Key Words
Antiphospholipid syndrome · Antiphospholipid antibodies · Lupus anticoagulants · Thrombosis

Abstract
Antiphospholipid antibodies are a wide and heterogeneous group of immunoglobulins, whose presence in patients with arterial and venous thrombosis, and obstetrical complications defines the antiphospholipid syndrome. We systematically reviewed published articles on this syndrome to investigate the association between thrombosis and the most common antiphospholipid antibodies. Lupus anticoagulants were a clear risk factor for thrombosis, irrespective of the site and type of thrombosis, the presence of systemic lupus erythematosus, and the methods used to detect them. Anticardiolipin and anti 2-glycoprotein I antibodies were possible risk factors of thrombosis, at least in some selected situations. Conversely, the measurement of antiprothrombin antibodies was not helpful to define the patient’s risk of thrombosis. These results are mainly due to the still far from optimal standardization of the methods to detect the various antiphospholipid antibodies.

Introduction
The combination of arterial and venous thromboembolic events, obstetric complications, and antiphospholipid antibodies defines the antiphospholipid syndrome [1]. Two forms have been described: the "primary" syndrome [2], where there is no evidence of an underlying disease, and the "secondary" syndrome, mainly in the setting of systemic lupus erythematosus [3]. In 1998, an international panel of experts met in Sapporo to establish the classification criteria for definite antiphospholipid syndrome [1]. Thrombosis may occur in any tissue or organ, and, with the sole exception of superficial venous thrombosis, must be objectively confirmed by imaging or ultrasound studies or histopathology. In case of histopathologic evaluation, inflammation should not be found in the vessel wall.

Antiphospholipid antibodies are a wide and heterogeneous family of IgG, and/or IgM, or less frequently also IgA, immunoglobulins, long considered to react with negatively charged phospholipids. Lupus anticoagulants and anticardiolipin antibodies were the first two such antibodies to be described. Lupus anticoagulants and anticardiolipin antibodies were the first two such antibodies to be described. Lupus anticoagulants behave as acquired inhibitors of coagulation, prolonging phospholipid-dependent coagulation [4], and anticardiolipin antibodies are usually identified by immunoassays with cardiolipin or other anionic phospholipids in solid phase [5].

The "Sapporo" laboratory criteria for definite antiphos-
pholipid syndrome require lupus anticoagulants and/or anti-cardiolipin antibodies to be present on two or more occasions at least six weeks apart [1]; lupus anticoagulants must be diagnosed according to the criteria proposed by the Scientific Subcommittee of Standardization of Lupus Anticoagulants/Phospholipid-Dependent Antibodies [6]; anticardiolipin antibodies must be measured by a "standardised" ELISA for β2-glycoprotein I-dependent antibodies; IgG and/or IgM anticardiolipin antibodies have to be present at medium or high titres. According to the Sapporo criteria, definite antiphospholipid syndrome is established when at least one clinical and one laboratory criteria are met.

**Antigens and Proposed Mechanisms of Action of Antiphospholipid Antibodies**

In the 1990s, work from different laboratories made it clear that lupus anticoagulants and anticardiolipin antibodies do not recognize anionic phospholipids, as long believed, but plasma proteins bound to suitable anionic surfaces. Among them, β2-glycoprotein I, and prothrombin are the most common and investigated antigenic targets. β2-glycoprotein I is required by the great majority of anticardiolipin antibodies to react with cardiolipin in immunoassays [7-9], whereas lupus anticoagulant activity in phospholipid-dependent coagulation tests is caused by subgroups of both antibodies [10-12].

For most antiphospholipid antibodies immune recognition does not occur in free solution, but requires antigen binding to a suitable anionic, non necessarily phospholipid, surface [7,8,12-16]. Whether this is due to the exposure of cryptic epitopes, or to the low-affinity nature of most antiphospholipid antibodies has not yet been fully elucidated. We [13,14] and others [15,16] have demonstrated that most anti β2-glycoprotein I and antiprothrombin antibodies are low affinity. In both cases, in fact, the engagement of two adjacent antigen molecules by the F(ab)2 portion of one IgG antiphospholipid antibody molecule at an anionic (phospholipid) surface leads to a substantial reduction of the dissociation constant of the antigen from the surface. The stable trimolecular complexes formed in this way interfere with the proper assembly of the phospholipid-dependent coagulation systems [13,14]. Interference of antiphospholipid antibodies with these reactions provides a rational explanation of the lupus anticoagulant effect exerted by anti β2-glycoprotein I and antiprothrombin antibodies. β2-glycoprotein I, in fact, slightly inhibits prothrombin activation by the prothrombinase complex [11], and factor X activation by the tenase complex [17]. Anti β2-glycoprotein I antibodies amplify and accelerate the former effect 11, and diminish the latter one [17]. On the other side, by reducing prothrombin availability, antiprothrombin antibodies directly inhibit prothrombin activation [10], and, provided the presence of human prothrombin, calcium ions ad anionic phospholipids, they also hamper factor X activation by the tenase complex 18. Thus, anti β2-glycoprotein I and antiprothrombin antibodies affect the same coagulation reactions through completely different mechanisms (Figure 1). In most patients, lupus anticoagulant activity is caused by a combination of anti-β2-glycoprotein I, and antiprothrombin antibodies [19]. In their study of 28 lupus anticoagulant-positive plasma samples, Horbach et al. [19] showed that the anticoagulant activity was totally dependent on antiprothrombin or anti β2-glycoprotein I antibodies in 4 and 7 cases, respectively. In the other 17 plasmas both antibodies contributed to lupus anticoagulant activity. The same group also demonstrated that it is relatively easy to distinguish between the two inhibitors by adding phospholipid vesicles composed either of pure cardiolipin or of a mixture of phosphatidylserine/phosphatidylcholine to the lupus anticoagulant screening test in plasma: the former neutralize β2-glycoprotein I-dependent lupus anticoagulants, the latter both antibodies [20].

![Fig. 1. Sites of action of anti 2-glycoprotein I and antiprothrombin antibodies in the phospholipid-dependent coagulation reactions. The X signs indicate the inhibition of the enzymatic reaction by the antibodies complexed with the respective antigens.](image-url)
Other antigenic targets of antiphospholipid antibodies are listed in Table 1. As most of these proteins are involved in the initiation and control of blood coagulation, it is conceivable that antibodies that reduce their availability or hamper their function may affect the pro- and anti-coagulant balance, thus increasing the thrombotic risk of antiphospholipid-positive patients. Several murine models of antiphospholipid syndrome support the possibility that antiphospholipid antibodies play a causative role in the pathogenesis of the antiphospholipid syndrome-related complications. However, no definite demonstration of such a role has yet been convincingly given in humans.

Table 1. Antigenic targets of antiphospholipid antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2-glycoprotein I</td>
<td>(Human) prothrombin (activated) protein C</td>
</tr>
<tr>
<td>Protein S</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>Oxidized low-density lipoproteins</td>
<td>Factor XII</td>
</tr>
<tr>
<td>High- and low-molecular weight kininogens</td>
<td>Factor VII/VIIa</td>
</tr>
<tr>
<td>Complement components H and C4b</td>
<td></td>
</tr>
</tbody>
</table>

**Laboratory Detection of Antiphospholipid Antibodies**

Laboratory diagnosis of antiphospholipid antibodies is based on coagulation tests for lupus anticoagulants, and in-house or commercially available enzyme-linked immunosorbent assays (ELISA) for anticardiolipin, anti-β2-glycoprotein I, and antiprothrombin antibodies. Here, we tackle some of the open problems in antiphospholipid testing.

With respect to lupus anticoagulants, their heterogeneous nature makes it necessary to perform more than one coagulation assay to reach a proper diagnosis. As no single test is 100% sensitive and specific, a large variety of "screening" and "confirmatory" assays has been proposed during the last 20 years. The Subcommittee of Standardization of Lupus Anticoagulants/Phospholipid-dependent Antibodies, and the British Committee for Standards in Haematology set criteria and guidelines, which have been repeatedly updated [6,21]. Nevertheless, the diagnosis of lupus anticoagulants may remain cumbersome and difficult. This is partly due to the high degree of heterogeneity in reagents, methods, instructions, and the methods used to express the results, as pointed out by national and international surveys [22-24]. The first French interlaboratory survey on lupus anticoagulants reported a correct diagnosis by only 41% of more than 4,000 laboratories [22]. A plasma containing anti-factor VIII antibodies was misdiagnosed as lupus anticoagulant-positive by 20% of the laboratories participating in a survey of the European Concerted Action on Thrombophilia (ECAT) [23]. In a National External Quality Assessment Scheme (NEQAS) performed in the United Kingdom more than half laboratories failed to identify a weak lupus anticoagulants, and 26% of them misdiagnosed a factor IX deficiency for lupus anticoagulants [24]. These data are, at least, discouraging, but leave room for improvement. In fact, adoption of common guidelines may increase diagnostic accuracy of lupus anticoagulant testing, as suggested by a recent NEQAS survey [25]: a remarkably low rate of false-positive results (3%) was observed, even though the prevalence of false-negative results was still 18%. Also the availability of plasmas containing monoclonal antibodies to β2-glycoprotein I and prothrombin may help to overcome the lack of internationally accepted reference and control materials [26]. Plasmas spiked with different amounts of monoclonal antibodies to human β2-glycoprotein I were evaluated in the first ECAT survey on lupus anticoagulants [27]: 82% of 59 laboratories correctly identified the strongly positive sample, and 37% of them the weakly positive one. In principle, monoclonal antibodies might be used also to quantify lupus anticoagulants and discriminate the clinically relevant subtypes. Studies along this line of research are in progress [26].

With respect to anticardiolipin, anti-β2-glycoprotein I, and antiprothrombin antibody measurement, the situation is not better. Most laboratories now commonly use the so-called "Harris" or "Louisville" standards and express anticardiolipin results in units, even though a multicenter study did not show any advantage [28]. Despite several standardization workshops on anticardiolipin antibodies have been performed since 1987 [29-32], a recent international survey organized by the European Forum on antiphospholipid antibodies showed an agreement with the "consensus" values of 25% for G and 0% for M anticardiolipin antibodies among 24 Centres [33]. Similar, disappointing results have been shown by other groups of investigators [34]. Things do not really change in the case of anti-β2-glycoprotein I antibodies, as shown by the European Forum on antiphospholipid antibodies: the prevalence of concordant results was 37% for IgG and 27% for IgM antibodies among 21 Centres [35]. Anyway, it must be stressed that the majority of the samples were in the low-positive range, and that the agreement on anti-β2-glycoprotein I results with high- and medium-positive samples was better. The use of an uniform way to deter-
mine the cut-off of positivity and the method of β2-glycoprotein I purification were identified among the main variables that influence the ELISA outcome. So far, no national or international surveys have been performed for antiprothrombin antibodies. Recently, Donohoe et al. [36] attempted to find the optimal assay conditions for their detection, identifying a number of pre-analytical and analytical factors that may influence the test outcome. Heterogeneity in reagents, calibrators, assay conditions, and methods used to calculate the results were again, the main variables responsible for ELISA discrepancies.

Besides the problems of assay sensitivity to the different antiphospholipid antibodies, their specificity to the clinical events of the antiphospholipid syndrome raises important concerns. In fact, antiphospholipid antibodies have been identified in the course of infections, administration of certain drugs, and neoplastic diseases [37-42]. In general, these antibodies are associated neither with thrombosis nor obstetric complications. Antiphospholipid antibodies may be found also in apparently normal subjects, with a prevalence dependent on the type of antibody and the population under investigation. Most data deal with anticardiolipin antibodies, which have been reported in 1-9% of blood donors [43,44], and in 12-52% of apparently healthy elderly aged more than 65 years [45,46]. In most cases, the antibodies are transient, have a low titre, and are not associated with thrombosis. Few studies investigated the presence of lupus anticoagulants in healthy individuals: figures ranging from 0 to 3.6% were reported in blood donors [44,47].

In conclusion, the coagulation tests and ELISAs presently in use to detect the different antiphospholipid antibodies are far from being properly standardized. The development of common protocol procedures and the production of reference materials are in progress, and, hopefully, will help improving harmonization among laboratories.

**Clinical Relevance of Antiphospholipid Antibodies**

Taken into account the heterogeneity of antiphospholipid antibodies, the lack of laboratory standardization, and that not all antibodies are associated with the clinical events of the antiphospholipid syndrome, the best way to validate optimal screening tests is to look for their correlations in clinical trials. We undertook this task, to support the inclusion of lupus anticoagulants, anticardiolipin, anti-β2-glycoprotein I, and antiprothrombin antibodies as laboratory criteria for the antiphospholipid syndrome in relation to thrombosis. Despite the large body of publications, it was impossible to perform a meta-analysis, because the studies were too different in terms of enrolment criteria, clinical end-points, design, and laboratory tests used to detect the antibodies. We, thus choose to perform a systematic review. For lupus anticoagulants and anticardiolipin antibodies we focused on prospective, cross-sectional, case-control, and ambispective studies because they provide different but complementary information about how long the antibody has been present and the risk of thrombosis. This selection was virtually impossible for studies on anti-β2-glycoprotein I and antiprothrombin antibodies, which were mostly retrospective. Retrospective studies have a low level of evidence, because they do not provide the objective documentation of thrombosis, a temporal sequence between measurement of the antibodies and occurrence of the events, and the presence of a control group.

**Lupus Anticoagulant and Anticardiolipin Antibodies**

We made a systematic computer-assisted (MEDLINE) search of the literature published in the English language from 1988 through 2001 on lupus anticoagulants and anticardiolipin antibodies [48]. Twenty-five studies were selected for their prospective (n=9), ambispective (n=2), cross-sectional (n=3), and case-control (n=11) design. They provided on enabled us calculate the odds ratio (OR) with 95% confidence interval (CI) of lupus anticoagulants and anticardiolipin antibodies for arterial and/or venous thrombosis in 4,184 patients and 3,151 controls (Table 2).

Five studies compared lupus anticoagulants with anticardiolipin antibodies: the OR with 95% CI of lupus anticoagulants for thrombosis was always significant. None of them found anticardiolipin antibodies were associated with thrombosis. Four studies analysed only lupus anticoagulants: the OR with 95% CI was always significant. Sixteen studies served to assess 28 associations between anticardiolipin antibodies and thrombosis: the OR with 95% CI was significant in 15 cases.

**Table 2.** Strength of the association between lupus anticoagulants, anticardiolipin antibodies and thrombosis.

<table>
<thead>
<tr>
<th>Type of thrombosis</th>
<th>Lupus Anticoagulants</th>
<th>Anticardiolipin antibodies*</th>
<th>Odds Ratio range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial</td>
<td>2/2</td>
<td>13/19</td>
<td>8.65 - 10.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n.s. - 18</td>
<td>2.51</td>
</tr>
<tr>
<td>Venous</td>
<td>5/5</td>
<td>2/12</td>
<td>4.09 - 16.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n.s. - 2.51</td>
<td>1/2</td>
</tr>
<tr>
<td>Any*</td>
<td>2/2</td>
<td>1/2</td>
<td>5.71 - 7.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n.s. - 3.66</td>
<td>n.s. * not significant.</td>
</tr>
</tbody>
</table>

*No distinction was possible between arterial and venous thrombosis; **No distinction was made between anticardiolipin isotypes; n.s. * not significant.
The review formally established that lupus anticoagulants are strong risk factors for thrombosis, irrespective of its site and type, and presence of systemic lupus erythematosus. The tests used to detect lupus anticoagulants are another relevant issue, in the light of the many efforts aimed at establishing whether single tests or panel of assays stand up better than others in terms of the clinical relevance of lupus anticoagulants. Using in-house tests, our group reported that the dilute Russell's viper venom time (dRVVT) is more strongly associated with thrombosis than the kaolin clotting time [49]. We [50] and others [51,52] subsequently confirmed and extended these findings to commercially available dRVVTs. However, the retrospective nature of these studies weakens the strength of the reported associations, and calls for caution when interpreting their results. Although it is yet impossible to foresee whether and which coagulation test(s) predicts the risk of thrombosis, our systematic review underscores that the diagnosis of lupus anticoagulants is relevant to the single patient rather than the tests used to reach it.

Anticardiolipin antibodies were not such strong risk factors as lupus anticoagulants, unless the G isotype, and medium or high titres were considered. Separate analysis of the different types of thrombosis showed anticardiolipin antibodies were associated with cerebral stroke and myocardial infarction, but not with deep vein thrombosis.

In conclusion, the systematic review confirmed the inclusion of lupus anticoagulants as laboratory criterion of the antiphospholipid syndrome in relation to arterial and venous thrombosis. Uncertainty still exists with respect to anticardiolipin antibodies.

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**Antibodies to beta-2-glycoprotein I and Antiprothrombin Antibodies**

Since their first description, anti beta-2-glycoprotein I and antiprothrombin antibodies have been amply studied for their relationship with thrombosis. Investigators are now inclined to consider them good candidate laboratory criteria for the antiphospholipid syndrome, together with or possibly as replacement for lupus anticoagulants and anticardiolipin antibodies.

To contribute to this issue, we extended our computer-assisted search of the literature (MEDLINE) to studies on anti beta-2-glycoprotein I and antiprothrombin antibodies [53]. All series of ten or more patients were classified according to the antiphospholipid antibody type and underlying disease, and information about study design and assay methods was recorded. Thirty-two articles were retrieved: cross-sectional and case-control studies gave information on 1,324 patients and 1,973 controls, and retrospective studies contributed another 3,778 patients. All but four studies employed in-house assays, mostly ELISA.

Systemic lupus erythematosus, the antiphospholipid syndrome, and the presence of lupus anticoagulants and/or anticardiolipin antibodies were the enrolment criteria in 26 studies. This makes it difficult to establish the relative roles of anti beta-2-glycoprotein I and antiprothrombin antibodies as independent risk factors of thrombosis. Only 11 studies did multivariate analysis using logistic regression, which allows a summary of the risk assessment, given the joint contribution of each risk factor.

The OR with 95% CI for thrombosis were available or could be calculated in 28 studies on 4,394 patients and 1,973 controls for anti beta-2-glycoprotein I antibodies, and in 17 studies on 2,339 patients and 613 controls for antiprothrombin antibodies.

**Table 3. Strength of the association between anti beta-2-glycoprotein I and antiprothrombin antibodies and thrombosis: analysis in relation to the type of thrombosis.**

<table>
<thead>
<tr>
<th>Type of thrombosis</th>
<th>Antibodies* to beta-2-glycoprotein I</th>
<th>Prothrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of significant / Total N. of available associations</td>
<td>Odds Ratio range</td>
</tr>
<tr>
<td>Arterial</td>
<td>5/17</td>
<td>n.s. - 3.4</td>
</tr>
<tr>
<td></td>
<td>n.s. - 8.3</td>
<td></td>
</tr>
<tr>
<td>Venous</td>
<td>12/21</td>
<td>n.s. - 11.5</td>
</tr>
<tr>
<td></td>
<td>n.s. - 19.0</td>
<td></td>
</tr>
<tr>
<td>Any*</td>
<td>17/22</td>
<td>n.s. - 4.4</td>
</tr>
<tr>
<td></td>
<td>n.s. - 27.1</td>
<td></td>
</tr>
</tbody>
</table>

*No distinction was possible between arterial and venous thrombosis; **No distinction was made between isotypes; n.s. = not significant.

**Table 4. Strength of the association between anti beta-2-glycoprotein I and antiprothrombin antibodies and thrombosis: analysis in relation to the antibody isotype.**

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Antibodies* to beta-2-glycoprotein I</th>
<th>Prothrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of significant / Total N. of available associations</td>
<td>Odds Ratio range</td>
</tr>
<tr>
<td>G</td>
<td>20/33</td>
<td>n.s. - 11.5</td>
</tr>
<tr>
<td>M</td>
<td>7/15</td>
<td>2/14</td>
</tr>
<tr>
<td>A</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Any*</td>
<td>4/9</td>
<td>4/8</td>
</tr>
</tbody>
</table>

*No distinction was possible between isotypes; **No distinction was possible between type of thrombosis.

Sixty associations between anti beta-2-glycoprotein I antibodies and thrombosis were available: overall, 34 of them reached significance. They were 5 of 17 associations with arterial thrombosis, 12 of 21 with venous events, and 17 of 22 with any type of thrombosis (which means that no distinction between arterial and venous thrombosis was possible) (Table 3). The association between anti beta-2-glycoprotein I and thrombosis was analysed also in relation to the anti-
body isotype (Table 4). Overall, IgG anti β2-glycoprotein I antibodies seemed more consistently associated with thrombosis than IgM antibodies. IgA anti β2-glycoprotein I antibodies were always significantly associated with thrombosis. However, as only one study was available, no definite conclusion can be reached about the clinical significance of this isotype. The lack of reference materials to quantify anti β2-glycoprotein I antibodies meant we could not assess whether the risk correlated with their titre.

Although these data suggest that IgG anti β2-glycoprotein I antibodies are associated with thrombosis, a number of issues raise concern. Firstly, significant associations were reported only by retrospective studies. Secondly, only a minority of studies confirmed these findings by multivariate analysis. Finally, when the antibodies were analysed in relation to the type of thrombosis, they were not associated with arterial, and only marginally with venous events. Based on these observations, the role of anti β2-glycoprotein I antibodies as laboratory criteria for the antiphospholipid syndrome remains to be established.

Forty-six associations between antiprothrombin antibodies and thrombosis were available for the review: only 17 (37%) reached significance (Tables 3 and 4). No clear association with thrombosis was found, irrespective of antibody isotype, site and type of event, and systemic lupus erythematosus. Whether the presence of antiprothrombin antibodies further increases the risk of thrombosis carried by lupus anticoagulants and, possibly, anticardiolipin antibodies has still to be defined. Also the utility of their detection in clinical practice remains to be established.

Conclusions

We reviewed a wide selection of clinical studies on antiphospholipid antibodies, and formed the opinion that they are, at best, inconclusive. Only lupus anticoagulants were consistently associated with thrombosis, which implies that measuring them is helpful to define the patients’ risk of arterial and venous thrombosis, and to guide therapeuetic management. The results of studies on anticardiolipin and anti β2-glycoprotein I antibodies are less convincing and partly controversial, but they leave open the possibility that their measurement too may be practical and useful, at least in some situations. At present, there does not seem to be any role for measurement of antiprothrombin antibodies.

We foresee the need for well-designed clinical trials, to help establish which, if any, among the various antibodies, are risk factors for the antiphospholipid syndrome. To accomplish this, standardization or at least harmonization of the methods used to detect the antiphospholipid antibodies is mandatory. Without it, any clinical study will be criticisable and unable to reach firm conclusions.

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