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EDITORIALE

Laboratory diagnosis of antiphospholipid syndrome

La diagnosi di laboratorio della sindrome da antifosfolipidi

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Diagnosis of antiphosholipid syndrome (APS) is based on laboratory detection of antiphospholipid (aPL) antibodies in patients with documented thrombosis or in women with pregnancy morbidity. Recently, both clinical and laboratory criteria were revised on the basis of an international consensus conference held in Sydney (1). The previous international consensus statement of one clinical and one laboratory criterion to diagnose APS was maintained (2) but time-lapse between the previous thromboembolism and laboratory diagnosis should not exceed 5 years. Moreover, laboratory tests should not be performed in the 12 weeks following the event to avoid any interference of the acute phase of the disease. Thus, laboratory evaluation of venous thromboembolism (VTE) should not be requested during the hospital stay as tests may be false-positive with no influence on the treatment regimen. The situation is different, however, when testing for aPL in patients with cerebral ischemia (TIA/stroke) or thrombosis-related arterial events. In our opinion, an early marked aPL positivity may induce clinicians to switch treatment from antiplatelet drugs to oral anticoagulants. Moreover, diagnosis cannot be delayed when there is a suspicion of catastrophic APS. New criteria correctly stated that thrombosis must be confirmed by objective validated tests (i.e.unequivocal findings of appropriate imaging studies or histopathology). Furthermore, two subgroups of APS patients should be recognized, according to:

a) the presence, or
b) the absence of additional risk factors for thrombosis.

Clinical criteria related to pregnancy morbidity were unchanged from the previous consensus but a better definition of preeclampsia/eclampsia and placental insufficiency was reported.

Laboratory criteria were lupus anticoagulant (LAC), anticardiolipin (aCL) antibodies and anti β_2-Glycoprotein I (aβ_2GPI) antibodies of IgG and/or IgM isotype. The introduction of the latter criterion was made after a majority voting. To be considered positive each test had to be confirmed at least 12 weeks apart. LAC should be detected according to the guidelines of the International Society on Thrombosis and Hemostasis (3) and aCL/ab$_2$GPI antibodies measured by a standardized enzyme-linked immunosorbent assay (4, 5). The last consensus conference did not specify criteria for LAC positivity. However, it did introduce those for aCL antibody positivity (i.e. >40 GPL or >40 MPL, or a value over the 99th percentile for normal subjects) and aβ_2GPI antibody positivity (value over the 99th percentile for normal subjects).

Finally, the most important note was that investigators are strongly advised to classify APS patients into one of the following categories: category I when more than one laboratory criteria is present (any combination), category IIa when lupus anticoagulant is present alone, category IIb when anti-cardiolipin antibodies are present alone, category IIc when anti-β_2 glycoprotein-I antibodies are present alone.

Many studies have shown that among the tests exploring the presence of antiphospholipid antibod-

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ies, LAC is the strongest risk factor for thrombosis. Therefore, in our opinion, the first screening test to detect the presence of antiphospholipid (aPL) antibodies should be a coagulation test. In an analysis of the literature published between 1988 through 2001, a clear association between LAC positivity and thrombosis (OR range 5.71-7.3) was shown (6). Although grouping different studies (retrospective, ambispective, prospective) may influence the quality of results, the strong association of LAC with thrombosis may suggest that this test is the only one to rely on for a diagnosis of APS. Analyzing the studies between 1988 through 2001 it was found that the number of significant associations between aCL antibodies and thrombosis were found in only 6 out of 13 studies and the number of significant associations between aβ_2GPI antibodies and thrombosis were 10 out of 13 (7). Association with thrombosis is thus not significant for aCL nor for aβ_2GPI antibodies. In a cohort study our group found a significant association with thrombosis for LAC and aβ_2GPI antibodies and no association with aCL titre of more than 40 GPL or MPL (8).

It is not clear from Sidney consensus conference if diagnosis of APS could be made performing a single test. If this the case, frequent false positive and to lesser extent false negative results can be obtained in each test and this aspect will be analyzed. In our opinion all the three tests should be performed and patients classified according to their antiphospholipis antibody profile.

PROBLEMS IN EVALUATING RESULTS OF EACH LABORATORY TEST

Lupus anticoagulant

Unfortunately this test is not standardised and reference material is not available. Absence of reference material comes from the non specificity of involved antibodies formerly identified in anti β_2GPI and anti prothrombin antibodies. The culprit anitibodies were recently believed to be those directed against β_2GPI, but only those directed against the domain 1 of the molecule (9).

We have tested the performance of Clinical Laboratories in the frame of the Italian Federation of Thrombosis Centers (FCSA) by using affinity purified IgG with LAC activity strongly positive in aCL and aβ_2GPI assays (10). Three of the six samples were positive at high, moderate and low intensity (the same batch of IgG was diluted 1:2 and 1:4 with normal plasma) and three samples were negative. In one negative plasma sample heparin was added and another plasma was negative but contained reduced levels of vitamin k-dependent coagulation factors. It was found that most laboratories were able to detect LAC and half of them were able to differentiate the intensity of positive samples. In the same way most laboratories excluded LAC in the negative sample while false positive results were reported by around 25% of laboratories for heparinized normal plasma and for 'anticoagulated' plasma. What happens when a false positive diagnosis of LAC is made? Diagnosis of APS in the presence of clinical criteria is made and long term oral anticoagulant treatment is prescribed. Diagnosis of LAC is thus critical and every effort should be made to render this assay more accurate.

To better understand the real life in Italian Laboratories concerning LAC diagnosis we asked the participants to collect LAC positive plasma and to send it to our reference laboratory for further confirmation (11). We have received 301 LAC positive plasma samples and found that 71 were false positive. This latter group significantly differed from that in which LAC was confirmed in patients who were older and were first diagnosed in the lab and LAC was appraised as mild. Moreover more false positive LAC were found in patients on oral anticoagulant treatment.

LAC potency is an interesting characteristic to be considered when evaluating a positive LAC. It has been demonstrated that increasing the cut-off levels for LAC positivity results in a selection of patients with thromboembolic events (12). Furthermore, LAC potency is significantly stronger when both coagulation tests employed diagnosed the presence of the inhibitor (11).

ACL antibodies

In a survey on the performance of Italian laboratories to identify positive and negative aCL and aβ_2GPI samples we found a correct interpretation of high positive and negative samples by both ELISAs (13). Nonetheless, the high variability of reported data using the same commercial kit (cases in which the same sample was negative for a centre and highly positive for another centre were common) remains a major problem that only a consensus on the part of laboratories and manufacturers to utilize standard, uniform materials and procedures can hope to overcome. Therefore, there are many difficulties connected to standardization and

reference material (4). Moreover, in the healthy elderly population, the detection of positive tests is not rare (14) and a correction for the age of aCL cut-off levels should be considered (15).

Anti β_2-glycoprotein I antibodies

Previous studies have demonstrated marked differences from laboratory to laboratory in the materials and procedures utilized, which is the cause no doubt of the variance in results (15). High variability using the same commercial kit has been demonstrated by our group (13).

INTERPRETATION OF ANTIPHOSPHOLIPID ANTIBODY PROFILES

The report of the Sydney consensus conference clearly states that a single positive test among the three laboratory criteria allows diagnosis of APS to be made. In this way laboratories performing a single test could diagnose APS without knowing results of the other two tests. In addition to the fact that the amount of false positive results of a single test is not negligible, the possibility of classifying patients in category I (multiple positivity - high risk patients) is not met. Therefore all the three tests must be performed and pathologists and clinicians should draw conclusion on the basis of laboratory profiles and clinical events.

Profiles with a single positive test

• [positive LAC; negative aCL; negative aβ2GPI] In patients with positive LAC and normal aCL and aβ_2GPI, a false positive LAC should be taken into consideration. If LAC positive only is confirmed, these patients may be considered at low risk of thrombosis (8, 11).

• [negative LAC; positive aCL; negative aβ2GPI] aCL ELISA suffer from interpretation problems especially when it is the only positive test out of those determining the presence of aPL antibodies. Moreover, when isotypes from ELISAs obtained for aCL positive patients were considered, we have shown that only the IgG isotype was associated with the presence of a previous thromboembolic event or obstetric complications (8). Autoimmune anticardiolipin antibodies are directed against β_2-glycoprotein I which is the relevant autoantigen in APS. When aCL is positive but the same aβ_2GPI isotype is negative then the aCL test may be false-positive or the aCL may bind to bovine β_2GPI or directly to cardiolipin. In 8 patients with suspected APS in which aCL was the only positive test, we found that 5 individuals had antibodies to bovine instead of to human β_2GPI (17). These subjects may be incorrectly classified as APS patients in the absence of autoimmune antibodies (i.e. anti human β_2GPI). It would seem from these data that human β_2GPI-dependency of aCL should be assayed using the combined testing by ELISA of aCL and anti human β_2GPI antibodies. This approach would avoid overdiagnosing APS by identifying only patients with an autoimmune disease. Nevertheless, the Sydney consensus statement (6) confirmed that positivity of aCL, based on a single positive test result, remains a criterion for the diagnosis of APS and did not specify how to test for autoimmune aCL. Though a better definition of the threshold for positive aCL (>40 GPL or MPL units, or >99th percentile) was introduced (1), the role of aCL as the sole positive test to diagnose APS was not discussed. We have recently shown that when individual tests (LAC, aCL, aβ_2GPI) were considered in a multivariate analysis taking age, gender, the presence of SLE or other autoimmune diseases and established risk factors for venous and arterial thromboembolism into account, aCL antibodies were not an independent risk factor for thrombosis (8).

• [negative LAC; negative aCL; positive aβ_2GPI] As suggested at the 48th SSC/ISTH meeting held in Boston in June, 2002 (18), positivity of anti-human β_2GPI antibodies should be included in the laboratory criteria of APS, as it identifies LA-positive patients at risk for thrombosis (19,9) and autoimmune aCL (17). In the Sydney consensus statement (1) it was indeed decided (by the majority) that IgG and IgM aβ_2GPI (in title >99th percentile) should be included as part of the modified Sapporo criteria. There is evidence that aβ_2GPI antibodies are an independent risk factor for thrombosis and pregnancy loss (20-22) but a recent metanalysis of available studies was unable to reach a clear conclusion (7). It is important to underline that apparently only some antibodies to a specific domain of β_2GPI express LAC activity and correlate strongly with thromboembolic events. Therefore, IgG aβ_2GPI are associated with thrombosis only in a subset of patients. Other autoantibodies to β_2GPI may not be pathogenic and this might explain why studies on their detection have not produced uniform results (7). In those cases (from 2% to 10%) in which aβ_2GPI is the only positivity detected in patients with clinical manifestations of the antiphospholipid syndrome (23, 24), aβ_2GPI may not

be pathogenic as these antibodies do not recognize β_2GPI bound to an anionic PL surface. To homogenize test results from various laboratories aβ_2GPI antibodies should be tested following the indications of the Standardization Group of the European Forum on antiphospholipid antibodies (16).

Table I - Interpretation of most frequent aPL profiles.

LAC	*aCL*	*β2GPI*	*Thrombosis*	*Pregnancy loss*
+	+	+	+++	+++
-	+	+	+	++
-	+	-	-/?	-/?
-	-	+	-/?	-/?

Profiles with multiple positive tests

• [negative LAC; positive aCL; positive aβ_2GPI]
The simultaneous positivity of aCL and aβ_2GPI of the same isotype is very helpful as it excludes the presence of infective antibodies and confirms the presence of relevant autoimmune antibodies. We have found that this aPL profile (IgG isotype for both tests) is associated with thrombosis but the association is much stronger with pregnancy morbidity (8). Titre of aβ_2GPI antibodies is significantly lower than that of triple positive patients and this probably explains the absence of LAC activity in plasma samples.

• [positive LAC; positive aCL; positive aβ_2GPI]
A full positive pattern appears to reflect the presence of significant amounts of autoantibodies to human β_2GPI with a consequent increased risk of thrombosis-related events or obstetric complications (25). These patients should be classified as a high risk, homogeneous group of APS for whom treatment efficacy should be documented by specific clinical trials and new therapeutic procedures should be considered (26).

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