## Asymptomatic carriers presenting a high-risk antiphospholipid antibody profile.

### I. Introduction

Antiphospholipid antibodies (aPLs) constitute a heterogeneous group of auto-antibodies associated with thrombosis related events, and define the anti-phospholipid syndrome (APS) (22). APS is an autoimmune disease characterized by venous or arterial thrombosis and/or obstetric complications. Clinical criteria associated with the persistent positivity of at least one aPL, such as Lupus Anticoagulant (LA) or antibodies directed against Cardiolipin (aCL) or β2-Glycoprotein I (aβ2GPI) of IgG or IgM isotype, is necessary for APS diagnosis (6). Functional tests, based on clotting time are used for the detection of LA, described as the most correlated marker to clinical manifestations (30). APS mainly affecting young people, is a severe disease, which requires long-term anticoagulant therapy, and despite this, thrombotic recurrences are frequent.

aPLs are not only used for APS diagnosis, they also have an important role in the pathophysiology of the disease. A two hit hypothesis has been suggested to explain why thrombotic events occur only occasionally in spite of the persistent presence of autoantibodies (22). aPL (first hit) increase the thrombotic risk and clot takes place in the presence of another thrombophilic condition, such as infectious or inflammatory diseases (second hit) (22).

It is now widely recognized that multiple positivity of aPL is more frequently associated with thrombotic or obstetrical events than single one (38, 41). Pengo et al demonstrated that among multiple positivity, triple positivity which is defined as the association of LA, aCL and aβ2GPI, irrespective of their isotype, represents the aPL profile associated with the highest risk of thromboembolic events occurrence and severe pregnancy morbidity (64).

However, triple positivity is also described in asymptomatic “carriers” raising the dilemma of whether to initiate or not a primary thromboprophylaxis. To better stratify the thrombotic risk, an appropriate interpretation of aPL positivity has been challenging. To this end, we performed a retrospective study on 7111 samples to select triple positive patients presenting or not thrombotic related events. Among them a comparison was made between

"clinical" and "carrier" patients leading us to propose a score to stratify the thrombotic risk. This score was then tested in a follow up study performed on triple positive "carriers".

## II. Material and methods

### 1. Patients and study design

In this retrospective study, we analysed over a 2-year period, consecutive venous blood samples, for LA, IgG/IgM aCL and IgG/IgM aβ2GPI antibodies routine detection. Samples were sent to immunology and hemostasis hospital laboratories. Reasons for testing were mainly an autoimmune disorder, recent venous or arterial thrombosis, previous pregnancy morbidity, pregnancy monitoring, and exploration of prolonged partial thromboplastin time.

7111 samples were tested, corresponding with 6500 patients. Subjects were grouped according to their laboratory profile. We considered LA positive if at least one of the two tests performed was positive (PTT-LA and dRVVT), aCL or aβ2GPI positive if IgG or IgM isotype test detection was positive. We obtained 528 patients with a single test positive (208 LA positive, 157 aCL positive and 163 aβ2GPI positive), 116 patients double positive (33 LA & aβ2GPI positive, 38 LA & aCL positive, and 45 aCL and aβ2GPI positive) and 93 triple positive patients (Fig. 1).

Exclusion criteria were an age younger than 18 years old, or older than 80 years old; and evidence of no aPL persistence explained by an infection.

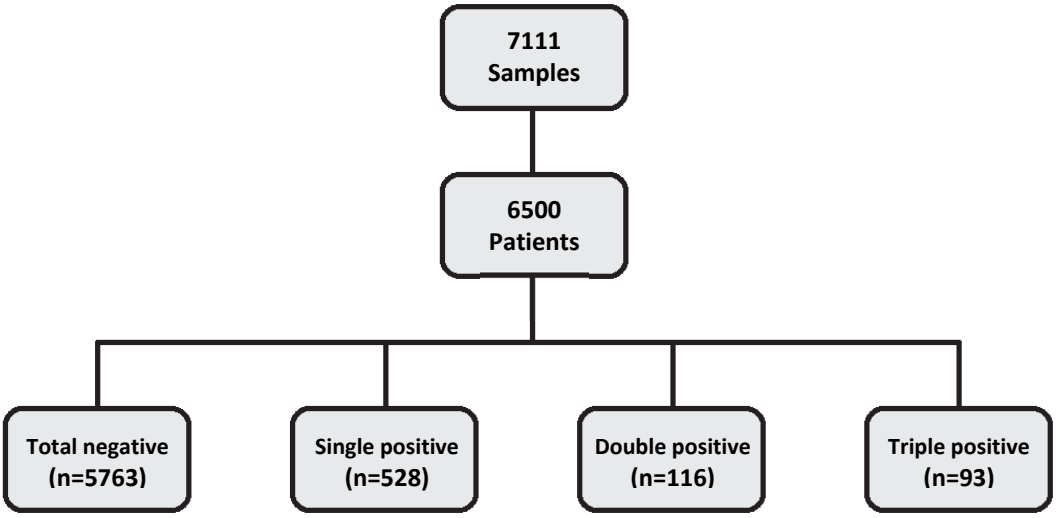


*Fig. 1 Patients selection*

## 2. Data collection

The following clinical data were retrospectively systematically collected : (i) history of venous or arterial thromboembolism (such as deep vein thrombosis, pulmonary embolism, stroke, transient ischemic attack, myocardial infarction); (ii) history of obstetrical complications; (iii) clinical manifestations not included in revised criteria (6); (iv) arterial risk factors (smoking habit, hypercholesterolemia, high blood pressure, diabetes, obesity, family history), (v) venous risk factors (combined oral contraceptive, pregnancy, neoplasia, thrombophilia, family history).

*NB: Concerning « family history » we were looking for: history of venous thromboembolism (VTE) in first-degree relative (parent or sibling); or history of myocardial infarction or sudden cardiac death in a first-degree relative men younger than 55 years old or in a first-degree relative women younger than 65 years old; or history of stroke in a first degree relative (parent or sibling) before 45 years old.*

Furthermore, we systematically collected those biological data : (i) persistence of aPL; (ii) persistence of triple positivity; (iii) presence (or absence) of antinuclear antibodies; (iv) presence (or absence) of anti-DNA; (v) ABO blood group status, considering only “O blood groups” versus “non-O blood groups”, since it is well established that O blood group status is a risk factor for thrombosis (75), (vi) platelets, (vii) hemoglobin, with Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin Concentration (MCHC), (viii) white blood cells and neutrophils, (ix) bilirubin, LDH and haptoglobin, to evaluate a potential hemolysis.

## 3. Laboratory tests

Lupus anticoagulant (LAC) was determined as recommended by International Society of Thrombosis and Haemostasis (ISTH) using two clotting times: Partial Thromboplastin Time-Lupus Anticoagulant (PTT-LA) by STAGO® and diluted Russel Viper Venom Time (dRVVT) by STAGO®. We calculated the Rosner Index (RI) and we considered positive when RI > 15. Results of dRVVT were expressed with a Normalized Ratio (NR) (positive value, > 1.2).

IgG and IgM aβ2GPI were measured with two specific ORGENTEC diagnostika® kits ELISA using purified B2GPI directly bound to the microwells. The same cut off was set at 8 UI/mL (76). The assays were performed following the manufacturer’s instructions.

aCL was determined with two home-made ELISA as previously described (77). The aCL positivity is concluded if test results are higher than 22 UI/mL for IgG and 10 UI/mL for IgM (calculated using the 99$^{th}$ percentile of the geometric mean for 100 blood donors).

### 4. Statistical analysis

Statistical analysis was performed using R software. Continuous variables were compared by Student's t test and categorical data by the $\chi^2$ test or Fisher test. Estimation of odds ratio for each variable was calculated using univariate logistic regression. Variables with p-values < 0.20 were considered as candidates for the multivariate analysis. For multivariate analysis, we considered two or most cardiovascular risk factors (including high blood pressure, diabetes, obesity, dyslipidemia, smoking habit, combined oestroprogestative contraceptive and age older than 45 years old). All tests were two-sided. $p<0.05$ was retained for significance.

## III. Results

Over a 2-year period, we selected 93 patients presenting a triple positivity, defined as the association of LA, IgG and/or IgM aCL, and IgG and/or IgM aβ2GPI. Among them, 5 patients were excluded according to criteria as defined in material and methods. As shown in Table 1, we constituted two groups of patients based on their clinical history.

The first one called "clinical group" is composed by 51 patients (58%) with history of recurrent superficial thrombosis (n=2) and thromboembolic events or obstetrical complications as defined by revised criteria of APS (n=49) (6). In the second group of 37 subjects (42%), named "carriers group", patients have never had such events. Some patients presented clinical features such as chorea, APS nephropathy, rheumatic mitral valve disease, migraine headache, or less than 2 early miscarriages.

A comparison between the two groups was performed according to their demographic and clinical data, but also to their cardiovascular risk factors including high blood pressure, diabetes, obesity, dyslipidemia, smoking habit, combined œstroprogestative contraceptive and age older than 45 years old.

| | Carriers patients (No(%)) | Clinical patients (No(%)) | p-value (Odds Ratio; Confidence Interval) |
|---|---|---|---|
| **Clinical history** | | | |
| No | 37 (100%) | 0 (0%) | < 0.0001 |
| Yes | 0 (0%) | 51 (100%) | |
| **Age** | 48.14y ± 15.98 | 46.06 ± 14.33 | 0,5233 (OR 0.99; IC 0.96-1.02) |
| **Weight** | 69.59 ± 17.21 | 72.15 ± 20.87 | 0,5651 (OR 1.01; IC 0.98-1.03) |
| **BMI** | 26.08 ± 6.49 | 25.88 ± 6.78 | 0,9054 (OR 1.00; IC 0.92-1.07) |
| **Gender** | | | |
| Men | 7(19%) | 17(33%) | 0,1339 (OR 0.47; IC 0.16-1.24) |
| Women | 30(81) | 34(67%) | |
| **ABO Blood group status** | | | |
| O blood group | 12(40%) | 16(38%) | 0,8702 (OR 1.08; IC 0.41-2.83) |
| Non O blood group | 18(60%) | 26(62%) | |
| **Cardiovascular risk factor** | | | |
| < 2 | 24(65%) | 22(43%) | **0,0440 (OR 2.43; IC 1.03-5.95)** |
| ≥ 2 | 13(35%) | 29(57%) | |
| **Triple positivity persistence** | | | |
| No | 2(7%) | 2(5%) | 1,0000 (OR 1.46; IC 0.17-12.79) |
| Yes | 28(93%) | 41(95%) | |
| **Autoimmune disease associated** | | | |
| No | 17(46%) | 30(59%) | 0,2319 (OR 0.60; IC 0.25-1.39) |
| Yes | 20(54%) | 21(41%) | |
| **Lupus Erythematosus** | | | |
| No | 29(78%) | 38(75%) | 0,6743 (OR 1.24; IC 0.46-3.50) |
| Yes | 8(22%) | 13(25%) | |
| **Recent infectious disease** | | | |
| No | 22(81%) | 33(94%) | 0,2233 (OR 0.27; IC 0.04-1.36) |
| Yes | 5(19%) | 2(6%) | |
| **Anti-Nuclear Status** | | | |
| Negative | 8(22%) | 20(43%) | 0,0522 (OR 0.39; IC 0.14-1.00) |
| Positive | 28(78%) | 27(57%) | |
| **Anti-DNA status** | | | |
| Negative | 25(78%) | 27(82%) | 0,7098 (OR 0.79; IC 0.23-2.70) |
| Positive | 7(22%) | 6(18%) | |
| **Anemia** | | | |
| No | 30(83%) | 41(85%) | 0,7939 (OR 0.85; IC 0.26-2.90) |
| Yes | 6(17%) | 7(15%) | |
| **Thrombopenia** | | | |
| No | 22(61%) | 37(77%) | 0,1131 (OR 0.47; IC 0.18-1.20) |
| Yes | 14(39%) | 11(23%) | |
| **Leucopenia** | | | |
| No | 33(92%) | 46(96%) | 0,6470 (OR 0.48; IC 0.06-3.04) |
| Yes | 3(8%) | 2(4%) | |
| **Neutropenia** | | | |
| No | 36(100%) | 48(100%) | nc |
| **Hemolysis** | | | |
| No | 14(61%) | 28(70%) | 0,4592 (OR 0.67; IC 0.23-1.98) |
| Yes | 9(39%) | 12(30%) | |

*Table 1 Characteristics of carriers versus clinical triple positive patients.*

As expected, history of thrombotic event or obstetrical complications was significantly associated with “clinical group” ($p < 0.001$). No difference was found between the two groups in term of age, gender, weight nor BMI, ABO blood group, cell numeration, an association with an autoimmune disease, hemolysis neither an infection.

We can note that no difference was found concerning the persistence of triple positivity (Odds Ratio 1.46; Confidence Interval 0.17-12.79). A trend of significance is observed regarding anti-nuclear status, in favour of the carriers group.

Interestingly, having two or more cardiovascular risk factors was significantly associated with the “clinical group” (Odds Ratio 2.43, Confidence Interval 1.03-5.95). This result was confirmed in a multivariate analysis (Odds Ratio 2.93; Confidence Interval 1.13-7.56).

Regarding the biology and in particular aPL profile, although twenty seven profiles are possible, fourteen biological profiles were found in our study (Fig. 2). The most prevalent profile, called “Double-Double” is composed by the association of the two positive LA tests (with RI > 15 and NR > 1.2) and double IgG isotype for both aCL and aβ2GPI. This double-double profile, with an Odds Ratio at 3.9 (Confidence Interval 1.43-12.01) is significantly associated with the “clinical group”, and represents an independent risk factor for thrombosis or obstetrical complications. This result was confirmed in a multivariate analysis (Odds Ratio 3.59, Confidence Interval 1.23-10.50). No statistical analysis of the others groups were performed due to low enrolment.

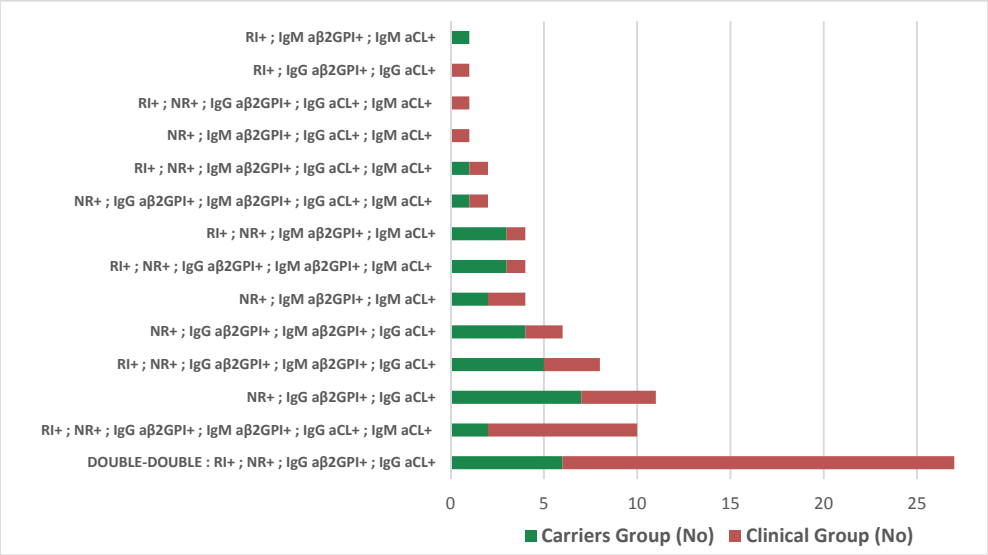


*Fig. 2 Biological profiles of carriers versus clinical triple positive patients.*

In order to better interpret aPLs positivity, each test was also individually analysed (Table 2). We found a significant association of IgM aβ2GPI with carriers patients and a trend of significance with the clinical group for Rosner Index and IgG aCL, with Odds Ratio at respectively 2.57 (CI 0.92-7.55) and at 2.96 (CI 0.93-10.47).

| | Carriers patients (No(%)) | Clinical patients (No(%)) | p-value (OR; CI) |
|---|---|---|---|
| **Rosner Index** | | | |
| < 15 | 12(36%) | 8(18%) | 0,07 (OR 2.57; CI 0.92-7.55) |
| > 15 | 21(64%) | 36(82%) | |
| **Normalized Ratio** | | | |
| < 1,2 | 1(3%) | 1(2%) | 1,00 (OR 1.43; CI 0.06-36.94) |
| > 1,2 | 35(97%) | 50(98%) | |
| **IgG aβ2GPI** | | | |
| < 8 UI/mL | 7(19%) | 5(10%) | 0,2187 (OR 2.15; CI 0.63-7.85) |
| > 8 UI/mL | 30(81%) | 46(90%) | |
| **IgM aβ2GPI** | | | |
| < 8 UI/mL | 13(35%) | 30(59%) | 0,0282 (OR 0.38; CI 0.15-0.90) |
| > 8 UI/mL | 24(65%) | 21(41%) | |
| **IgG aCL** | | | |
| < 22 UI/mL | 9(24%) | 5(10%) | 0,066 (OR 2.96; CI 0.93-10.47) |
| > 22 UI/mL | 28(76%) | 46(90%) | |
| **IgM aCL** | | | |
| < 10 UI/mL | 23(62%) | 34(67%) | 0,6623 (OR 0.82; CI 0.34-2.00) |
| > 10 UI/mL | 14(38%) | 17(33%) | |

*Table 2 aPL tests in carriers versus clinical triple positive patients.*

These results conducted us to determine new thresholds, presented in Table 3.

| | Carriers patients (No(%)) | Clinical patients (No(%)) | p-value (OR; CI) |
|---|---|---|---|
| **ROSNER INDEX** | | | |
| ≤ 27 | 27(82%) | 25(57%) | **0,0204 (OR 3,42; CI 1,23-10,66)** |
| > 27 | 6(18%) | 19(43%) | |
| **Normalized Ratio** | | | |
| ≤ 1,85 | 29(78%) | 30(60%) | 0,054 (OR 2,54; CI 1,00-6,94) |
| > 1,85 | 8(22%) | 21(41%) | |
| **IgG aB2GPI** | | | |
| ≤ 15,5 UI/mL | 10(27%) | 9(18%) | 0,2911 (OR 1,73; CI 0,62-4,89) |
| > 15,5 UI/mL | 27(73%) | 42(82%) | |
| **IgG aCL** | | | |
| ≤ 33,8 UI/mL | 14(38%) | 10(20%) | 0,058 (OR 2,50; CI 0,97-6,67) |
| > 33,8 UI/mL | 23(62%) | 41(80%) | |

*Table 3 Statistical analysis of new thresholds.*

Interestingly, with values above 27, Rosner Index became an independent risk factor for thrombosis related events (Odds Ratio 3.42, Confidence Interval 1.23-10.66). This result was also confirmed by a multivariate analysis (Odds Ratio 3.33, Confidence Interval 1.11-10.04).

At least, a combination of a Rosner Index > 27; a Normalized Ratio > 1.2, and double IgG isotype for both aCL and aβ2GPI antibodies was significantly associated with the "clinical group", showing that this combination of positive results represented an important risk factor for thrombosis or obstetrical complications (Odds Ratio 6.31, Confidence Interval 1.58-42.36). This profile was also identified in a multivariate analysis as an independent and an important risk factor for thrombosis or obstetrical morbidity (Odds Ratio 6.58, Confidence Interval 1.33-32.45).

Altogether our data led us to propose a score (Table 4) called "Score of triple aPLs positive patients" (STAPP) combining clinical data and biological results of aPLs. This score considered all risk factors highlighted in our study such as, having ≥ 2 cardiovascular risk factors, the double-double profile, and a high value of Rosner Index (>27). Like previously described, all this results were significantly associated with thrombotic events or obstetrical complications in our study. We assigned to each risk factor the same weighted points since their β-logistic regression coefficient values (calculated in multivariate analysis) were substantially equivalent.

| STAPP | Points |
|---|---|
| **≥ 2 Cardiovascular risk factors**<br>Including : High Blood Pressure, diabetes, dyslipidemia, obesity, smoking habit, Combined Oestroprogestative Contraceptive, age > 45 years old | 1 |
| **Double-Double profile**<br>Combination of Rosner Index positive, Normalized Ratio positive, Double IgG isotype for aCL and aβ2GPI antibodies | 1 |
| **Rosner Index > 27** | 1 |

*Table 4 "Score of triple antiphospholipid positive patients" (STAPP)*

The STAPP mean values were respectively 1.44 (± 0.97) and 0.72 (± 0.89) in “clinical” group and in “carriers” group, with an expected significance at p=0.0014. For STAPP values above 2, we obtained specificity at 84%, a positive predictive value at 82%, and an odds ratio at 5.65 (Confidence Interval 1.84-17.29).

To test the STPP, a follow up of 2 years was performed on 22 triple positive carriers. The mean Score at inclusion was 0.86 (+/-0.94) with a repartition as presented in Fig. 3, indicating that two patients presented the highest score at 3.

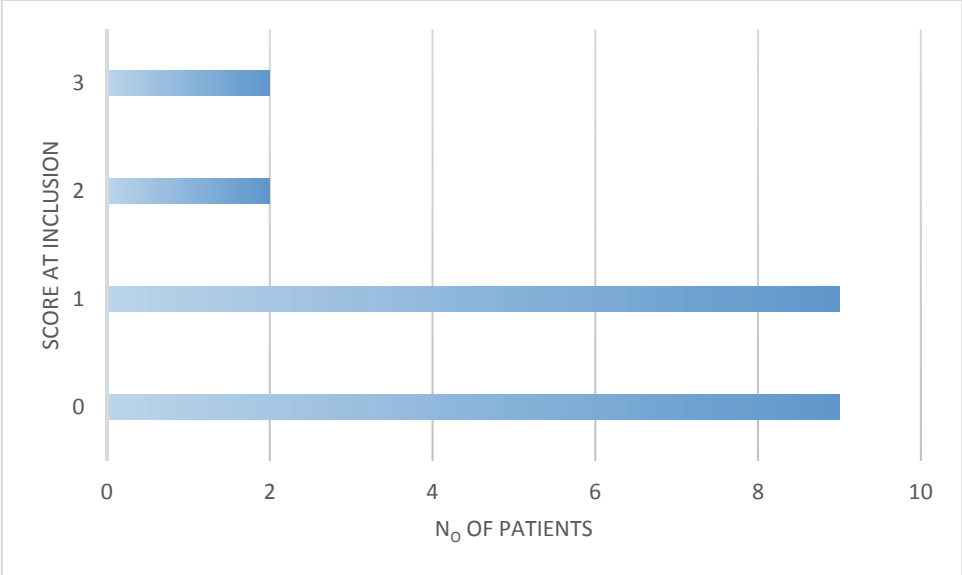


*Fig. 3 Distribution of STAPP of followed up patients at inclusion.*

New clinical events, particularly thrombotic or obstetrical complications were reported. Among the 22 patients only a woman experienced a transient ischemic attack, occurring 1.35 year after laboratory test inclusion. At inclusion, her STAPP was calculated at 3. This patient presented triple positivity persistence, two cardiovascular risk factors (age older than 45 years old and smoking habit), the double-double profile, and a high value of Rosner Index (> 27). At this event, an oral anticoagulant prophylaxis with vitamin K antagonist (VKA) has been proposed.

## IV. Discussion

Nowadays interpretation of aPL positivity represents an important challenge. The aPL positivity, and in particular aPL triple positivity confers the higher risk for both thrombosis and pregnancy morbidity. The quantification of such a risk may constitute a help for the clinicians to tailor the treatment.

We showed that among the triple positive patients, a combination of double IgG isotype for both aCL and aβ2GPI antibodies, a Normalized Ratio > 1.2 and a Rosner Index > 27 represents an important risk factor of thrombosis or obstetrical complications with an Odds Ratio higher than 6. Altogether these results led us to propose the STAPP, a Score of triple antiphospholipid positive patients.

To design our study, 7111 samples were retrospectively analysed to select triple positive patients. Among the 88 selected patients, we showed that 42% of triple positive patients have never experienced thrombotic events or obstetrical complications. To the best of our knowledge, such a proportion of “carriers” has never been described. This may be due to a large subject’s recruitment from various medical specialities. Anyway, this percentage highlights the high prevalence of aPL “carriers” and the need to anticipate the clinical risk in this group of patients.

Pengo et al. have improved the specificity of laboratory results by considering biological profile of patients. He demonstrated that triple positivity of aPLs tests remains the antibody profile with the highest thrombotic risk (64). In the same way and more precisely, we showed that the profile composed by IgG aCL, IgG aβ2GPI and LA positivity according to two tests (PTT-LA expressed with Rosner Index, dRVVT expressed with normalized ratio) and called double-double profile displays a higher risk. Our study agrees also with the fact that IgG isotype is the isotype mainly correlated with clinical events (78, 79). Interestingly, no difference was observed between the two groups of triple positive patients, “clinical” and “carriers”, according to the persistence of aPLs. In agreement with Pengo et al. (65), we observed that triple positivity is persistent in almost all cases (95%), also making us think that, in triple positive patients, an aPLs persistence confirmation at least twelve weeks apart is not necessary (80). However, triple positivity persistence does not appear to be a risk factor for thrombosis.

In addition, we evidenced that triple positive patients presenting at least two cardiovascular risk factors (including high blood pressure, diabetes, obesity, dyslipidemia, smoking habit, combined oestroprogestative contraceptive and age older than 45 years old) have an increased risk for thrombosis or obstetrical complications. Several studies demonstrated that cardiovascular risk factors increase the thrombotic risk in aPLs carriers, particularly an age older than 45 years old (64), hypertension (81-83), hypercholesterolemia

(71, 83), smoking (82). Patients with multiple risk factors are closely monitored in high-risk situations such as surgery, pregnancy, and prolonged immobilization. In the same way, women with multiple thrombosis risk are strongly advised to avoid combined oral contraceptive or hormonal replacement therapy. We conclude with our study that consideration of clinical data with biological results remains critical.

To get further in aPL interpretation, each aPLs antibody test (PTT-LA, dRVVT, ELISAs for IgG and IgM aCL antibody detection, and ELISAs for IgG and IgM aβ2GPI antibody detection) was analysed. Further studies are required to better interpret the significance of positivity for IgM isotype of aβ2GPI in carriers patients. Interestingly, we showed that a Rosner Index established at the threshold at 27 represents an independent and increased risk for thromboembolism events or obstetrical complications. This result is consistent with data from the literature since LA is the conventional marker most correlated to the clinic of APS (30). However, dRVVT is described as the most specific, sensitive and robust test to detect lupus anticoagulant (84). Although studies are necessary to confirm ours results, we can hypothesize that in triple aPLs positive patients, the Rosner Index displays a very interesting asset.

Moreover, a combination of Rosner Index higher than 27 with the double-double profile reached the main Odds Ratio of our study, with a value greater than 6. This is a high value compared with those observed in literature considering all antiphospholipid carrier patients, irrespective of their profiles (66, 71, 85-88). Even in high risk population of triple positive patients, Pengo et al. calculated lower odds ratio for male gender (OR 4.4) and cardiovascular risk factors associated (OR 3.3) (66).

The consideration of all these results led us to propose the Score of Triple Antiphospholipid Positive Patients (STAPP). Two scores have already been proposed: the Antiphospholipid Score (68) and the Global APS Score (GAPPS) (69, 71). Only the GAPSS includes clinical data (hyperlipidemia and arterial hypertension) and aPLs specificities (IgG/IgM aCL; IgG/IgM aβ2GPI; LA; IgG/IgM anti-prothrombin/phosphatidylserine complex) (71). The originality of STAPP is that it constitutes the first proposal to manage the triple positive patients. STAPP also associates clinical data and aPL results, and its advantage is that it includes aPL routinely tested. Therefore STAPP is easy to elaborate. In addition, STAPP was tested in a follow up study showing that the only one patient, who had a thrombotic event, had a maximal score (3/3).

To conclude, we evidenced that among triple aPLs patients, having two or more cardiovascular risk factors, a double-double profile and a high Rosner Index represents an increased risk for thrombosis related events. We demonstrated that the risk profile of triple aPLs patients can be assessed by STAPP which can be calculated in routine practice. Although prospective studies are now necessary to validate this score, STAPP could help clinicians to stratify the clinical risk of triple-positive patients and possibly initiate a primary prophylaxis.