



## RATIONAL LABORATORY DIAGNOSTICS OF ANTIPHOSPHOLIPID ANTIBODIES: ANTI-CARDIOLIPIN, ANTI- $\beta$ 2-GLYCOPROTEIN I, ANTI-PROTHROMBIN AND ANTI-ANNEXIN V ANTIBODIES

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### Abstract

A possible co-appearance of anticardiolipin (aCL), anti- $\beta$ 2-glycoprotein I (anti- $\beta$ 2-GPI), anti-prothrombin (aPT) and anti-annexin V (aANXV) antibodies of IgG, IgM and IgA class were studied in 58 patients with SLE alone and 32 patients APS in the view of rational laboratory diagnostics. The presence of anti-phospholipid antibodies (aPL) were defined by our in-house ELISA methods. Out of 17 aCL negative SLE patients 6 had other antigenically defined aPL antibodies. In 13 patients only IgA but not IgG and IgM anti- $\beta$ 2GPI were detected. Different combinations of aPL subsets were equally distributed in APS and SLE groups. The prevalence of aANXV were similar in APS and SLE patients which was not the case with other aPL. Our findings support the idea of measuring additional subsets of aPL (aPT and aANXV) in unclear cases. IgA (either aCL or anti- $\beta$ 2-GPI) improved neither the diagnostic specificity nor diagnostic sensitivity, but only increased the frequency of the total anti- $\beta$ 2-GPI.

## INTRODUCTION

Antiphospholipid antibodies (aPL) are a heterogeneous family of antibodies against phospholipid-binding proteins or phospholipids alone. Antigenically not defined, the so-called anticardiolipin antibodies (aCL) are used as the laboratory criterion for the diagnosis of antiphospholipid syndrome (APS), clinically characterized by venous or arterial thrombosis, and recurrent spontaneous abortion (1). However, even introduced decenies ago, detection of aPL is not always reproducible for many reasons. To achieve a univocal diagnostic definition of APS, efforts were made within the European Forum on Antiphospholipid Antibodies to reduce the inter- and/or intra-laboratory variability of the diagnostic (2).

The most studied aPL subset are antibodies against  $\beta$ 2-glycoprotein I (anti- $\beta$ 2-GPI) (3) with a higher clinical specificity for APS than aCL and recently described as laboratory criterion for classification of APS (4). Antibodies against other target proteins have also been studied: prothrombin (5), annexin V (6), protein C, protein S (5), high- and low-molecular weight kininogens (7), thrombomodulin (8). For some of them close associations with aCL were found (9 - 12).

The knowledge that some of aPL subsets do (not) occur simultaneously could

- a) help to rationalize screening analysis,
- b) could contribute some valuable information about possible antigen associations or antibody spreading.

The aim of our study was to determine a possible co-appearance of anticardiolipin (aCL), anti-β2-GPI, anti-prothrombin (aPT) and anti-annexin V (aANXV) antibodies of IgG, IgM and IgA class in patients with SLE and/or APS in the view of rational laboratory diagnostics.

## **SUBJECTS AND METHODS**

### **Patients and controls**

We evaluated 90 Caucasian patients with systemic autoimmune disorders (85 females and 5 males, mean age 40.2±13.7 years, range 18 – 78 years) who visited the University Medical Centre, Department of Rheumatology between 1991 and 2001: 58 with SLE alone, 32 with APS (10 with primary, 22 with secondary to SLE). Patients with SLE fulfilled the revised criteria by the American College of Rheumatology (13), patients with APS fulfilled the "Sapporo" criteria (1). AT and VT were objectively verified at the time of the occurrence.

As we described previously (14), sera from 434 blood donors, were used as controls in relevant ELISA tests. Selected positive sera from patients with SLE and/or APS were used as internal standards for aPT and aANXV ELISA. All sera were stored at - 40°C until analysed.

### **Anticardiolipin ELISA**

IgG, IgM and IgA aCL were measured according to the standard aCL ELISA using an animal serum as described by Harris and co-workers (15), with some modifications which we reported elsewhere (16).

### **Anti-β2-glycoprotein I ELISA**

IgG, IgM and IgA anti-β2-GPI were measured by our in-house method as described earlier (16). The titre of anti-β2-GPI in each sample was derived from the standard curve according to the defined dilutions of monoclonal antibodies (13).

### **Anti-prothrombin ELISA**

IgG, IgM and IgA aPT were measured by ELISA using phosphatidylserine as described elsewhere(18).

### **Anti-annexin V ELISA**

IgG, IgM and IgA aANXV were measured by in house ELISA: 50 µl of human placental annexin V (Sigma-Aldrich, Wien, Austria), dissolved to 8 mg/l in phosphate buffered saline (PBS), pH 7.4 were used to coat microtitre plates (Costar High Binding EIA/RIA plates, Cambridge, USA). After 2 hours of incubation at room temperature (20-24°C), the wells were washed once with 300 µl PBS containing 0.1 % Tween 20 (PBS-Tween) and blocked with 150 µl of 1% BSA in PBS-Tween for 30 minutes at room temperature. A preliminary series of experiments indicated that the optimal dilution of sera was 1:100 for IgG and IgA and 1:200 for IgM (data not shown). 50 µl of serum samples, diluted in PBS-Tween containing 0.1 % BSA, were incubated for 30 minutes at room temperature. After four washes, 50 µl of an appropriate dilution of alkaline phosphatase conjugated goat anti-human IgG, IgM or IgA second antibodies in PBS-Tween were added. Following 30-minute incubation at room temperature and four washes, 100 µl of 1 g/l p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8 were added into each well. OD was measured at 450 nm by a microtitre plate reader until optimal fitting to the predicted OD of internal standards was obtained.

### **Determination of positivity**

aCL: positive/negative values were defined by statistical evaluation of data obtained: i/ for IgG and IgM from 147 blood donors and calibrated against the reference sera from KAPS studies (19) as we reported earlier (20) and ii/ for IgA from 52 blood donors as we reported earlier (21). The levels of positivity for IgG and IgM (used as the criteria for APS) were defined as low, moderate and high according to the results of the inter-laboratory testing in KAPS study (19, 20).

Anti- $\beta$ 2-GPI (IgG, IgM, IgA): positive/negative values were defined according to the statistical analysis of data obtained from 434 blood donors as we described recently (14).

aPT (IgG, IgM, IgA): values above 98th percentile of 66 blood donors were considered positive.

aANXV (IgG, IgM, IgA): values above 98th percentile of 140 blood donors were considered positive (22).

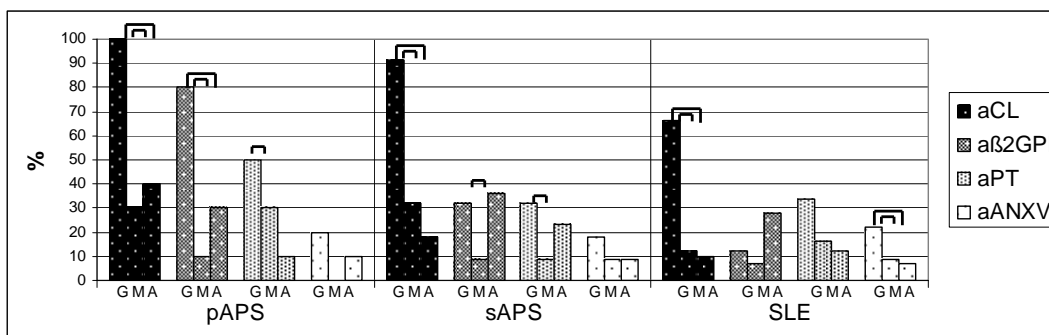
### Statistical analysis

Statistical analysis was performed using the hypothesis test with the proportions from independent groups (z test). A probability value of  $p \leq 0.05$  was considered statistically significant.

## RESULTS

In all three groups of patients (pAPS, sAPS, SLE), the frequency of positive cases for each type of antibody of any isotype was significantly higher than in the control group of blood donors (data not shown). The frequency of the three isotypes of the detected antibodies in each of the three groups is shown in **Figure 1**. IgG was the most frequently found isotype in all four types antibodies.

Figure 1. The presence of tested antibodies by isotypes. Data are shown as the percentage of cases positive for a particular isotype in each clinical group of patients. All marked associations showed statistically significant differences ( $p < 0.05$ ).



The numbers of positive samples in individual groups of patients are shown in **Table 2**. aCL antibodies were found more frequently in pAPS (10/10) and sAPS (22/22) than in SLE patients (41/58). aPT were significantly more often in the pAPS group (7/10) than in SLE (15/58) or sAPS (7/22). The prevalences of a $\beta$ 2GPI and aANXV antibodies did not differ between the patients' groups.

Different combinations of simultaneous occurrence of antibodies regardless of the isotype are shown in the **Table 1**. The most common antibody pair, aCL and anti- $\beta$ 2-GPI was found together alone or with other measured aPL in 33/79 aPL positive patients, aPT was always in combinations with other aPL, while aANXV were found in 2 cases alone and in 24 cases together with other antibodies. aCL alone were found in 9/32 APS patients (primary or secondary), while in other 23 cases other aPL were present as well. There was no statistically significant difference between the group of 32 patients with pAPS or sAPS who had in average 2.3 subsets of aPL and the group of 37 aCL positive SLE patients, who had in average 2.2 subsets of aPL, regardless of the isotype. From 5 patients with the combination of all four tested antibodies, 3 had SLE, one had sAPS and one had pAPS. Anti- $\beta$ 2-GPI and aPT were more frequently found in the pAPS group (70%, both) than in SLE alone (34% and 26%, respectively). The prevalence of aANXV antibodies did not differ between the patients' groups (**Figure 2**).

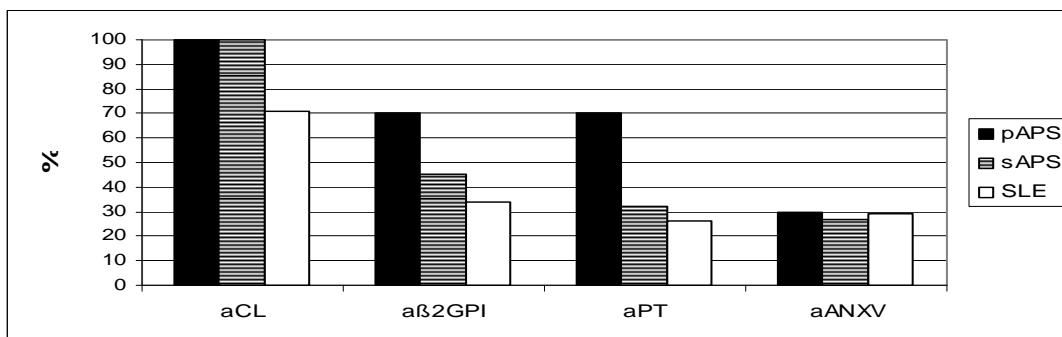
Table 1. Occurrence of aCL, a $\beta$ 2GPI, aPT and aANXV antibodies in 90 patients. Not detected combinations are omitted.

No. of antibodies	Type of combination	No. of cases
none		11
one	aCL	23
	a $\beta$ 2GPI	2
	aANXV	2
two	aCL + a $\beta$ 2GPI	9
	aCL + aANXV	9
	a $\beta$ 2GPI + aPT	1
	aCL + aPT	4
three	aCL + a $\beta$ 2GPI + aPT	14
	aCL + a $\beta$ 2GPI + aANXV	5
	aCL + aANXV + aPT	4
	a $\beta$ 2GPI + aANXV + aPT	1
four	aCL + a $\beta$ 2GPI + aPT + aANXV	5

Table 2. The presence of different antibodies in patients with primary APS, secondary APS to SLE and SLE without APS. Data are given as number (percentage) of positive cases.

Diagnosis	aCL	a $\beta$ 2GPI	aPT	aANXV
pAPS n=10	10 (100)	7 (70)	7 (70)	3 (30)
sAPS n=22	22 (100)	10 (45)	7 (32)	6 (27)
SLE n=58	41 (71)	20 (34)	15 (26)	17 (29)

Figure 2. Antibodies and clinical diagnoses. Percentages of positive cases (any of Ig class) are shown.



When aCL were of IgG class (67 cases), anti- $\beta$ 2-GPI, aPT and aANXV were also of IgG class in 20, 20, and 16 cases, respectively. In 18/29 IgG aCL positive APS patients at least one of other tested aPL (anti- $\beta$ 2-GPI, aPT, aANXV) was of IgG class also. On the other hand, in 10/66 IgG aCL positive patients (regardless of the diagnosis) IgM or IgA but no IgG of anti- $\beta$ 2-GPI, aPT and/or aANXV were found. Three of them had APS (one primary and two secondary).

Comparing the presence of particular antibody type with clinical features venous or arterial thrombosis (VT or AT), abortuses (AB), thrombocytopenia (T) and central nervous system involvement (CNS) some differences were observed. Patients with moderate or high IgG and/or IgM aCL had significantly more often CNS involvement than those with low or absent aCL (18/40 and 6/50, respectively). IgG a $\beta$ 2GPI were significantly associated with VT, AT, AB, CNS and T. The presence of elevated IgG and/or IgM a $\beta$ 2GPI showed a significant association with AT, AB and CNS (7/21 versus 9/69, 5/15 versus 6/56 and 15/21 versus 9/69, respectively). IgA a $\beta$ 2GPI did not improve the clinical specificity of a $\beta$ 2GPI. Elevated IgG aPT antibodies were significantly associated with AT and CNS (8/23 versus 8/67 and 10/23 versus 14/67, respectively). IgA aPT did not improve the clinical specificity of these antibodies. IgG aANXV positive patients experienced significantly less AB and CNS (0/15 and 2/19, respectively) when compared with aANXV negative patients (10/56 and 22/71, respectively). A similar association for AB was still seen when all three isotypes were considered.

## DISCUSSION

Autoimmune aPL comprise a heterogeneous group of autoantibodies which bind in vitro not only to phospholipids but predominantly to their complex with plasma proteins (23 - 26). They are detected either by solid-phase binding assays or functional phospholipid-dependent coagulant assays. aCL and lupus anticoagulant tests are widely performed to screen the aPL antibodies which are associated with thrombotic complications in patients with APS and/or SLE. It has been reported that the screening tests might miss some cases (27, 28). On the other hand, their relatively low diagnostic specificity provoked a debate about the substitution of aCL with antigenically determined aPL e.g. anti-β2-GPI (29 - 34), which resulted in inclusion of the latter into laboratory criterion for APS (4). Even more, recently completed multicentric study suggested that the detection of IgG antibodies, directed against domain I of beta2GPI, proved to be more strongly associated with thrombosis and obstetric complications than those detected using the standard anti-β2GPI antibody assay (35).

On the other hand, 9 patients in our study with definite APS would have been overlooked and therefore not diagnosed as APS if only antigenically specific aPL had been used instead of aCL. Despite growing interest for the use of antigenic specific aPL, aCL ELISA remains mostly used screening test for the diagnosis of APS. The aCL test is not as specific as the anti-beta2GPI test, but it is very clinically sensitive and together with the LA test should capture the majority of the APS patients (36). aCL and anti-β2-GPI are a heterogeneous group of antibodies with different clinical significances and can be present in different autoimmune diseases as well as in infectious diseases (37).

Among our 16 aCL negative SLE patients five had other antigenically defined aPL antibodies. Two of them fulfilled the clinical criteria for APS. These results spoke in favor of detecting other antigenically specific aPL in SLE patients and could be successfully used in the diagnosis of the so-called seronegative APS (27, 28) in spite of the classification criteria (1). Similarly Inanc et al (9) reported of a discrepancy between anti-β2-GPI and aCL. Namely, some of their patients with SLE and pAPS were positive for IgG anti-β2-GPI but not for aCL. A group of such patients is particularly important from the therapeutic point of view (using acetylsalicylic acid and/or anticoagulants), however a final decision how to treat them has not been reached so far.

Our results do not support the diagnostic value of the IgA aCL and/or anti-β2-GPI for APS. However we found 12 IgA anti-β2-GPI positive patients (10 with SLE, 2 with sAPS) without IgG and IgM isotypes anti-β2-GPI evidently increasing the prevalence of anti-β2-GPI in SLE population. On the opposite, Tsutsumi et al. (38) reported that measuring IgA anti-β2-GPI in addition to IgG could increase the clinical sensitivity of the test for APS. The reason could be the ethnic origin, which was already reported as an important factor in the distribution of different isotypes of anti-β2-GPI and aCL (32), or using only anti-β2-GPI as criterium for APS.

Several groups have studied different combinations of aPL with respect to thromboembolic events (9 – 11). We were not able to demonstrate any significant difference between groups of patient with APS and SLE, concerning the combination(s) of different aPL, but differences could be observed comparing the presence of antigenically defined aPL associated with particular clinical features. This is in agreement with conclusion of Pengo and co-workers that the analysis of a complete antiphospholipid antibody profile can better determine patients at risk as compared to a single test (39). Even more, some recent studies including multicentric study in the frame of European Forum on aPL clearly show, that beside the presence of aPL, their avidity is important for recognising patient at risk for thrombosis (17, 40, 41).

A statistically significant higher prevalence of either anti-β2-GPI or aPT were found in pAPS patients compared to SLE, which was not true for aANXV. These results supported the idea that aANXV represented a special group of autoantibodies of potentially protective function (22, 42 - 44). We did not find a significant association between the positivity for aANXV and expression of clinical features, what is in concordance with the results of Ogawa et al (45). On the other hand, patients with elevated IgG aANXV experienced less AB than patients without IgG aANXV which is in disagreement with the report of Kaburaki et al (46). The reason could be in selected group of patients or in dissimilarities of the determination methods (47).

Our findings support the extension of aPL determination from screening measurement of aCL to the measurement of antigenically defined aPL in unclear cases with a clinical picture of suspected APS. IgA (either aCL or anti-β2-GPI) improved neither the diagnostic specificity nor diagnostic sensitivity, but only increased the frequency of the total anti-β2-GPI which deserves further investigations.

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