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ORIGINAL ARTICLE

# Intensity of immune/clotting assays relate to multiple antiphospholipid antibody positivity in thrombotic primary antiphospholipid syndrome

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## Abstract

The dual positivity (DP) and triple positivity (TP) concepts bypass the poor comparability of immune/clotting assay for the laboratory classification of antiphospholipid syndrome (APS)**.** To evaluate intensity of immune/clotting assays and DP/TP through different clinical severity groups (CSG) as follows: (1) non-thrombotic asymptomatic carriers of aPL (N-THR), thrombotic primary APS (THR), deceased (D) for recurrent and fatal thrombosis. Activated partial thromboplastin time ratio (aPTTr), dilute Russell viper venom time ratio (DRVVTr), IgG/IgM anticardiolipin (aCL) and anti β-2-glycoprotein-I (aβ2GPI). Participants: 33 N-THR, 64 THR and 11 D. The frequency of DP and TP (DRVVTr or aPTTr partnered with respective IgG aCL or aβ2GPI) increased across CSG ($p=0.006$ and $p=0.003$); mean DRVVTr and IgG aCL/aβ2GPI were always greater in TP versus non-TP within each CSG and progressively increased across the CSG. The intensity of individual lupus anticoagulants partnered with their corresponding IgG aPL related to the frequency of multiple positivity throughout CSG suggesting that of intensity of immune/clotting assays and multiple positivity are the different faces of the same diagnostic coin in our thrombotic PAPS cohort.

**Keywords** Dual and triple positivity · Antiphospholipid · Lupus anticoagulant

## Introduction

The primary antiphospholipid syndrome (PAPS) defines arterial and venous thrombosis in the presence and persistence of antiphospholipid antibodies (aPL) measured by immune and clotting assays in the absence of any underlying autoimmune or chronic inflammatory disorder [1]. The concept of double positivity (DP) [2] and triple positivity [TP] [3] for the laboratory diagnosis of APS has been introduced to obviate the lack of standardisation of the immunoassays [4]: in particular, TP patients that simultaneously harbour a lupus anticoagulant (LA) alongside anticardiolipin (aCL) and anti-β-2-glycoprotein antibodies (aβ2GPI) seem more likely to present greater disease severity than APS patients who lack TP [3]. We, therefore, investigated in our cohort of PAPS whether titres of immune assay and strength of individual LA related to the DP/TP concept across three clinical severity groups (CSG): non-thrombotic carriers of antiphospholipid antibodies (N-THR), thrombotic primary APS (THR) patients and primary APS patients deceased (D) for recurrent and fatal occlusions.

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## Materials and methods

### Participants

The study was carried out on 108 participants found positive for aPL on two separate occasions, 6 weeks or 3 months apart, according to evolving guidelines [1, 2]. Participants were evaluated initially for a history of any thrombosis or for a prolongation of an activated partial thromboplastin time, suggesting a possible lupus anticoagulant (LA). Because our interest was mainly on vascular involvement (arterial and venous occlusions, atherosclerosis), the cohort did not include women in whom aPL was sought as part of their obstetric assessment and women with obstetric morbidity (intrauterine growth retardation, miscarriages, with or without thrombosis). Therefore, our exclusion criteria were: (1) history of obstetric morbidity, aPL positivity in women whose aPL was sought as part of an obstetric assessment, (2) secondary or systemic lupus-related APS and positive aPL in relation to any acute or chronic autoimmune and/or neoplastic disorder, to avoid the oxidative and inflammatory status that contributes to coagulation activation in these conditions. The ethics committee of the Cardarelli Hospital (Naples, Italy) and San Giuseppe Moscati Hospital (Avellino, Italy) granted approval for this study that was carried out according the principles of the declaration of Helsinki. Table 1 shows the demographics and clinical characteristics of the cohort.

Nine patients in the D group passed away for unprovoked vascular occlusions while on optimal oral anticoagulation: ischaemic stroke ($n=6$), (three of these patients at presentation suffered an ischemic stroke, one a right atrial thrombus and two a deep vein thrombosis (DVT); myocardial infarction ($n=1$) (the initial event of this patient was a DVT); saddle pulmonary embolism ($n=1$) and Budd-Chiari syndrome ($n=1$) (both suffered DVT as first occlusive events). Two patients in the D group passed away for provoked events: one for myocardial infarction with recurrent ischaemic stroke after aortic valve replacement and the other for post-partum catastrophic antiphospholipid syndrome with DVT as an initial event.

### Blood samples

Blood samples for clotting assays were collected by venepuncture in 1/10 volume of 0.109 M trisodium citrate (Beckton-Dickinson, Milano, Italy). Platelet poor plasma was obtained after centrifuging twice at 2500 × g for 10 min at room temperature to obtain platelet-free plasma that was aliquoted and frozen at − 70 ºC until use. Serum was prepared after blood was collected into plastic tubes (Beckton-Dickinson, Milano, Italy), left to clot for 2 h at room temperature, spun at 1000 × g for 10 min and frozen in aliquots of 0.4 ml at − 80 ºC until use. For thrombotic patients, blood samples were taken from three to six months after the thrombotic event and after 3 weeks of anti-vitamin K antagonists (warfarin in our case) cessation, covered by a

**Table 1** Demographics of the cohort according to clinical status

| | N-THR | | THR | | D | |
|---|---|---|---|---|---|---|
| No | 33 | | 64 | | 11 | |
| M/F | 5/26 | | 22/42 | | 5/6 | |
| Age (mean ± SD) | 48.5 ± 15.8 | | 46.3 ± 13.7 | | 55.5 ± 8.9 | |
| Age 1st event (mean ± SD) | na | | 33.9 ± 12.5 | | 40.1 ± 16.3 | |
| FUP, years (mean ± SD) | 12 ± 3.7 | | 10.3 ± 3.7 | | 7.9 ± 2.7 | |
| | No | % | No | % | No | % |
| Thrombosis number prior to diagnosis | NA | | 64 | | 11 | |
| No 1 | | | 42 | 65.6 | 0 | 36.3 |
| No 2 | | | 15 | 23.4 | 7 | 36.3 |
| No 3 | | | 7 | 10.9 | 2 | 9.1 |
| No 4 | | | 0 | 0 | 2 | 18.1 |
| Thrombosis type | | | | | | |
| Arterial | | | 18 | 28.1 | 7 | 63.6 |
| Venous | | | 45 | 70.3 | 2 | 18.1 |
| Arterial + venous | | | 1 | 1.5 | 2 | 18.1 |
| Anticoagulant | | | | | | |
| Warfarin | 0 | | 64 | 100 | 11 | 100 |
| Aspirin | 4 | 12 | 0 | 0 | 1 | 9 |

*N-THR* non thrombotic; *THR* thrombotic primary antiphospholipid antibody syndrome; *D* deceased; *No* number; *FUP* follow-up; *NA* not applicable

prophylactic dose of low-molecular-weight heparin withheld 36/48 h before the sampling; the second confirmatory blood sample was taken after 6 or 12 weeks (according to evolving guidelines) and managed in the same way [5, 6]. For non-thrombotic participants, the blood sample was taken at first visit, then repeated as above.

### Antiphospholipid antibodies measurement

LA was detected by the silica clotting time (S-aPTT) employing synthetic phospholipids and the dilute Russel viper time (DRVVT), both screen and confirm, performed on an ACL TOP-500 coagulometer (all reagents and equipment by Instrumentation Laboratory, Milano, Italy); the upper cut-offs for each assay were set at the 99th percentile from testing 122 plasmas from 81 females and 41 males (mean age $45 \pm 18$) who were healthy hospital and laboratory personnel. A clotting time ratio between patient and control sample greater than 1.20 for the S-aPTT (range 0.86–1.20) and 1.18 for the DRVVT (range 0.90–1.18) indicated an abnormal result. Antiphospholipid antibodies were measured by commercially available immune assays: IgG/IgM anticardiolipin antibodies (aCL) (Menarini Diagnostica, Milano Italy) and IgG/IgM anti-β-2-glycoprotein-I (aβ2GPI) antibodies (Corgenix, Bloomfield, Colorado, USA). Normal ranges were established using the same 122 healthy hospital personnel as above, with a cut-off for positivity at the 99th percentile [5, 6]. The inter- and intra-coefficient of variability for all the immune assays ranged between 3.1% and 4.1%.

## Statistics

Data are expressed as mean and standard deviations; for univariate and multivariate regression analyses, immunoassay data were log-transformed because they violated the normality assumption; comparisons between groups were performed by non-parametric tests.

## Results

### Relationship between clotting and immune assays

By univariate regression, the aPTT related to IgG aCL ($p = 0.004$) (Fig. 1a) but neither to IgG β2GPI (Fig. 1c) nor to IgM aCL/β2GPI (not shown). The DRVVT related to all immune assays: IgG aCL ($p < 0.0001$) (Fig. 1b), IgG β2GPI ($p < 0.0001$) (Fig. 1d), IgM aCL ($p < 0.006$) (Fig. 1e) and IgM β2GPI ($p = 0.0002$) (Fig. 1f). DRVVT and aPTT were interrelated ($r^2 = 0.23$, $p < 0.0001$) (figure not shown).

By multivariate regression, with IgG aCL, age and sex as independent variables and aPTT ratio as the dependent variable, the assumption of the univariate regression did not change (Table 2A); by multivariate regression, with IgG aCL, IgG β2GPI, IgM aCL, IgM β2GPI alongside age and sex as independent variables and DRVVT ratio as the dependent variable, IgG aCL and to a lesser extent IgM aβ2GPI independently predicted DRVVTr (Table 2B).

### Average lupus anticoagulant ratios and antibody titres by triple and non-triple positivity by clinical severity groups

Within each CSG, we averaged DRVVTr, aPTTr and partnering average IgG aCL and IgG β2GPI titres according to TP or non-TP status; within each CSG, the mean DRVVTr and its partnering IgG aPL were always greater in TP than in non-TP groups, though not significant in the deceased group for the paucity of numbers (Table 3A). The same applied to the mean aPTTr and partnering average IgG aPL (Table 3B). Of the two LA assays, the average DRVVTr within the TP patients was progressively higher throughout the three CSG ($p = 0.05$), while the average aPTTr was not ($p = 0.09$).

### Frequency of dual and triple positive tests by clinical severity groups

Table 4 shows all the partnering aPL combinations derived from participants in the CSG. Panel A shows any LA, aCL, aβ2GPI in isolation and in combination as DP and TP; the frequency of DP and TP increases progressively through the severity groups. Once we split the LA into the relevant assays, we have: (panel B) showing the DRVVT with aCL and aβ2GPI of either isotype in isolation and in combination; DP and TP were progressively more common through the CSG when DRVVT partnered with the IgG aPL but not the IgM aPL isoptype; (panel C) showing the aPTT with aCL and aβ2GPI of either isotype in isolation and in combination: also, here, DP and TP were progressively more common through the severity groups when aPTT partnered with the IgG aPL but not the IgM aPL isotype.

## Discussion

Weakly positive aPL tests, just above the cut-off for positivity, may not have clinical significance in isolation but may bear clinical weight in association with each other. Hence, the introduction of the DP and TP concepts stems from the lack of standardisation of aCL and aβ2GPI [7] and from the need to harmonize laboratory classification criteria for APS that may help in conducting and comparing studies worldwide [4]. Intuitively, the higher the aPL titre, the more likely a DRVVT and/or an aPTT ratio might be positive and vice versa; the DRVVTr is very sensitive to the LA activity

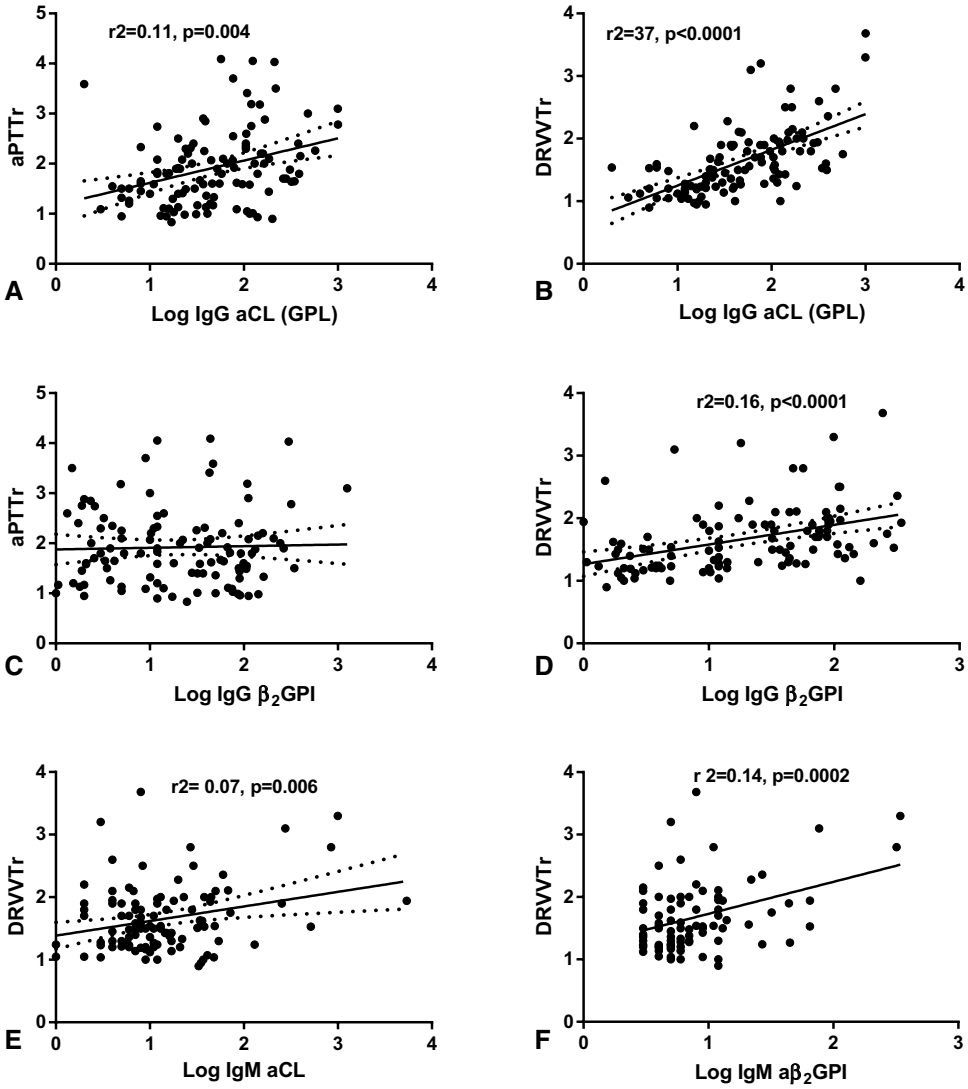


**Fig. 1** Relationships between immune and clotting assays: (**a**) regression between log IgG aCL and aPTTr; (**b**) regression between log IgG aCL and DRVVTr; (**c**) regression between log IgG a$\beta_2$GPI and aPTTr; (**d**) regression between log IgG a$\beta_2$GPI and DRVVTr; (**e**) regression between log IgM and DRVVTr; (**f**) regression between log IgM a$\beta_2$GPI and DRVVTr

of aβ2GPI [8] and may associate to aβ2GPI of all immunoglobulin isotypes [9, 10].

When looking at average values, within each CSG, the TP showed a higher mean DRVVT and aPTT ratios than the non-TP group, though this was not always matched by the average IgG aCL/aβ2GPI titres; moreover, the average DRVVTr and IgG aβ2GPI titres of the TP combination were progressively higher across the CSG.

When looking at the frequencies of multiple positivity, DP and TP expressed as any LA and/or any aPL isotype were progressively more common across the different CSG; once these frequencies were recalculated according to subtype of LA assay and by the two IgG isotypes, the same pattern appeared in partnership with the IgG but not with the IgM isotype. Indeed, a positive IgM aCL or IgM aβ2GPI, whether in isolation or in combination with DRVVT/aPTT, was poorly represented across the CSG; it is known that IgM aCL has a questionable relationship with thrombosis [11].

In keeping with others [12, 13], we found that TP and DP relate to disease severity, though the means of the individual tests making up the TP and DP also progressively increased across the CSG, accounting for the increasing multiple positive frequency. The risk of thrombosis associated with aPL increases on a linear scale [14], with a likely early threshold effect at 40GPL for IgG aCL [15], and reflects the linear relation between IgG aPL titre and DRVVT ratio [16]; hence, IgG aPL and DRVVT may be regarded as risk factors in addition to being diagnostic tests [17].

Our approach is similar to that of Otomo et al. who constructed an aPL-score (aPL-S) based on the strength of LA ratio and their mixing ratio as well as on the categorisation in titres of different aPL; their results indicate that the higher the aPL-S, the greater the risk of thrombosis [18]. A later study confirmed this aPL-S concept though its final score was based also on the presence of arterial hypertension and hyperlipidaemia [19].

**Table 2** Multivariate regression evaluating the independent predictors of the activated partial thromboplastin time and dilute Russell viper venom time ratios

| A | | | | |
|---|---|---|---|---|
| aPTTr | | | | |
| Independent variables | $\beta$ | SD | $t$ | $p$ value |
| Log IgG aCL | 0.3608 | 1.32 | 2.818 | 0.0058 |
| Age | −0.0047 | 0.05 | −0.917 | 0.3615 |
| Sex | 0.1731 | 1.60 | 1.119 | 0.2659 |
| B | | | | |
| DRVVTr | | | | |
| Independent variables | $\beta$ | SD | $t$ | $p$ value |
| Log IgG aCL | 0.5152 | 1.17 | 4.523 | < 0.0001 |
| Log IgM a$\beta_2$GPI | 0.3752 | 1.88 | 2.069 | 0.0415 |
| Log IgG a$\beta_2$GPI | −0.0086 | 0.90 | −0.0996 | 0.9209 |
| Log IgM aCL | −0.0201 | 1.20 | −0.173 | 0.8627 |
| Age | 0.0018 | 0.03 | 0.530 | 0.5974 |
| Sex | 0.1553 | 1.61 | 1.542 | 0.1268 |

*aPTTr* activated partial thromboplastin time ratio; *IgG* immunoglobulin G antibody; *aCL* anticardiolipin; *SD* standard deviation; *DRVVTr* dilute Russell viper venom time ratio; *IgM* immunoglobulin M antibody; *a$\beta_2$GPI* anti beta-2-glycoprotein-I

**Table 3** Mean lupus anticoagulant ratios and titres of matching immune assays by triple and non-triple positivity by clinical severity groups

| A | N-THR | | | THR | | | D | | |
|---|---|---|---|---|---|---|---|---|---|
| DRVVTr + IgG | TP | NTP | $p$ | TP | NTP | $p$ | TP | NTP | $p$ |
| No | 7 | 26 | | 34 | 30 | | 7 | 4 | |
| DRVVTr ($\bar{x} \pm$ SD) | 1.61 ± 0.2 | 1.21 ± 0.2 | 0.001 | 1.82 ± 0.4 | 1.49 ± 0.3 | 0.0006 | 2.47 ± 0.8 | 2.40 ± 0.6 | 0.8 |
| IgGaCL ($\bar{x} \pm$ SD) | 163 ± 75 | 210 ± 13 | < 0.0001 | 153 ± 125 | 36 ± 51 | < 0.0001 | 445 ± 400 | 113 ± 139 | 0.07 |
| IgGa$\beta_2$GPI ($\bar{x} \pm$ SD) | 71 ± 267 | 4 ± 3 | < 0.0001 | 90 ± 76 | 16 ± 30 | < 0.0001 | 153 ± 103 | 10 ± 8.5 | 0.006 |
| B | N-THR | | | THR | | | D | | |
| aPTTr + IgG | TP | NTP | $p$ | TP | NTP | $p$ | TP | NTP | $p$ |
| No | 5 | 28 | | 29 | 35 | | 7 | 4 | |
| aPTTr ($\bar{x} \pm$ SD) | 2.59 ± 1.0 | 1.57 ± 0.7 | 0.02 | 2.31 ± 0.7 | 1.7 ± 0.53 | 0.0005 | 2.31 ± 0.5 | 1.93 ± 0.32 | 0.2 |
| IgGaCL ($\bar{x} \pm$ SD) | 169 ± 83 | 24 ± 27 | < 0.0001 | 162 ± 131 | 45 ± 56 | < 0.0001 | 445 ± 440 | 113 ± 139 | 0.07 |
| IgGa$\beta_2$GPI ($\bar{x} \pm$ SD) | 63 ± 23 | 6 ± 9 | < 0.0001 | 86 ± 67 | 22 ± 33 | < 0.0001 | 153 ± 103 | 10 ± 8.5 | 0.006 |

*N-THR* non thrombotic persistently positive for antiphospholipid antibodies; *THR* thrombotic primary antiphospholipid syndrome; *D* deceased thrombotic primary antiphospholipid syndrome; *DRVVTr* dilute Russell viper venom time ratio *TP* triple positivity; *NTP* non triple positivity; *No* numbers; *IgG* immunoglobulin G antibody; *aCL* anticardiolipin; *a$\beta_2$GPI* anti beta-2-glycoprotein-I. *aPTTr* activated partial thromboplastin time ratio

Our study was carried out on primary thrombotic APS and persistent carriers of aPL in the absence of any underlying autoimmune or inflammatory disease. Detractors may see this as a limitation, purists may view it as a strength, given the homogeneous population, though the low numbers in our deceased primary APS group may lessen the power of our calculations. Nevertheless, our data show that intensity of clotting assays and titres of immune and/or clotting assays relate to multiple positivity and they may be viewed as different faces of the same diagnostic coin [20, 21]. Further co-operative efforts are needed to harmonize the concepts discussed herein to refine the laboratory diagnostic criteria for APS and make them more comparable across clinicians and investigators. Finally, the poor representation of the IgM aCL and a$\beta_2$GPI across our CSG confirms the uselessness of the IgM aPL assay [22].

**Table 4** Frequency of laboratory classification categories for antiphospholipid syndrome by clinical severity status

| A | N-THR $n=33$ | | THR $n=64$ | | D $n=11$ | | $p$ value |
|---|---|---|---|---|---|---|---|
| | No | % | No | % | No | % | |
| LA + aCL + a$\beta_2$GPI | | | | | | | |
| LA only | 18 | 56.2 | 13 | 20.3 | 0 | 0 | ns |
| aCL only | 1 | 3.2 | 0 | 0 | 0 | 0 | ns |
| a$\beta_2$GPI only | 0 | 0 | 0 | 0 | 0 | 0 | ns |
| DP | 13 | 40.6 | 51 | 79.6 | 11 | 100 | < 0.0001 |
| TP | 8 | 25 | 35 | 54.6 | 11 | 100 | < 0.0001 |
| B | | | | | | | |
| DRVVT + IgG | | | | | | | |
| DRVVTr only | 8 | 25.0 | 13 | 20.3 | 1 | 9.1 | ns |
| aCL only | 0 | 0 | 0 | 0 | 0 | 0 | ns |
| $\beta_2$GPI only | 0 | 0 | 0 | 0 | 0 | 0 | ns |
| DP | 12 | 37.5 | 51 | 79.6 | 10 | 90.9 | < 0.0001 |
| TP | 7 | 21.8 | 34 | 53.1 | 7 | 63.6 | 0.006 |
| DRVVT + IgM | | | | | | | |
| DRVVTr only | 16 | 50.0 | 44 | 68.7 | 8 | 72.7 | ns |
| aCL only | 1 | 3.1 | 1 | 1.5 | 0 | 0 | ns |
| $\beta_2$GPI only | 0 | 0 | 0 | 0 | 0 | 0 | ns |
| DP | 4 | 12.5 | 12 | 18.7 | 3 | 27.2 | ns |
| TP | 1 | 3.1 | 5 | 7.8 | 2 | 18 | ns |
| C | | | | | | | |
| aPTT + IgG | | | | | | | |
| aPTTr only | 13 | 40.6 | 19 | 29.6 | 2 | 18.2 | ns |
| aCL only | 1 | 3.1 | 0 | 0 | 0 | 0 | ns |
| $\beta_2$GPI only | 0 | 0 | 2 | 3.1 | 0 | 0 | ns |
| DP | 9 | 28.1 | 43 | 67.1 | 9 | 81.8 | < 0.0001 |
| TP | 5 | 15.6 | 29 | 45.3 | 7 | 63.6 | 0.003 |
| aPTT + IgM | | | | | | | |
| aPTTr only | 21 | 65.6 | 40 | 62.5 | 8 | 72.7 | ns |
| aCL only | 2 | 6.2 | 0 | 0 | 0 | 0 | ns |
| $\beta_2$GPI only | 0 | 0 | 1 | 1.5 | 0 | 0 | ns |
| DP | 2 | 6.2 | 11 | 17.1 | 3 | 27.2 | ns |
| TP | 1 | 3.1 | 1 | 1.5 | 1 | 9 | ns |

*N-THR* non thrombotic; *THR* thrombotic primary antiphospholipid antibody syndrome; *D* deceased; *No* number; *LA* lupus anticoagulant; *aCL* anticardiolipin; *a$\beta_2$GPI* anti beta-2-glycoprotein-I. *aPTTr* activated partial thromboplastin time ratio; *IgG* immunoglobulin G antibody; *IgM* immunoglobulin M antibody; *DRVVTr* dilute Russell viper venom time ratio; *DP* double positivity; *TP* triple positivity; *ns* not significant

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## Compliance with ethical standards

**Conflict of interest** None of the authors have any conflict of interest to disclose.

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