatidylserine dependent coagulation pathways, including neutralization of activated FX and inhibition of coagulation initiation by tissue factor pathway inhibitor (3). APC inhibition by antiphospholipid antibodies potentially also occurs via β_2 -GPI-independent mechanisms, such as by antibody-prothrombin complexes, competing with APC for binding to phospholipids, during inactivation of factor (F)Va or FVIIIa.

Recently, global plasma coagulation tests are being applied progressively to monitor overall coagulation. Hence, thrombin generation allows the recording of a thrombogram, via a high throughput approach, with acceptable experimental error, using a fluorogenic thrombin substrate and continuous calibration in each individual sample (11). The use of calibrated automated thrombography allows definition of several thrombin generation parameters, such as the lag time, time-to-peak, endogenous thrombin potential and peak height. A recent study found that supplementing pooled normal plasma with immunoglobulins from LAC patients induces a wide range of enhancing or inhibitory effects on the endogenous thrombin potential (12). We have, therefore, investigated via thrombin generation measurements in the plasma of LAC patients whether calibrated automated thrombography has added value in the diagnosis of APS. As part of this characterization, thrombin generation parameters were determined in the presence of APC (13) and upon addition to plasma of the strong LAC-positive monoclonal anti- β_2 -GPI antibody 23H9 (4). Analytical criteria were defined for the detection of APS, also in patients on oral anticoagulants, in an effort to facilitate the currently established laboratory diagnostic criteria for APS, via use of a single integrated test.

Materials and methods

Subject selection and plasma collection

A control population consisted of plasma from healthy adult volunteers ($n=30$). Patients were selected from individuals referred to our thrombophilia centers or referred for autoimmune disease testing. Peripheral venous blood and plasmas were collected, as recently described (13) from patients, referred for LAC screening. After LAC analysis, all samples were kept at -80°C , until further analysis. Plasmas were also collected from known LAC-positive patients, prior to ($n=48$) or during ($n=12$) anti-vitamin K antagonist (VKA) therapy. As controls for those patients on VKA therapy, additional plasma samples ($n=9$) were collected from patients on long-term anticoagulant therapy, but negative for LAC; their international normalised ratio (INR) values ranged from 2.05–3.05. A normal plasma pool was made, by mixing equal volumes of plasma from healthy individuals ($n=40$). Permission to analyze patient samples was given by the Ethical Committees of the Ghent and Leuven University Hospitals.

Persistent LAC were defined, according to the revised ISTH Sapporo criteria, as LAC-positivity on at least two occasions, at least 12 weeks apart (2). Transient LAC patients were positive on a first and negative on a second occasion. Thrombotic episodes

or pregnancy morbidity were retrospectively identified upon consultation of medical records. Paediatric samples were excluded from analysis, since normal values differ between adults and children (13).

Automated measurement of thrombin generation

Thrombin generation was triggered in platelet-poor plasma in the presence of an intermediate concentration of tissue factor (5 pM) and a relatively high concentration of phospholipids (4 μM). In these conditions, thrombin generation depends to a small degree on FVIII and FIX but not on FXI and is phospholipid-controlled, at concentrations too low to trigger contact activation (14). Thrombin generation was performed in normal plasma, in the normal plasma pool and in 1:1 mixtures of patient plasma and normal plasma pool, in the presence of 1 μM phospholipids, to raise the phospholipid dependence of the assay. The tissue factor concentration was 5 pM. Calibrated Automated Thrombography[®] was performed as recently reported (13). Experiments were done in triplicate (controls) or duplicate (patients) and lag time, peak height of thrombin formation, time to peak and endogenous thrombin potential (14) were analyzed. Thrombin generation was also performed in the presence of human APC (0–7.5 nM, Rossix, Mölndal, Sweden), spiked in normal plasma pool and in plasma of all 48 non-anticoagulated and of 12 LAC-positive anticoagulated patients. To this end, patient samples were mixed 1:1 with normal plasma pool. The anticoagulant effect of APC was expressed as the *APC-ratio* = [(peak height/lag time without APC) – (residual peak height/lag time with APC)]/[peak height/lag time without APC]. The APC-ratio expresses thrombin generation inhibition, relative to thrombin generation, in the absence of APC. The APC-ratio, determined in the 1:1 plasma mixtures was then compared to that measured for normal pooled plasma, to evaluate the patient antibody-induced resistance to APC.

Thrombin generation was also studied in normal plasma pool, in normal and patient plasmas, in the presence of the potent LAC monoclonal anti- β_2 -GPI domain 2 antibody 23H9 (0–150 $\mu\text{g/ml}$) (4). The 23H9-induced dimerization of β_2 -GPI mimics the effect of patient antibody- β_2 -GPI complexes (4). For those experiments, where 23H9 was added to 1:1 plasma mixtures, the anticoagulant effect of 23H9 was expressed as the *23H9-ratio* = [(peak height/lag time without 23H9) – (residual peak height/lag time with 23H9)]/[peak height/lag time without 23H9]. This ratio expresses thrombin generation inhibition, relative to thrombin generation, in the absence of 23H9. The 23H9-ratio was then compared to that measured for normal pooled plasma, to evaluate the patient antibody-induced resistance to 23H9.

Alternative thrombin generation measurements were performed in a purified system, with the prothrombin complex concentrate PPSB[®] (prothrombin = FII, proconvertin = FVII, Stuart-Prower-Factor = FX, and antihemophilic globulin B = FIX from CLB, Amsterdam, The Netherlands).

Routine measurements

Screening for deficiency of antithrombin, protein C (functional), protein S (free antigen), activated protein C resistance and the prothrombin gene GA20210 mutation was carried out using standard methods (15).

Lupus anticoagulant assays

Plasmas were qualified as LAC-positive, by considering the ISTH-criteria in a four-step procedure (16). Samples normal in all LAC-sensitive screening tests are reported as LAC-negative, without further mixing or confirmation testing. Samples with prolonged screening test results were further analyzed via mixing and confirmation tests. As screening assays, a sensitive activated partial thromboplastin time (aPTT) (PTT-LA, Diagnostica Stago, Asnières, France), in combination with the diluted Russell's viper venom time (dRVVT) (LA-screen, Gradiopore Ltd., Australia), or a dilute prothrombin time (dPT) (Dade Innovin®, Dade Behring, Marburg, Germany) in combination with a dRVVT (LAC-screen, Instrumentation Laboratories, Lexington, MA, USA) and silica clotting time (SCT) (Instrumentation Laboratories) were used. Finally, a heparin sensitive thrombin time was performed to exclude samples with prolonged screening tests, due to heparin, before further LAC testing and specific coagulation factor dosage was performed to confirm or exclude factor deficiency, due to VKA treatment (17).

Mixing studies were done when screening tests were prolonged. Reference values were calculated as described and were confirmed by retrospective receiver-operator characteristics

(ROC) analysis (2, 17). We performed mixing tests in a 1:1 ratio with normal plasma pool (17). In samples with less than 70% correction for at least one mixing test or a positive Rosner index, the presence of an inhibitor was concluded (18). To correlate thrombin generation parameters and mixing tests, mixing tests were expressed as the ratio between the clotting times of the mixed patient plasma and the clotting time of a normal plasma (pool, when indicated), analyzed in the same run. Likewise, thrombin generation parameters were normalized by division of the results for the 1:1 plasma mixtures and those measured for the plasma pool.

Confirmatory tests were done by aPTT (StacLOT-LA, Diagnostica Stago), dRVVT (LA-confirm, Gradiopore Ltd.), dPT and SCT, as described (17). Interpretation of the confirmatory tests is based on the comparison of clotting times by testing the sample in a phospholipid insensitive coagulation assay and by repetition of the clotting time after addition of extra phospholipids. Since there are no guidelines to interpret confirmation tests, own cut-off values were calculated and compared to literature data (2, 19). Samples with a positive screening test, a mixing test result compatible with the presence of an inhibitor and a positive confirmation test in at least one test system are reported as positive in this study.

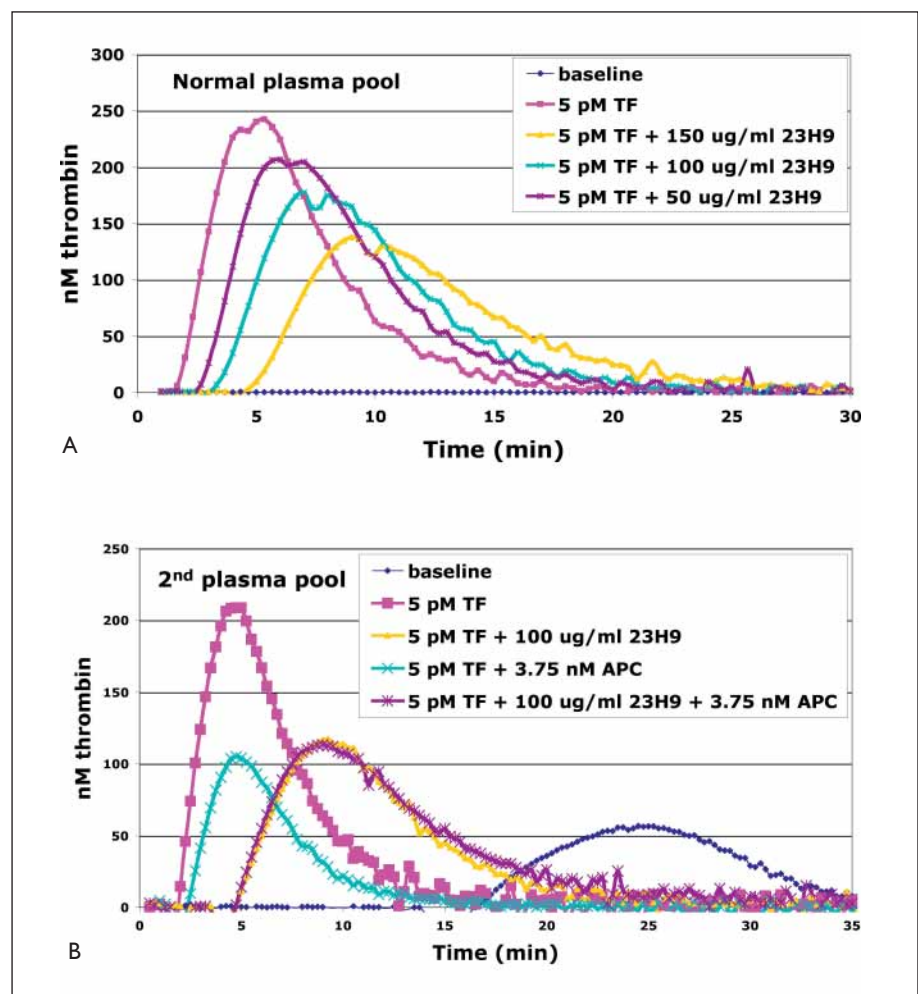


Figure 1: Anticoagulant properties of 23H9 and APC in thrombin generation. Individual thrombograms, as a function of the indicated concentrations of 23H9, added to normal plasma (A, B) and of APC (B), initiated by 5 pM tissue factor and 4 μ M of a phosphatidylcholine (PC) and phosphatidylserine (PS) mixture (PC/PS: 7/3).

Statistical analysis

Data were analysed using MedCalc® Version 7.1.0.0 (MedCalc Software, Mariakerke, Belgium). Comparison of results and correlation analysis were done using Pearson's correlation and linear regression analysis. Results were presented as Box-and-Whisker plots or histograms.

Results

Monoclonal anti- β_2 -GPI antibody 23H9 in thrombin generation

First, the anticoagulant properties of the LAC-positive antibody 23H9 (0–150 $\mu\text{g/ml}$) were investigated in thrombin generation, upon its spiking in pooled normal plasma. When thrombin generation was triggered by 5 pM tissue factor and a standard phospholipid concentration (4 μM), 23H9 prolonged the lag time in a concentration-dependent manner, approximately two-fold in the

presence of 100 $\mu\text{g/ml}$ 23H9, and slightly more at 150 $\mu\text{g/ml}$ (Fig. 1). At the same time, at 100 $\mu\text{g/ml}$ 23H9, the peak height of the thrombogram was reduced by about 30%. Figure 1B also shows how the addition of APC to plasma reduces thrombin generation (via degradation of coagulation cofactors Va and VIIIa). 23H9 (100 $\mu\text{g/ml}$) completely inactivated APC (Fig. 1). Hence, in agreement with recent data (20–22), 23H9- β_2 GPI antibody complexes strongly interfered with the anticoagulant activity of APC, during calibrated automated thrombography.

In a reconstituted system of thrombin generation, consisting of phospholipids (4 μM), the coagulation factor concentrate PPSB (1/400 final dilution) and added purified β_2 -GPI (150 $\mu\text{g/ml}$), the separately added β_2 -GPI or 23H9 had no effect. In combination, they retarded lag time up to two-fold and reduced peak height up to 40%, for [23H9] = 50–100 $\mu\text{g/ml}$ (not shown), confirming that the anticoagulant effect of 23H9 resulted from complex formation with β_2 -GPI.

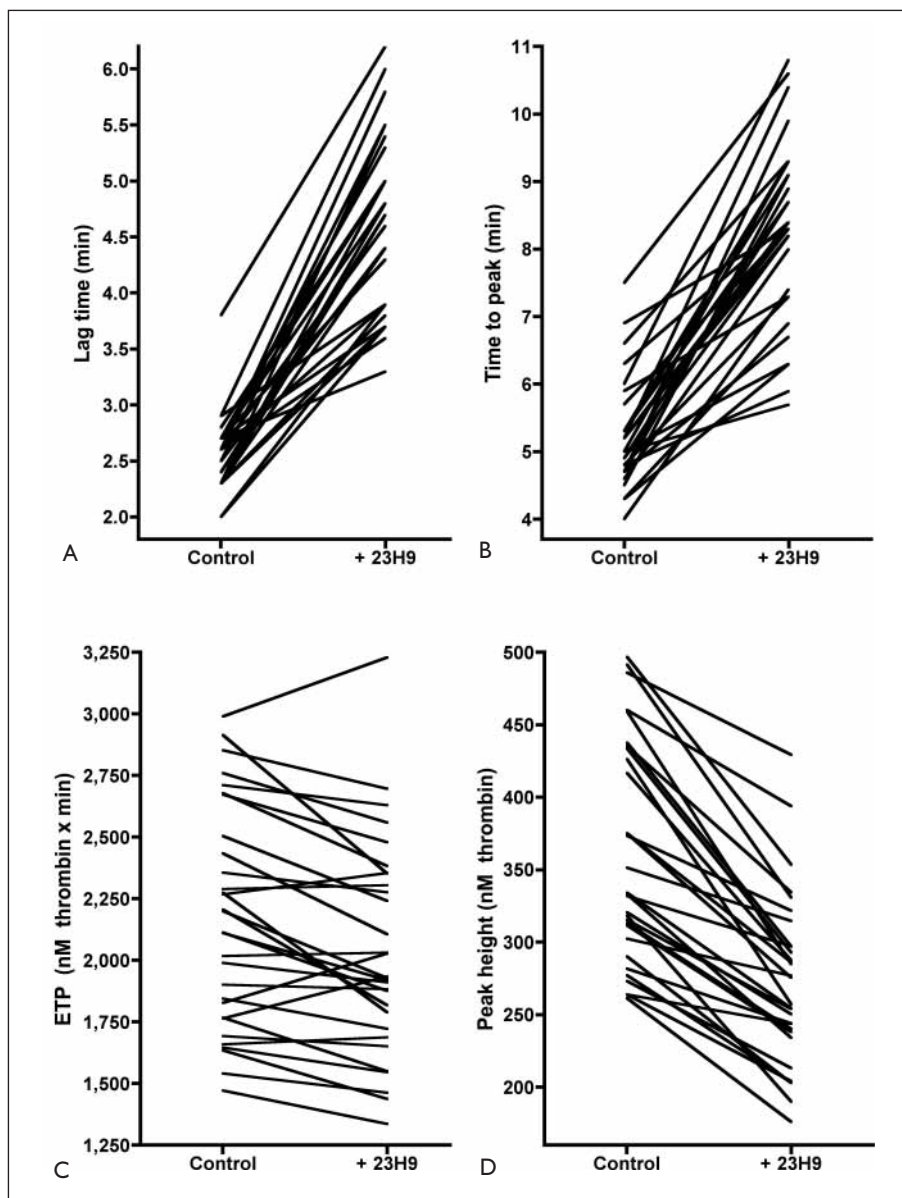
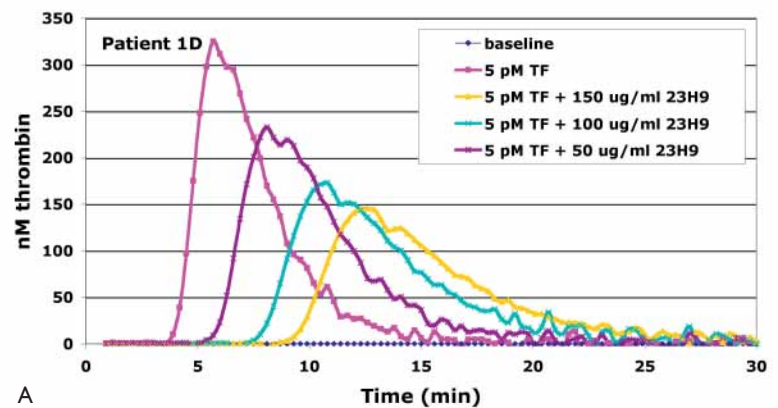


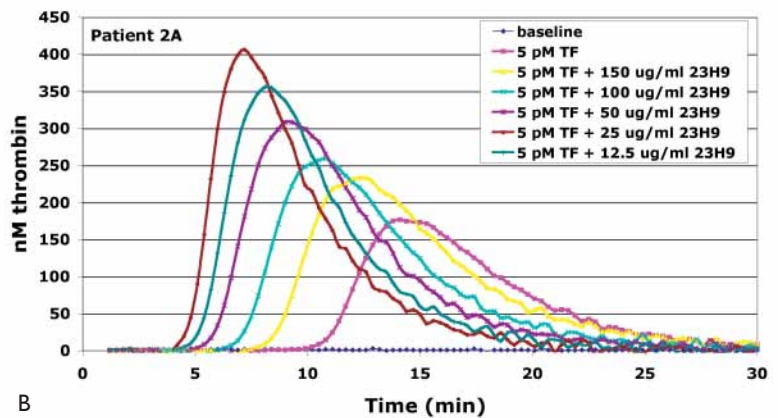
Figure 2: Identification of thrombin generation parameters in 23H9-induced APS. Individual line diagrams for the four major thrombin generation parameters indicated in the y-axes, measured in the plasma of 30 healthy controls, before and after addition of 150 $\mu\text{g/ml}$ 23H9, as indicated (ETP: endogenous thrombin potential).

The anticoagulant effect was most potent at 150 $\mu\text{g/ml}$ 23H9. Therefore, the anticoagulant effect of 150 $\mu\text{g/ml}$ 23H9 was analyzed for all thrombin generation parameters in 30 healthy donor plasmas (Fig. 2). Inhibition resulted in a significant prolongation of the mean lag time (2.5 ± 0.33 to 4.6 ± 0.76 min, $p < 0.0001$) and time to peak (5.27 ± 0.78 to 8.3 ± 1.34 min, $p < 0.0001$). The mean peak height was significantly lowered (360.6 ± 75.6 to 272.7 ± 58.8 nM thrombin, $p < 0.0001$). The mean endogenous thrombin potential was not significantly influenced (2771 ± 444 to $2037 \pm$

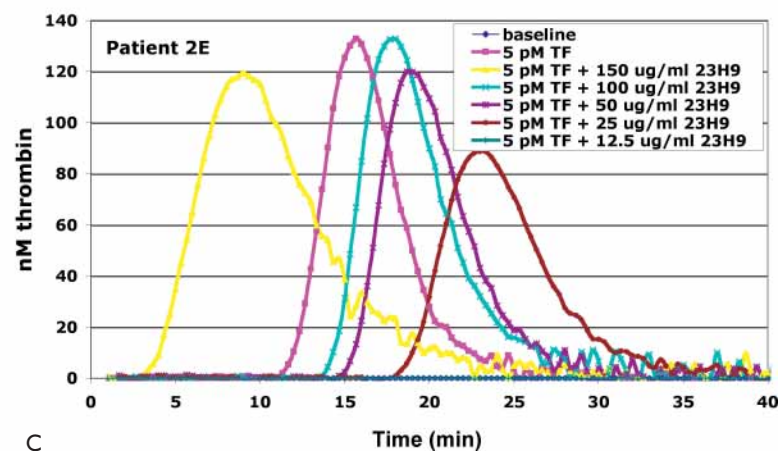
432 nM thrombin.min) and was even raised by 23H9 in some samples (Fig. 2). Because in all normal individuals ($n=30$), the lag time and peak height was significantly affected by 23H9, these two parameters were combined in a single index. Indeed, the peak height/lag time ratio was affected more (145.8 ± 36.4 to 61.77 ± 20.3 nM thrombin/min, $p < 0.0001$) by 23H9 than each thrombin generation parameter separately. This index was, therefore, applied as a measure of the LAC effect in plasma (see below).



A



B



C

Figure 3: Interference on the thrombogram profile between 23H9 and LAC in plasma from APS patients. Individual thrombograms, as a function of added 23H9, at the indicated concentrations, initiated by 5 pM tissue factor, supplemented with 4 μM PC/PS, in plasma samples of a transiently LAC-positive patient (A) and of two LAC-positive patients with evidence of thrombosis (B, C).

Patient antibody-23H9 interference

The activity of APC was inhibited by 23H9. To investigate whether the anticoagulant activity of 23H9 and that of patient antibodies were mutually exclusive, thrombin generation was measured in the plasma of LAC-positive patients, upon spiking with 23H9 (0–150 µg/ml). Figure 3 shows a series of thrombograms, upon addition of 23H9. For a transiently positive patient without thromboembolic complications, additive effects were noted for 23H9, at the time where she was LAC-positive (Fig. 3A). When she had become LAC-negative, the response to 23H9 had normalized (not shown), a tendency confirmed for other transiently positive patients.

In contrast, in patients with persistent LAC and evidence of thrombosis, 23H9 reacted strongly and with divergent types of response. In the example of Figure 3B, low [23H9] disrupted anticoagulant immune complexes, shortening lag time and normalizing peak height. Increasing [23H9] then restored the expected effect for 23H9, i.e. doubling the lag time, accompanied by an almost halving of the peak height (Fig. 3B). Finally, Figure 3C shows the thrombogram for a strongly LAC-positive plasma, with demonstrated anti-prothrombin antibodies. The strongly retarded lag time and reduced peak height in this patient were interfered with by 23H9 in a more complex reactivity pattern, in which low [23H9] compromise thrombin formation further, but higher concentrations increasingly restored it. In another sample of the same patient, taken seven weeks later, lag time and peak height equalled 13 min and 190 nM thrombin, respectively, but high [23H9] only mildly reduced thrombin formation (not shown). These findings illustrate the large interindividual variation and fluctuations over time in antibody titers and manifestation in LAC-positive patients, probably reflecting heterogeneity of implicated antigens, epitopes and antibody concentration. Nevertheless, they illustrated that, similarly to APC, 23H9 was a useful reagent in the study of the anticoagulant properties of patient LAC, when tested at relevant concentrations.

Thrombin generation in LAC patient plasmas:

Analytical conditions

We have investigated whether lowering the phospholipid concentration in the plasma sample would raise the sensitivity of the LAC-detection via calibrated automated thrombography. Figure 4A shows that in normal plasma, the peak height is proportional to the concentration of PC/PS and that the relative effect of 100 µg/ml 23H9 on thrombin generation improved at PC/PS concentrations below 4 µM. Because thrombin generation was too much compromised at 250 nM, the further analysis of patient samples was carried out at 1 µM PC/PS, together with tissue factor, at 5 pM. Figure 4B and C confirm that the same effect was observed in LAC patient plasma, spiked with 100 µg/ml 23H9, i.e. peak height and lag time were more affected by 23H9 at 1 µM than at 4 µM. Therefore, subsequent thrombin generation measurements were done in the presence of 1 µM PC/PS.

Plasma characteristics of anticoagulated and non-anticoagulated LAC patients

We wanted to determine whether thrombin generation has value in LAC-detection in plasmas from patients, including those on oral anticoagulants. Therefore, 48 non-anticoagulated and 12

anticoagulated LAC-positive plasmas were selected. The normalized dPT, dRVVT and SCT distribution in screening, confirmation and mixing tests of the selected samples is shown in detail in Figure 5, for both categories of patient samples. The dRVVT has the highest sensitivity in LAC screening, mixing and confirmation tests; a positive dRVVT mixing test for the patients on oral anticoagulants illustrates that these samples were LAC-positive (Fig. 5). When both patient categories were compiled, Table 1 confirms that all patients were LAC-positive and that the dRVVT has the best overall sensitivity for the LAC-detection, irrespective of the anticoagulant treatment status of the patient.

Before investigating in LAC-positive plasma samples whether thrombin generation could be performed in plasma from patients on oral anticoagulants, we have evaluated whether 1:1 mixing of LAC-negative plasma from patients on oral anticoagulants (n=9) with normal plasma would normalize the anticoagulant properties of such plasma. Figure 6A shows how the mixing procedure almost normalized the peak height/lag time index in the 1:1 plasma mixtures (mean peak height/lag time around 80 nM thrombin/min), compared to the normal plasma pool (around 100 nM thrombin/min). When these tests were done in the presence of 23H9 (100 µg/ml) and/or APC (3.75 nM) (Fig. 6A), the same degree of inhibition was found in the mixtures as in the normal plasma control, whereas 23H9 inhibited APC, as expected. Hence, the 1:1 mixing procedure appeared to almost completely correct the effects of the oral anticoagulants and 23H9 and APC affected the peak height/lag time ratio in these mixtures, to the same degree as in normal plasma.

Therefore, these analytical conditions were applied for the analysis of the 60 confirmed LAC-positive patients. Figure 6B shows the normalized results for lag time, peak height and peak height/lag time ratio, measured in 1:1 plasma mixtures, for the 48 non-anticoagulated and 12 anticoagulated plasmas, each. This representation reveals a similar distribution of values in the anticoagulated and non-anticoagulated plasmas. Repetitive determination in the normal plasma pool uncovered a coefficient of variation (CV) of 13% (n=8) for the normalized peak height/lag time ratio. Consequently, the corresponding cut-off value was not set at 1 but at 0.87 (Fig. 6B), revealing a reduced peak height/lag time ratio in 47/48 non-anticoagulated and 12/12 anticoagulated plasmas.

This analysis showed that LAC-positive samples could be analyzed via thrombin generation, irrespectively of the degree of anticoagulation (for INR<3). Hence, Figure 7A shows the normalized thrombin generation parameters, pooled for all 60 samples. As expected from their prolonged clotting times, all samples showed a prolonged lag time (normalized range: 1.16–18.2; mean ± standard deviation [SD]: 2.7 ± 3 ; median: 1.8), but with wide distribution. Although in normal plasma, 23H9, in addition to prolonging the lag time, produced a concomitant dose-dependent reduction of the peak height, the peak height was not reduced in all plasma samples (normalized range: 0.01–1.2; mean ± SD: 0.87 ± 0.25 ; median: 0.93). This analysis excludes an automatic coupling between lag time prolongation and peak height reduction, in agreement with our finding that the lag time and peak height provide independent information in thrombin generation, i.e. do not correlate (not shown). However, when combined in a single index, i.e. the peak height/lag time

ratio (normalized range: 0.0005–0.93; mean \pm SD: 0.5 ± 0.24 ; median: 0.53), this index showed a Gaussian distribution (Fig. 7A insert), when analyzed according to the D'Agostino-Pearson test ($p=0.265$) and was reduced (<0.87) in 59/60 patients. Comparison of the normalized peak height/lag time ratio to the normalised result of mixing studies for dPT, dRVVT and SCT (Fig. 7B-D) showed correlation coefficients of -0.586 ($p<0.0001$), -0.678 ($p<0.0001$), -0.715 ($p<0.0001$), respectively.

APC in thrombin generation in LAC patient 1:1 plasma mixtures

Analysis in 25 healthy donor plasmas revealed $51 \pm 23\%$ inhibition of the endogenous thrombin potential and $52 \pm 22\%$ inhibition of the peak height, by 3.75 nM APC, although slight adjustments of this concentration was necessary to maintain about 50% inhibition of peak height in normal plasma, for APC present in different batches. The lag time was hardly affected. Cor-

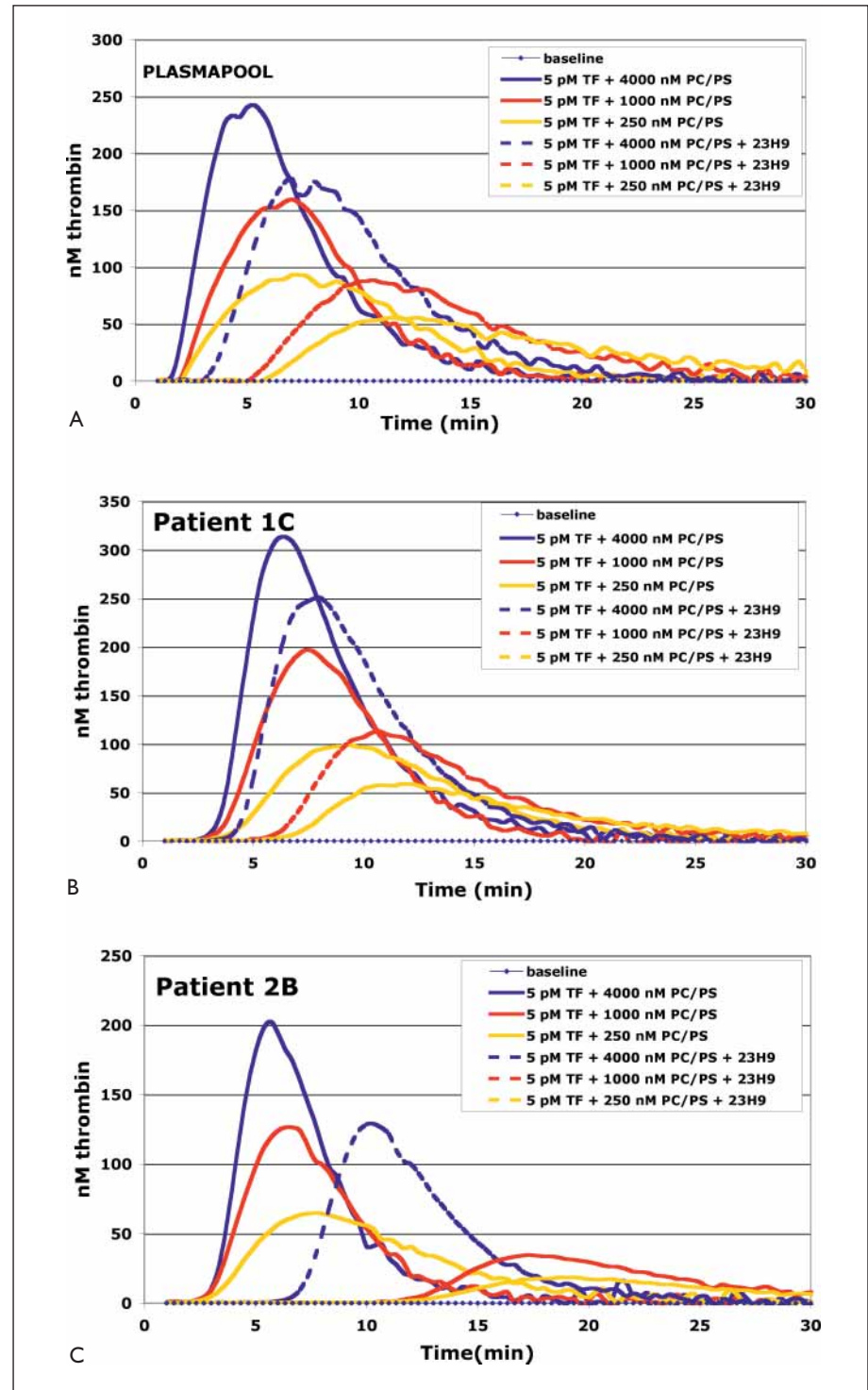


Figure 4: Phospholipid dependence of 23H9-induced anticoagulant activity. Individual thrombograms, with and without 23H9, added at 100 $\mu\text{g}/\text{ml}$, initiated by 5 pM tissue factor and measured in the presence of the indicated concentrations of a phosphatidylcholine (PC) and phosphatidylserine (PS) mixture (PC/PS: 7/3), in a normal plasma pool (A) and in LAC-positive plasmas (B, C).

respondingly, the mean APC-ratio for normal pooled plasma equalled 0.51, with an inter-assay coefficient of variation (CV) of 9.1%, therefore with lower and upper limits of 0.46 and 0.56, respectively. Hence, an APC-ratio >0.56 corresponds to more inhibition by APC, and a value <0.46 to less inhibition, or resistance to APC. We found that of the non-anticoagulated LAC pa-

tients 5/48 (10.4%) manifested an APC-ratio >0.56 and 3/48 (6.3%) had a ratio between 0.46 and 0.56. However, 40/48 (83.3%) patients showed an APC-ratio <0.46 , i.e. were resistant to APC. Only 1/9 anticoagulated LAC plasmas showed an APC-ratio <0.46 .

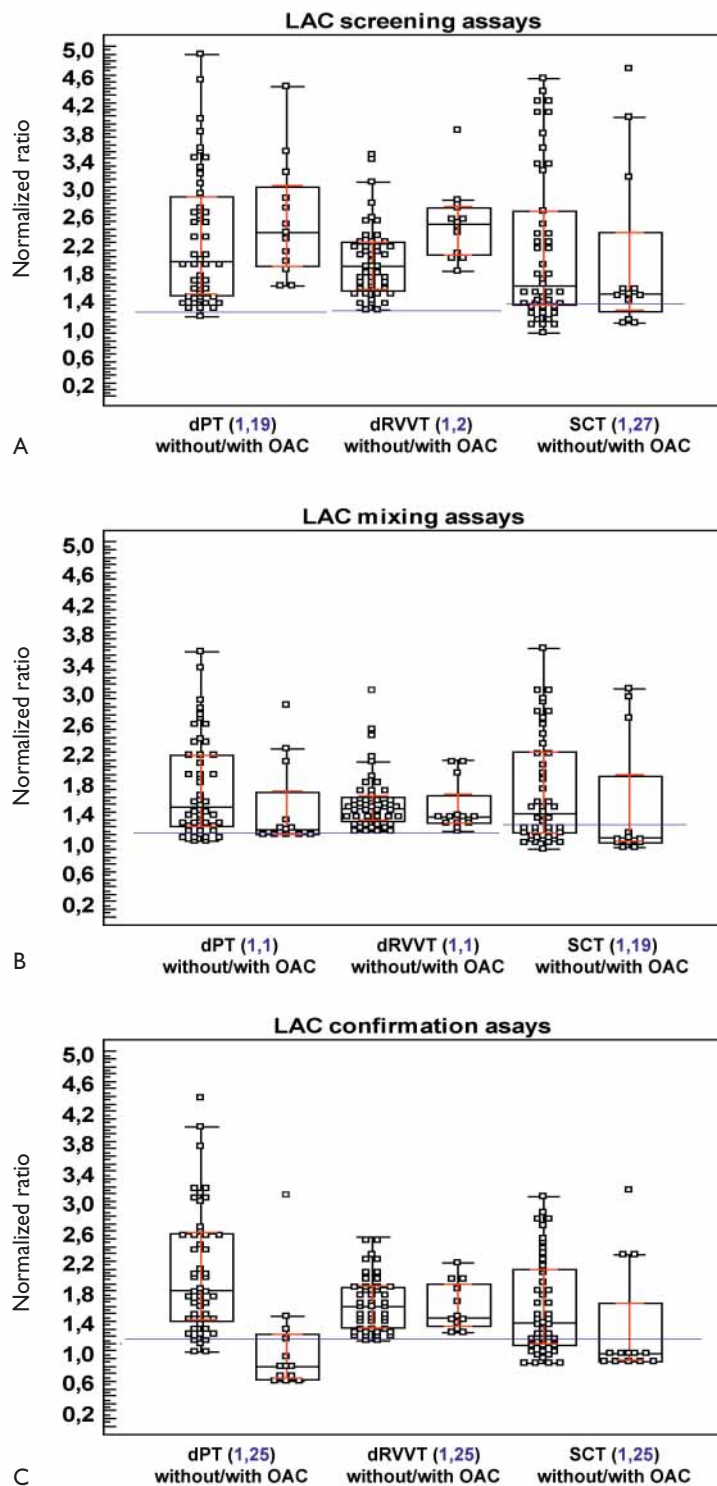


Figure 5: Distribution of the LAC-positive plasma sample characteristics. Box- and Whisker plots for normalized screening, confirmation and mixing test, as indicated, measured in 48 non-anticoagulated (left bar in each test, i.e. without OAC) and 12 anticoagulated (right bar in each test, i.e. with OAC) plasma samples from randomly selected LAC patients. The central box represents the 25 and 75 percentile and the middle line the median. The vertical black line extends from the minimum to the maximum value, outliers being displayed as separate points. The red lines represent the 1 SD group intervals. The blue lines indicate the cut-off value, which are also shown in parentheses.

Table 1: Sensitivity of LAC detection in 60 LAC-positive antiphospholipid patient plasma samples, expressed as positive normalized test result during screening, confirmation and mixing tests. Results were pooled for 48 non-anticoagulated and 10–12 anticoagulated plasmas.

	Screening + confirmation + mixing test	Screening	Mixing	Confirmation
dPT (n=60)	41 (66%)	59 (98%)	54 (90%)	43 (71%)
dRVVT (n=58)	52 (89%)	58 (100%)	58 (100%)	52 (89%)
SCT (n=58)	28 (48%)	46 (79%)	29 (50%)	30 (51%)

23H9 in thrombin generation in LAC patient 1:1 plasma mixtures

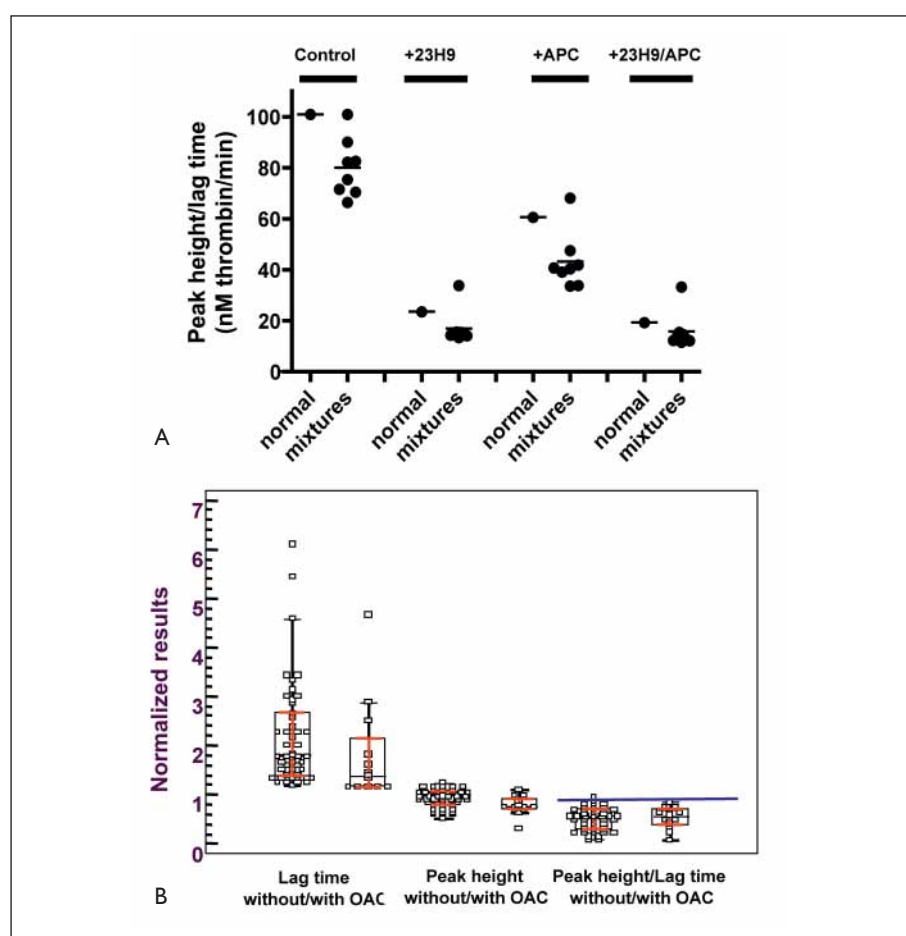
The 23H9-ratio was determined in the presence of 50 µg/ml (non-anticoagulated patient group) or 100 µg/ml (anticoagulated patient group) 23H9. The mean 23H9-ratio and inter-assay CV of the normal plasma pool were 0.69 (50 µg/ml 23H9) and 0.89 (100 µg/ml 23H9) and 12.3%, respectively. The lower limit was therefore set at 0.61, and the upper limit at 0.77 (50 µg/ml 23H9) and at 0.79 and 1, respectively (100 µg/ml). Patients with a 23H9-ratio higher than the upper limit show an enhanced effect for 23H9; a value lower than the lower limit means less effect compared to that in the normal plasma pool, i.e. resistance to 23H9. In the non-anticoagulated LAC plasmas, 1/48 (2.1%), 10/48 (20.8%) and 36/48 (75%), showed 23H9-ratios >0.77, between 0.61 and 0.77 and <0.61, respectively. In 3/12 (25%) anticoagulated LAC plasmas, a value <0.79 was found; all other patients were within the range for normal plasma. Or, 75% of the

non-anticoagulated and 25% of the anticoagulated samples showed resistance to 23H9.

Discussion

APS is an important cause of acquired thrombophilia. However, thrombogenicity assessment in APS patients remains a challenge. Laboratory tests in APS have poor predictive value for the thrombotic phenotype (2), and, in spite of their refinement, the test procedures in the laboratory detection of LAC, have essentially not changed over the last 30 years. Diagnosis relies on a combination of tests, since no single test has sufficient specificity and sensitivity (2). Therefore, the search for better performing coagulation assays in LAC testing continues; we presently studied whether calibrated automated thrombography can contribute to the diagnosis of APS. Since also thrombin generation relies on negative phospholipid surfaces, this study analyzed

Figure 6: Thrombin generation parameters in 1:1 plasma mixtures. Peak height/lag time ratios for “normal” plasma or for “mixtures” of LAC-negative plasma from patients on oral anticoagulants, mixed 1:1 with normal plasma pool (A), measured with 5 pM tissue factor, at 1 µM phospholipids. The anticoagulant effect of 23H9 (100 µg/ml), APC (3.75 nM) and their combination is also shown, in normal plasma and the 1:1 plasma mixtures. Box-and Whisker plots for the lag time, peak height and peak height/lag time ratio, measured in 48 non-anticoagulated (left bar in each test, i.e. without OAC) and 12 anticoagulated (right bar in each test, i.e. with OAC) plasma samples from LAC patients, in 1:1 mixtures with a normal plasma pool and after normalization (b); the blue lines represents the cut-off values for the peak height/lag time ration (0.87). Box parameters are as in Figure 5.



whether thrombin generation parameters can reliably diagnose LAC in patient plasma, based on the rationale that a single test may provide more accurate information than the multitude of clotting assays required in LAC testing, today (23, 24).

Previous thrombin formation tests by a chromogenic assay in APS patients showed inhibition of thrombin generation in the presence of anti- β_2 GPI antibodies (25, 26). This inhibition strongly correlated with a history of clinical manifestations (26). Thrombin generation in platelet-poor plasma seemed less sensitive to LAC than measurements in platelet-rich plasma, since the endogenous thrombin potential was only inhibited in part of the patients (12). Our present findings confirm, upon addition to 30 normal plasmas of the LAC monoclonal anti- β_2 GPI antibody 23H9 (4), that not all plasmas responded with an inhibition of this parameter. We found that rather lag time and peak height are the parameters of choice to monitor the anticoagulant effect of antiphospholipid antibodies.

When tested in β_2 -GPI antibody ELISAs, only a minority of our LAC patient samples tested positive (data not shown), questioning the sensitivity of such assays. In contrast, during thrombin generation in 1:1 plasma mixtures, either their plasma peak height (thrombin concentration) was lowered or their lag time delayed, or both. The prolongation of the lag time is not surprising, in view of the anticoagulant effect of LAC in the various clotting assays performed to select our patient plasmas. Yet, in

line with previous reports, thrombin generation parameters showed large intra-individual variations in the control group (13, 14), even larger in the patient LAC populations, reflecting the heterogeneity of LAC (12). In addition, not all LAC patients simultaneously manifested lag time prolongation and peak height reduction. This can be understood by the existence of initiation and propagation pathways of coagulation and potential interference by antiphospholipid antibodies with each phase, independently (11). Combining the independent information measured in the peak height (with reduced nominator) and the lag time (with enlarged denominator) in a ratio, seems a more powerful parameter for the detection of LAC patients because it contains more information than the lag time per se. This ratio was affected in all patient samples studied and was more normally distributed throughout the patient population tested than the lag time.

In relation to their thrombogenic risk, the assessment of LAC-activity is the best test in the detection of antiphospholipid antibodies (27). However, all coagulation tests for LAC are prolonged by oral anticoagulants, as well as by heparin. The current guidelines recommend to dilute patient plasma with normal plasma, at INR values above 3 (2). Evaluation of this strategy during thrombin generation measurements in 1:1 mixtures of anticoagulated patient plasma and normal plasma, showed that it effectively neutralized anticoagulant effects. To raise the sensitivity of the assay, recommended phospholipid concentrations

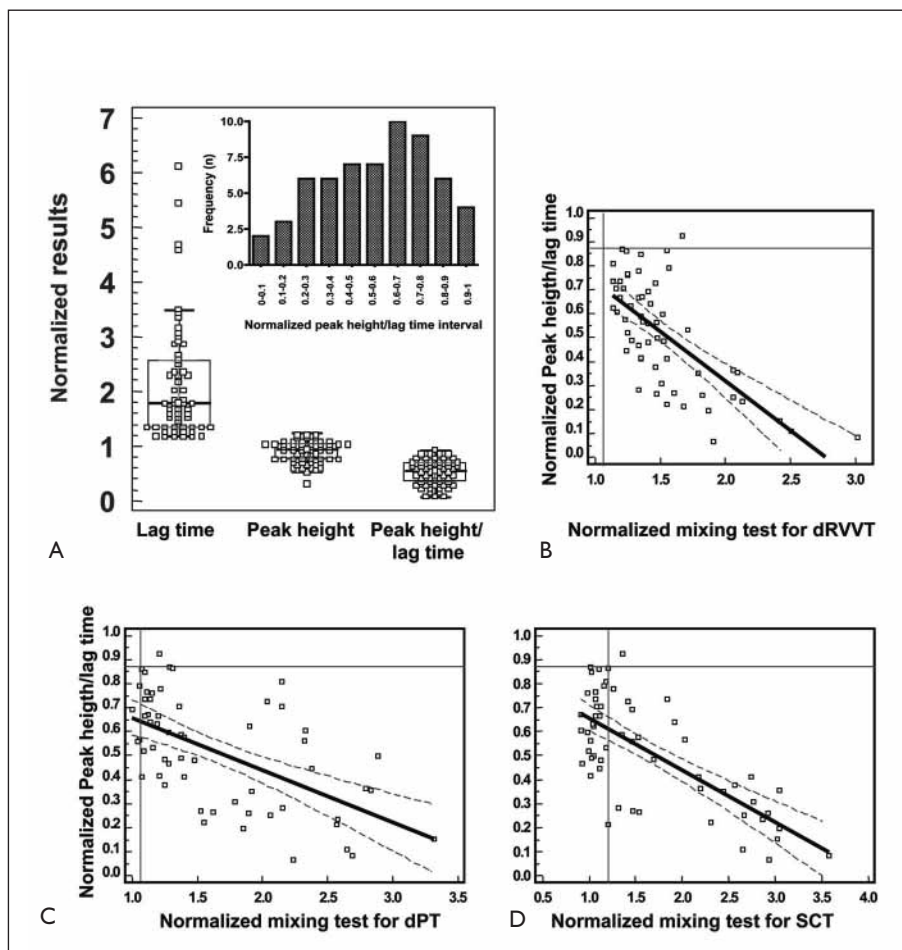


Figure 7: Relation between normalized peak height/lag time ratio and normalized mixing test results. Box-and-Whisker plots for the lag time, peak height and peak height/lag time ratio, for all 60 LAC-positive patients, in 1:1 mixtures with normal plasma and after normalization (A); the insert shows the normalized peak height/lag time distribution histogram for these samples. Linear regression analysis of normalized peak height/lag time ratios for LAC plasmas, mixed 1:1 with a normal plasma pool ($n=60$) versus the normalized result in mixing tests for dRVVT (B), dPT (C) and SCT (D). Thin lines represent calculated 99% percentile cut-off values for all parameters (0.87 in TG, 1.1 in dPT, 1.1 in dRVVT and 1.2 in SCT), respectively; corresponding Pearson correlation coefficients r with 95% confidence interval are -0.586 (-0.733 to -0.387) ($p<0.0001$, A), -0.678 (-0.797 to -0.509) ($p<0.0001$, B) and -0.715 (-0.821 to -0.560) ($p<0.0001$, C). Box parameters are as in Figure 5.

were lowered to 1 μ M. Following this strategy, and upon taking into account inter-assay variation, 59/60 (98%) selected LAC plasmas showed reduced normalized peak height/lag time ratios, irrespective of the intake of oral anticoagulants. A good correlation was found between the peak height/lag time ratio and the results of classical mixing tests, which are a part of the current three-step procedure for LAC testing. Furthermore, 59/60 patients were positive for both the normalized peak height/lag time ratio (<0.87) test and the mixing test on dRVVT (>1.1). The one patient with a high peak height/lag time ratio (0.93) comparable to that for normal plasma, was positive for all three classical mixing studies. These data suggest that a double assessment of LAC activity by thrombin generation and dRVVT in 1:1 mixed plasma samples may identify all APS patients, independently of their oral anticoagulant treatment status. They suggest a place for thrombin generation in diagnosis and monitoring of APS.

Our findings illustrated that LAC plasmas from thromboembolic patients contained potent anti- β_2 -GPI antibodies (even when not detected by currently used ELISAs), interfered with by 23H9 to a different degree from patient to patient, both qualitatively and quantitatively. Also, the mixing studies revealed a decreased response to a fixed concentration of added 23H9, in 75% of the non-anticoagulated LAC plasmas. However, in the anticoagulated group, 75% of the LAC-positive patients responded to 23H9 similarly as normal plasma. This confirmed what we observed in individual patients: antibody titers change with time. Acquired resistance to APC is a common feature of APS and may be independent of the presence of anti- β_2 GPI antibodies (28). Important resistance to the activity of the protein C system in the presence of LAC was demonstrated by thrombography before (21, 22). Regnault et al. only observed resistance to thrombomodulin (TM) and APC in the presence of phospholipids from the patient's platelets but not necessarily with phospholipids added to platelet-poor plasma, such as applied presently. Here, in 83% of the non-anticoagulated LAC plasmas, we observed resistance to APC, but this proportion was much lower in anticoagulated LAC plasmas. This finding substantiates the conclusion that antibody titers change with time. Measuring

What is known about this topic?

- Diagnosis of the antiphospholipid syndrome requires a multitude of laboratory tests.
- The laboratory diagnosis is only qualitative.

What does this paper add?

- Thrombin generation can detect the antiphospholipid syndrome in a single test.
- Normalization and definition of an index, defined as the ratio between the peak height and the lag-time allow to express the patient's antibody anticoagulant potency.
- The peak height/lag time ratio can be measured for 1:1 mixtures of normal plasma and plasma from patients on anticoagulant treatment.

and reporting a more precise index would, therefore, allow more quantitative monitoring, i.e. co-define the need for sustained anticoagulant therapy, in relation to the titer of antiphospholipid antibodies in patient plasma. The peak height/lag time ratio may provide such a sufficiently quantitative parameter to monitor APS, also during anticoagulant therapy.

So far, available information suggests that the laboratory measurement of thrombin production and activity in patients at risk for and in patients with significant thrombosis, does not provide information useful for clinical decision-making (29). Yet, our present analysis of the peak height/lag time ratio in patient samples and in 1:1 plasma mixtures shows that thrombin generation, in association with dRVVT is highly informative in the laboratory detection and monitoring of LAC.

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