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Fluctuation of Anti–Domain 1 and Anti–β_2-Glycoprotein I Antibody Titers Over Time in Patients With Persistently Positive Antiphospholipid Antibodies

Cecilia B. Chighizola,[1] Francesca Pregnolato,[1] Danieli Andrade,[2] Maria Tektonidou,[3] Vittorio Pengo,[4] Guillermo Ruiz-Irastorza,[5] H. Michael Belmont,[6] Maria Gerosa,[7] Paul Fortin,[8] D. Ware Branch,[9] Laura Andreoli,[10] Michelle A. Petri,[11] Ricard Cervera,[12] Jason S. Knight,[13] Rohan Willis,[14] Maria Efthymiou,[15] Hannah Cohen,[15] Doruk Erkan,[16] Maria Laura Bertolaccini,[17] on behalf of Antiphospholipid Syndrome Alliance for Clinical Trials and International Networking (APS ACTION)

Objective. The present study was undertaken to longitudinally evaluate titers of antibodies against β_2-glycoprotein I (anti-β_2GPI) and domain 1 (anti-D1), to identify predictors of variations in anti-β_2GPI and anti-D1 titers, and to clarify whether antibody titer fluctuations predict thrombosis in a large international cohort of patients who were persistently positive for antiphospholipid antibodies (aPL) in the APS ACTION Registry.

Methods. Patients with available blood samples from at least 4 time points (at baseline [year 1] and at years 2–4 of follow-up) were included. Detection of anti-β_2GPI and anti-D1 IgG antibodies was performed using chemiluminescence (BIO-FLASH; INOVA Diagnostics).

Results. Among 230 patients in the study cohort, anti-D1 and anti-β_2GPI titers decreased significantly over time ($P < 0.0001$ and $P = 0.010$, respectively). After adjustment for age, sex, and number of positive aPL tests, we found that the fluctuations in anti-D1 and anti-β_2GPI titer levels were associated with treatment with hydroxychloroquine (HCQ) at each time point. Treatment with HCQ, but not immunosuppressive agents, was associated with 1.3-fold and 1.4-fold decreases in anti-D1 and anti-β_2GPI titers, respectively. Incident vascular events were associated with 1.9-fold and 2.1-fold increases in anti-D1 and anti-β_2GPI titers, respectively. Anti-D1 and anti-β_2GPI titers at the time of thrombosis were lower compared to titers at other time points. A 1.6-fold decrease in anti-D1 titers and a 2-fold decrease in anti-β_2GPI titers conferred odds ratios for incident thrombosis of 6.0 (95% confidence interval [95% CI] 0.62–59.3) and 9.4 (95% CI 1.1–80.2), respectively.

Conclusion. Treatment with HCQ and incident vascular events in aPL-positive patients predicted significant anti-D1 and anti-β_2GPI titer fluctuations over time. Both anti-D1 and anti-β_2GPI titers decreased around the time of thrombosis, with potential clinical relevance.

INTRODUCTION

Antiphospholipid antibodies (aPL) provide the main acquired risk factor for both thrombosis and obstetric complications, the 2 clinical facets of antiphospholipid syndrome (APS) (1). The treatment of aPL-positive patients dictates a careful evaluation of the risk of future clinical events, with important therapeutic implications in terms of both primary and secondary

[1]Cecilia B. Chighizola, MD, PhD, Francesca Pregnolato, MStat, BSc: Pediatric Rheumatology Unit, ASST G. Pini-CTO, University of Milan, Milan, Italy; [2]Danieli Andrade, MD: Reumatologia, University of Sao Paulo, Sao Paulo, Brazil; [3]Maria Tektonidou, MD: First Department of Propaedeutic and Internal Medicine, Laiko Hospital, National and Kapodistrian University of Athens, Athens, Greece; [4]Vittorio Pengo, MD: Department of Cardiac Thoracic and Vascular Sciences, University of Padua, Padua, Italy; [5]Guillermo Ruiz-Irastorza, MD: Unidad de Investigación de Enfermedades Autoinmunes, Servicio de Medicina Interna, Universidad del País Vasco/Euskal Herriko Unibertsitatea, BioCruces Health Research Institute, Hospital Universitario Cruces, Barakaldo, Spain; [6]H. Michael Belmont, MD: Hospital for Joint Diseases, New York University, New York; [7]Maria Gerosa, MD, PhD: Clinical Rheumatology Unit, ASST G. Pini-CTO, University of Milan, Milan, Italy; [8]Paul Fortin, MD: Medicine, Université Laval, Quebec, Canada; [9]D. Ware Branch, MD: Department of Obstetrics, University of Utah and Intermountain Healthcare, Salt Lake City; [10]Laura Andreoli, MD, PhD: Rheumatology Unit, University of Brescia, Brescia, Italy; [11]Michelle A. Petri, MD: Rheumatology, Johns Hopkins University School of Medicine, Baltimore, Maryland; [12]Ricard Cervera, MD: Department of Autoimmune Diseases, Hospital Clínic de Barcelona, Barcelona, Spain; [13]Jason S. Knight, MD, PhD: Division of Rheumatology, University of Michigan, Ann Arbor; [14]Rohan Willis, MD: Internal Medicine, University of Texas Medical Branch, Galveston; [15]Maria Efthymiou, MD, Hannah Cohen, MD: Department of Haematology, University College London, London, UK; [16]Doruk Erkan, MD: Rheumatology, Hospital for Special Surgery, New York, New York; [17]Maria Laura Bertolaccini, PhD: Vascular Risk and Surgery Section, King's College London, London, UK.

thromboprophylaxis. The aPL profile provides the main determinant of APS clinical manifestations; because each aPL test conveys a characteristic specificity and sensitivity, clinicians consider the pattern of positive criteria for APS to include evaluation of all of the following components: presence of aPL(s) (namely, anticardiolipin antibodies [aCL], anti–β_2-glycoprotein I [anti-β_2GPI] antibodies, and/or lupus anticoagulant [LAC]), the number of positive aPL tests, the isotypes, and the antibody titers (1)

Notwithstanding this strategy, clinicians still face many difficulties in optimizing the treatment of aPL-positive patients and strongly advocate a further refining of the process of risk stratification. Indeed, despite similar aPL profiles and comparable conventional cardiovascular risk factors, some patients develop dramatic aPL-mediated clinical manifestations, whereas other patients remain asymptomatic throughout their lifespan. Research efforts have fostered the development of second-line testing tools, such as the characterization of domain reactivity of anti-β_2GPI antibodies, which are regarded as the true pathogenic antibody subset. With the ascertainment of their pathogenic role, attention has been generated on antibodies against domain 1 of β_2GPI (anti-D1) (2). Testing for anti-D1 antibodies could be useful, since positivity predicts aPL-associated manifestations; the anti-D1 antibody subset is highly prevalent in patients with thrombotic APS and frequently positive among women with pure obstetric manifestations, although anti-D1 antibodies are rarely detected in asymptomatic aPL carriers. Accordingly, positivity rates and titers of anti-D1 antibodies are highest among those patients with the most consistent risk of events, that is, those with triple aPL positivity (2).

Despite their consolidated prognostic role, to our knowledge, longitudinal data on anti-D1 antibody titers and comparisons between the longitudinal behavior of titers of antibodies directed against D1 and those targeting the β_2GPI whole molecule are not available. Thus, the aims of this large-scale prospective international study are 1) to assess the prevalence of anti-D1 antibody positivity in patients included in the Antiphospholipid Syndrome Alliance for Clinical Trials and International Networking (APS ACTION) clinical database and repository ("Registry"); 2) to evaluate the stability over time of anti-D1 and anti-β_2GPI IgG antibodies; 3) to identify predictors of the longitudinal fluctuation of anti-D1 and anti-β_2GPI antibody titers; and 4) to clarify whether the fluctuation of anti-D1 and anti-β_2GPI antibody titers carries a clinical significance in predicting thrombosis.

PATIENTS AND METHODS

Ethics statement. This study complies with the Declaration of Helsinki. The APS ACTION Registry was approved by the ethics committees of each participating center. The local ethics committee of the lead coordinating center approved the study (Hospital for Special Surgery Institutional Review Board, no. 2014-252).

APS ACTION Registry. The APS ACTION Registry includes persistently aPL-positive patients based on the revised Sapporo classification criteria for APS (1), with or without systemic autoimmune rheumatic diseases (SARDs), followed every 12 ± 3 months with collection of clinical and laboratory data and blood samples. Access to raw data is available upon request.

Data collection. Demographic data were collected at baseline (designated as year 1 [Y1]). The following clinical details were collected at Y1 and updated during follow-up (Y2, Y3, and Y4): concomitant SARD, conventional cardiovascular risk factors, medications, and aPL-related thrombotic and obstetric manifestations.

Detection of aPL using immunoassays. Detection of anti-D1 IgG, anti-β_2GPI IgG/IgM/IgA, and aCL IgG/IgM/IgA antibodies was performed with a chemiluminescence immunoassay exploiting the BIO-FLASH technology (Quanta Flash β_2GPI domain 1 IgG, Quanta Flash β_2GPI IgG/IgM/IgA, and Quanta Flash cardiolipin IgG/IgM/IgA; Inova Diagnostics) at Y1 and in follow-up samples at Y2–Y4. Threshold values to define anti-D1 and anti-β_2GPI positivity were based on the manufacturer's cutoff at 20 chemiluminescent units (CU). This threshold was established by the 99% percentile of 250 donors. Each APS ACTION core laboratory validated the manufacturer's cutoff by testing 20 local healthy subjects (3).

Samples were tested in 3 APS ACTION core laboratories following validation. At study inclusion, LAC, anti-β_2GPI IgG/IgM, and aCL IgG/IgM were tested in the core laboratories as previously described (4,5). Patients enrolled in the APS ACTION Registry were considered eligible for inclusion in this study when serum samples from at least 4 different time points (baseline Y1 and follow-up clinical and laboratory evaluations at Y2–Y4) were available for longitudinal anti-D1 and anti-β_2GPI antibody testing.

Statistical analysis. Descriptive statistics were generated for demographic, clinical, and laboratory data. As a result of the skewed distribution of anti-D1 and anti-β_2GPI antibody titers, results are expressed as geometric mean with 95% confidence interval (95% CI). Associations between variables were assessed by chi-square test and McNemar's chi-square test, as appropriate.

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Address correspondence via email to Cecilia B. Chighizola, MD, PhD, at cecilia.chighizola@unimi.it.

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The rates of anti-D1, anti-β_2GPI, and aCL antibody positivity were calculated for each time point for the whole study cohort and for patients categorized according to clinical features. Four positivity categories based on quartiles for anti-D1 antibody titers were identified: low positivity (<57.6 CU), intermediate-low positivity (57.6–165.7 CU), intermediate-high positivity (165.7–680.3 CU), and high positivity (<680.3 CU). Four positivity categories based on quartiles for anti-β_2GPI IgG antibody titers were also identified: low positivity (<116.9 CU), intermediate-low positivity (116.9–702.7 CU), intermediate-high positivity (702.7–2,254.6 CU), and high positivity (>2,254.6 CU).

Anti-D1 IgG, anti-β_2GPI IgG/IgM/IgA, and aCL IgG/IgM/IgA antibody titers within the same patient were compared by Friedman's test. Linear mixed-effects models for repeated measurement nested within each patient were built to identify predictors of anti-D1 and anti-β_2GPI IgG antibody titer fluctuations. To clarify whether fluctuations in anti-D1 and anti-β_2GPI IgG antibody titers were associated with thrombotic events, a case–crossover design was applied.

P values less than 0.05 were considered statistically significant. Data were analyzed using R version 4.0.5.

RESULTS

Identification of ≥1 positive anti-D1 antibody test in ~60% of the study cohort. Among the entire APS ACTION cohort, 1,942 samples from 515 patients were tested for anti-D1 and anti-β_2GPI IgG antibodies (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). Anti-D1 antibodies were tested at 4 time points (baseline Y1 and follow-up Y2–Y4) in 230 patients, and this sample comprised the included patients in our longitudinal study. The clinical and laboratory details of the 230 patients are listed in Table 1.

Of 230 patients, ≥1 positive anti-D1 antibody result was identified in 135 patients (58.7%); the remaining 95 patients had persistently negative results for anti-D1 antibodies both at baseline and during follow-up. Patients with ≥1 positive anti-D1 antibody result were significantly younger than those who tested negative for anti-D1 antibodies at all time points and had significantly higher positivity rates and titers of criteria aPL tests at baseline. Patients without anti-D1 antibodies were more frequently asymptomatic aPL carriers, and we found no difference in the distribution of associated SARDs between anti-D1 positive and negative subjects. Only 13 of 135 patients (9.6%) displayed anti-D1 antibody positivity at 1 time point, whereas most patients displayed persistent positivity for anti-D1 antibodies at all 4 time points (n = 100, 74.1%) (Supplementary Table 2, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). We found no difference in the rate of thrombosis between the 100 patients with positive anti-D1 antibodies at all 4 time points and the remaining patients ($\chi^2 = 0.836$, $P = 0.360$).

Among criteria aPL tests, IgG isotype was the most prevalent. Of the 230 included patients, 170 (73.9%) tested positive at least once for anti-β_2GPI IgG antibodies and 151 (65.6%) tested positive at least once for aCL IgG antibodies (Supplementary Tables 3 and 6, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). A positivity in IgM isotype emerged more frequently for the aCL antibody test compared with the anti-β_2GPI antibody test (n = 83 patients [36.1%] versus 73 patients [31.7%]) (Supplementary Tables 4 and 7, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). Noncriteria anti-β_2GPI and aCL IgA tests were found to be positive in 76 patients (33.0%) and 94 patients (40.9%), respectively (Supplementary Tables 5 and 8, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459).

Significant fluctuation over time in anti-D1 and anti-β_2GPI IgG antibody titers. Among the 135 patients with ≥1 anti-D1 positive result, anti-D1 titers varied significantly over time (Friedman statistic = 508.5, $P < 0.0001$). The anti-D1 geometric mean was 189.0 at Y1 (95% CI 141.2–253.1), 132.3 at Y2 (95% CI 97.4–179.7) (–15% versus Y1), 113.8 at Y3 (95% CI 83.8 – 154.4) (–17% versus Y2), and 109.2 at Y4 (95% CI 80.3–148.5) (–6% versus Y3 and – 38% versus Y1) (Figure 1A and Supplementary Figure 1A, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). Anti-D1 titers at baseline were significantly higher compared with results at Y4 ($P = 0.029$). The same fluctuation pattern was observed when patients were selected based on multiple anti-D1 positivities over time (Supplementary Table 2, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459).

Anti-β_2GPI titers correlated with anti-D1 titers at all time points, showing r = 0.804 at Y1 (95% CI 0.750–0.847; $P < 0.0001$), r = 0.836 at Y2 (95% CI 0.790–0.872; $P < 0.0001$); r = 0.831 at Y3 (95% CI 0.784–0.869; $P < 0.0001$), and r = 0.813 at Y4 (95% CI 0.763–0.854; $P < 0.0001$). Among the 170 patients with ≥1 anti-β_2GPI positive result, anti-β_2GPI titers were significantly reduced at Y4 compared with Y1 (Friedman statistic = 11.32, $P = 0.010$). The anti-β_2GPI geometric mean was 187.1 at Y1 (95% CI 14.5–1,586.5), 150.8 at Y2 (95% CI 11.1–1,379.2) (–9% versus Y1), 124.9 at Y3 (95% CI 12.2–1,304) (0% versus Y2), and 117.6 at Y4 (95% CI 8.7–1,136.6) (–2% versus Y3 and – 12% versus Y1) (Figure 1B and Supplementary Figure 1B, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). When patients were selected based on multiple anti-β_2GPI IgG positivities, a similar pattern of antibody titer variation emerged over time (Supplementary Table 3, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459).

Among the other aPL tests, a similar longitudinal variation of antibody titers was noted exclusively for aCL IgG (Supplementary Table 6, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). For the remaining assays, antibody titers significantly fluctuated over time but without a progressive decrease in titers

Table 1. Demographic, baseline clinical, and therapeutic details of 230 patients with positive antiphospholipid antibodies at study inclusion subgrouped according to anti-D1 antibody positivity in ≥1 determination*

	Total cohort (n = 230)	Anti-D1 positive (n = 135)	Anti-D1 negative (n = 95)	*P*
Age, mean ± SD years	45.0 ± 12.7	42.3 ± 11.8	48.8 ± 13.0	0.0001
Female sex	69.1 (159)	71.9 (97)	65.3 (62)	0.358
Race				
White	94.2 (179/190)	91.8 (101/110)	97.5 (78/80)	0.123
Other	5.8 (11/190)	8.2 (9/110)	2.5 (2/80)	
Diagnosis				
aPL carrier/PAPS	58.7 (135)	60.7 (82)	55.8 (53)	0.539
aPL+ SARDs/SAPS	41.3 (95)	39.3 (53)	44.2 (42)	
Phenotype				
aPL+ without APS	25.7 (59)	19.3 (26)	34.7 (33)	0.010
Thrombotic APS	53.9 (124)	54.1 (73)	53.7 (51)	
Obstetric APS	9.1 (21)	11.9 (16)	5.3 (5)	
Thrombotic and obstetric APS	11.3 (26)	14.8 (20)	6.3 (6)	
Patients with previous thrombosis†	65.2 (150)	68.9 (93)	60 (57)	0.163
No. of thrombotic events per patient				
1	35.2 (81)	38.5 (52)	30.5 (29)	
2	21.7 (50)	23.7 (32)	18.9 (18)	
3	5.6 (13)	3.7 (5)	8.4 (8)	
4	1.3 (3)	1.5 (2)	1 (1)	0.486
5	0.9 (2)	0.7 (1)	1 (1)	
6	0.4 (1)	0.7 (1)	0 (0)	
Thrombotic events				
Arterial thrombosis	43 (99)	40 (54)	47.4 (45)	
Venous thrombosis	53 (122)	60.7 (82)	42.1 (40)	
Small vessel thrombosis	11.3 (26)	10.4 (14)	12.6 (12)	0.159
CAPS	0.4 (1)	0 (0)	1 (1)	NP
Pregnancy complications				
Previous pregnancy	45.2 (104)	45.2 (61)	45.3 (43)	0.990
Previous pregnancy morbidity†	62.5 (65)	73.8 (45)	46.5 (20)	0.005
Pregnancy loss before 10 gw	29.8 (31)	27.8 (17)	32.5 (14)	0.265
No. of pregnancy losses before 10 gw				
1	22.1 (23)	26.2 (16)	16.3 (7)	
2	3.8 (4)	1.6 (1)	7.0 (3)	
3	2.9 (3)	0 (0)	7.0 (3)	0.064
4	3.8 (4)	0 (0)	9.3 (4)	
Pregnancy loss after 10 gw	36.5 (38)	49.2 (30)	18.6 (8)	0.001
Premature birth before 34 gw	20.2 (21)	31.1 (19)	4.6 (2)	0.0009
Associated autoimmune disease	44.3 (102)	43.7 (59)	45.3 (43)	0.054
Systemic lupus erythematosus	3 (70)	29.6 (40)	31.6 (30)	
SLE-like†	7.8 (18)	7.4 (10)	8.4 (8)	
UCTD	3 (7)	2.2 (3)	4.2 (4)	0.402
Organ-specific autoimmune disease	3 (7)	4.4 (6)	1 (1)	
aPL criteria tests‡	n = 227	n = 133	n = 94	
aCL, IgG (GPL)	63.0 (143)	89.5 (119)	25.5 (24)	<0.0001
aCL, IgM (MPL)	32.6 (74)	36.1 (48)	27.7 (26)	0.234
Anti-β_2GPI, IgG (SGU)	70.9 (161)	93.2 (124)	39.4 (37)	<0.0001
Anti-β_2GPI, IgM (SMU)	29.1 (66)	34.6 (46)	21.3 (20)	0.043
LAC (n = 99)	n = 173	n = 99	n = 74	
Positive	72.8 (126)	82.8 (82)	59.5 (44)	<0.001
Equivocal	12.1 (21)	5.1 (5)	21.6 (16)	
Not detected	6.9 (12)	3.0 (3)	12.2 (9)	
Negative	8.1 (14)	9.1 (9)	6.8 (5)	
Double/triple aPL positivity	73.6 (167/227)	93.2 (124/133)	45.8 (43/94)	<0.0001
Treatment				
Antiplatelets	54.3 (125)	50.4 (68)	60.0 (57)	0.191
Warfarin	54.3 (125)	60.0 (81)	46.3 (44)	0.055
LMWH	5.7 (13)	5.2 (7)	6.3 (6)	0.940
HCQ	54.3 (125)	52.6 (71)	56.8 (54)	0.615
Rituximab	0.4 (1)	0.7 (1)	0.0 (0)	NP
Other bDMARD	0.4 (1)	0.7 (1)	0.0 (0)	NP

(Continued)

Table 1. (*Cont'd*)

	Total cohort (n = 230)	Anti-D1 positive (n = 135)	Anti-D1 negative (n = 95)	*P*
Immunosuppressive agent				
Azathioprine	6.1 (14)	5.2 (7)	7.4 (7)	0.688
Cyclophosphamide	0.4 (1)	0.7 (1)	0.0 (0)	NP
Methotrexate	5.7 (13)	5.2 (7)	6.3 (6)	0.940
MMF	4.8 (11)	4.4 (6)	5.3 (5)	0.765
Prednisone	14.8 (34)	18.5 (25)	9.5 (9)	0.087
Cyclosporin A	1.7 (4)	2.2 (3)	1.1 (1)	0.644

* Except where otherwise indicated, values are the percentage of patients (number) per group. Anti-D1 = anti–domain 1; aPL = antiphospholipid antibody; PAPS = primary antiphospholipid syndrome (APS); SAPS = secondary APS; SARDs = systemic autoimmune rheumatic diseases; CAPS = catastrophic antiphospholipid syndrome; gw = gestational week; SLE = systemic lupus erythematosus; UCTD = undifferentiated connective tissue disease; aCL = anticardiolipin antibodies; GPL = aCL IgG units; MPL = aCL IgM units; anti-β_2GPI = anti–β_2 glycoprotein I antibodies; SGU = anti-β_2GPI IgG units; SMU = anti-β_2GPI IgM units; LAC = lupus anticoagulant; VKA = vitamin K antagonists; LMWH = low molecular weight heparin; HCQ = hydroxychloroquine; bDMARD = biologic disease-modifying antirheumatic drug; NP = not performed; MMF = mycophenolate mofetil.
† Patient could have presented with multiple clinical events.
‡ At study inclusion, aCL and anti-β_2GPI antibodies of IgG and IgM isotypes were tested by enzyme-linked immunosorbent assays in APS ACTION core laboratories.

(Supplementary Tables 4, 5, 7, and 8, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459).

Significant decrease over time in anti-D1 and anti-β_2GPI antibody positivity rates. Among 135 patients with ≥1 positive anti-D1 antibody result, anti-D1 titers over time significantly decreased in 79.3% (n = 107) of patients (mean change 86.5 CU [95% CI 62.3–120.0]) and increased in 18.5% (n = 25) of patients (mean change 65.1 CU [95% CI 32.2–131.5]). Any fluctuation of anti-D1 antibody titers was observed in 2.2% (n = 3) of samples. In 19.3% of 135 anti-D1–positive patients, anti-D1 results changed from positive to negative (n = 20) or from negative to positive (n = 6) (McNemar's χ^2 = 6.5, P = 0.011). When patients were examined according to the anti-D1 antibody titer quartile category, throughout follow-up, 63 patients (46.7%) remained in the same anti-D1 titer category, whereas 72 patients (53.3%) shifted titer categories, a change that was persistent in most cases (n = 52, 72.2%). A shift in titer categories occurred more frequently in patients included in the high anti-D1 antibody positivity category, whereas a change from anti-D1 positivity to negative test result was less frequent among patients with previous thrombosis (Table 2).

Among 170 patients who were positive at least once for anti-β_2GPI IgG, anti-β_2GPI antibody titers over time significantly decreased in 61.2% (n = 104) of patients (mean change 166.5 CU [95% CI 112.6–246.2]) and increased in 34.1% (n = 58) of patients (mean change 218.9 CU [95% CI 117.2–409.0]). Any fluctuation of anti-β_2GPI IgG antibody titers was observed in 4.1% (n = 7) of samples. In 7.1% (n = 12) of patients, the anti-β_2GPI IgG test result changed from positive to negative (n = 11, 6.5%) or from negative to positive (n = 1, 0.6%) (McNemar's χ^2 = 6.75, P = 0.009). A shift in result category was more frequently observed for anti-D1 compared with anti-β_2GPI antibodies (χ^2 = 9.18, P = 0.003). When patients were examined according to anti-β_2GPI antibody titer quartile category,

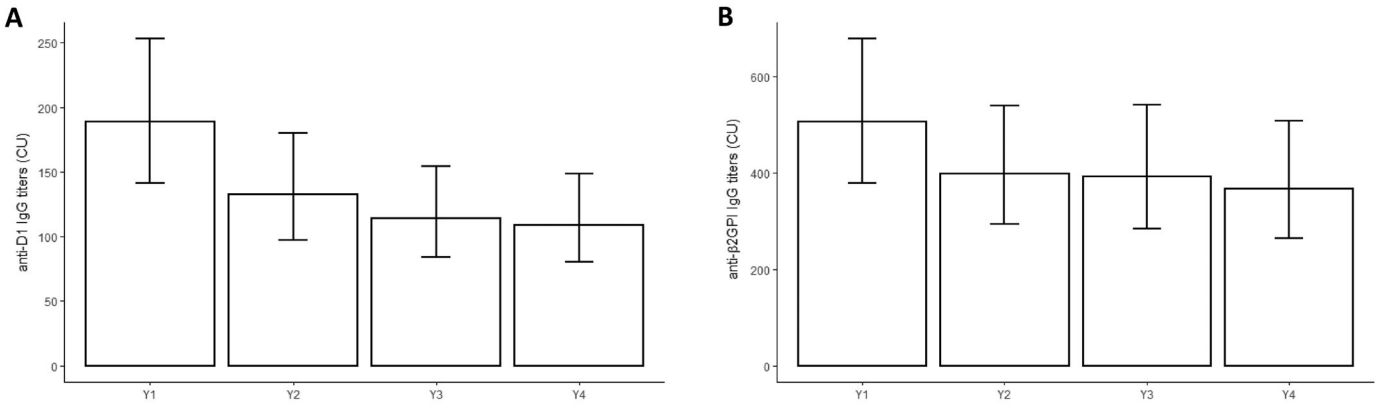

Figure 1. Longitudinal fluctuation of anti–domain 1 (anti-D1) and anti–β_2-glycoprotein I (anti-β_2GPI IgG) antibody titers in an international cohort of patients who were persistently positive for antiphospholipid antibodies. Bars show log-transformed levels of anti-D1 antibodies (**A**) and anti-β_2GPI antibodies (**B**) at years (Y) 1–4 in geometric mean chemiluminescent units (CU). Error bars represent 95% confidence intervals.

Table 2. Rates of anti-D1 antibody positivity at different time points in the total cohort of 230 patients and in patients subgrouped according to clinical and laboratory features*

	Anti-D1 positivity			
	Year 1	Year 2	Year 3	Year 4
Whole population (n = 230)	54.4 (125)	50.0 (115)	48.3 (111)	48.3 (111)
Low anti-D1 positivity	25.2 (34)	31.1 (42)	34.1 (46)	39.3 (53)
Intermediate-low anti-D1 positivity	25.2 (34)	23.7 (32)	25.2 (34)	22.2 (30)
Intermediate-high anti-D1 positivity	23.7 (32)	25.2 (34)	21.5 (29)	21.5 (29)
High anti-D1 positivity	25.9 (35)	20.0 (27)	19.3 (26)	17.0 (23)
Single aPL positivity (n = 60/227)†	6.7 (4)	6.7 (4)	3.3 (2)	5.0 (3)
Double/triple aPL positivity (n = 167/227)†	71.9 (120)	66.5 (111)	64.7 (108)	64.1 (107)
Associated SLE (n = 75/226)	49.3 (37)	42.7 (32)	41.3 (31)	41.3 (31)
No associated SLE (n = 151/226)	57.0 (86)	53.6 (81)	51.7 (78)	51.7 (78)
Previous thrombosis (n = 145)	57.9 (84)	55.2 (80)	54.5 (79)	54.5 (79)
No previous thrombosis (n = 85)	48.2 (41)	41.2 (35)	37.7 (32)	37.7 (32)

* Values are the percentage of patients (number) per group. See Table 1 for definitions.
† Data on the number of positive aPL tests were missing for 3 patients.

throughout follow-up, 105 patients (61.8%) remained in the same titer category, whereas 65 patients (38.2%) shifted titer categories, a change that was usually persistent (n = 41, 63%), although most samples (n = 105, 61.8%) remained in the same titer category.

Association between previous vascular events and higher anti-D1 and anti-β_2GPI antibody titers. Multivariable linear mixed-effects models were drawn to identify predictors of the longitudinal fluctuation of anti-D1 and anti-β_2GPI IgG titers. Patients with double and triple aPL positivity displayed 12.0-fold higher anti-D1 titers (95% CI 7.1–20.0), whereas presence of concomitant systemic lupus erythematosus (SLE) did not affect the anti-D1 titer fluctuation ($P = 0.531$) but was inserted in the model as a confounder (Table 3). Patients with previous thrombotic events had 1.9-fold higher (84%) anti-D1 antibody titers (95% CI 1.2–2.9). After adjustment for age and sex, we observed significant decreases in anti-D1 antibody titers over time, with the most marked decrease at Y1 of 21% (–1.3-fold, 95% CI –1.2 to –1.4; $P < 0.0001$). At Y4, the adjusted anti-D1 antibody titers were 1.5-fold lower compared with at Y1 (32% decrease, 95% CI –1.3 to –1.6; $P < 0.0001$).

Patients with double and triple aPL positivity displayed 32.4-fold higher anti-β_2GPI titers (95% CI 19.5–55.0; $P < 0.0001$), whereas those with concomitant SLE had 1.7-fold lower anti-β_2GPI antibody levels (95% CI –1.04 to –2.9; $P = 0.038$) (Table 4). Patients with previous thrombotic events had 2.1-fold higher anti-β_2GPI antibody titers (114%, 95% CI 1.4–3.4). After adjustment for age and sex, we observed significant decreases in anti-β_2GPI antibody titers over time, with the most marked decrease at Y1 of 16% (–1.2 fold, 95% CI –1.1 to –1.4; $P < 0.0001$). At Y4, the adjusted anti-β_2GPI antibody titers were 1.3-fold lower compared with at Y1 (21% decrease, 95% CI –1.1 to –1.4; $P < 0.001$).

Association between hydroxychloroquine and fluctuation of anti-D1 and anti-β_2GPI antibody titers. We observed an association between anti-D1 titer fluctuations

Table 3. Final multivariable linear mixed-effects model of predictors of fluctuations of anti-D1 antibody titers in the study patients*

Variable	Estimate of coefficient ± SE	Degrees of freedom	*t* value	*P*
Intercept	1.58 ± 0.23	662	6.88	<0.0001
Y2 versus Y1	−0.11 ± 0.02	662	−4.93	<0.0001
Y3 versus Y1	−0.16 ± 0.02	662	−7.35	<0.0001
Y4 versus Y1	−0.17 ± 0.02	662	−7.56	<0.0001
Age at enrollment (years)	-0.02 ± 0.004	217	−4.68	<0.0001
Sex	0.13 ± 0.11	217	1.18	0.240
SLE	−0.07 ± 0.11	217	−0.63	0.531
History of VE	0.27 ± 0.10	217	2.58	0.010
Incident VE	0.19 ± 0.11	662	1.75	0.080
HCQ	−0.10 ± 0.04	662	−2.30	0.022
Immunosuppressive agents	−0.11 ± 0.07	662	−1.55	0.123
Double/triple aPL positivity	1.08 ± 0.11	217	9.41	<0.0001
Incident VE × HCQ	−0.24 ± 0.14	662	−1.75	0.081

* Estimates of the regression coefficients are expressed as $\log_{10}$ of anti-D1 titers. Y1 = year 1; Y2 = year 2; Y3 = year 3; Y4 = year 4; VE = vascular event; VE × HCQ = interaction between VE and HCQ; see Table 1 for other definitions.

Table 4. Final multivariable linear mixed-effects model of predictors of the fluctuations of anti-β_2GI antibody titers*

Variable	Estimate of coefficient ± SE	Degrees of freedom	*t* value	*P*
Intercept	1.85 ± 0.23	659	7.98	<0.0001
Y2 versus Y1	−0.08 ± 0.03	659	−2.83	0.0048
Y3 versus Y1	−0.07 ± 0.03	659	−2.38	0.0174
Y4 versus Y1	−0.10 ± 0.03	659	−3.61	<0.001
Age at enrollment (years)	-0.02 ± 0.004	217	−4.15	<0.0001
Sex	0.08 ± 0.11	217	0.73	0.464
SLE	−0.24 ± 0.11	217	−2.09	0.038
History of VE	0.33 ± 0.10	217	3.22	0.002
Incident VE	0.20 ± 0.14	659	1.49	0.138
HCQ	−0.15 ± 0.05	659	−2.76	0.006
Immunosuppressive agents	−0.12 ± 0.08	659	−1.49	0.137
Double/triple aPL positivity	1.52 ± 0.11	217	13.25	<0.0001
Incident VE × HCQ	−0.32 ± 0.18	659	−1.81	0.070

* Estimates of the regression coefficients are expressed as $\log_{10}$ of anti-β_2GPI titers. Y1 = year 1; Y2 = year 2; Y3 = year 3; Y4 = year 4; VE = vascular event; VE × HCQ = interaction between VE and HCQ; see Table 1 for other definitions.

and HCQ treatment at any given time point (Table 3 and Supplementary Figure 2A, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). Of note, HCQ was associated with a 21% decrease in anti-D1 titers (1.3-fold reduction, 95% CI −1.1 to −1.5). Treatment with immunosuppressive agents did not affect anti-D1 titer fluctuation (P = 0.123) but was inserted in the model as a confounder, whereas treatment with biologic agents was not inserted as did not contribute to its fit.

We also observed an association between anti-β_2GPI titer fluctuation and HCQ treatment at any given time point (Table 4 and Supplementary Figure 2B, available at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). Of note, treatment with HCQ was associated with a 29% decrease in anti-β_2GPI titers (1.4-fold reduction, 95% CI −1.1 to −1.8). Treatment with immunosuppressive agents did not affect the fluctuation of anti-D1 titers (P = 0.137) but was inserted in the model as confounder. Treatment with biologic agents was not inserted as did not contribute to its fit.

Incidence of thrombotic events during follow-up. During follow-up, 17 new thrombotic events occurred in 15 patients (described in the Supplementary Material, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). Patients with triple aPL positivity and anti-D1 antibodies had a similar rate of thrombotic events compared with the remaining patients. In all but 1 patient, blood samples closer to the incident thrombosis were collected after the event, with median time between thrombosis and blood sample collection of 61 days (IQR 25.75–93.5).

During follow-up, of the 15 patients with new thrombotic events, 11 patients were positive for anti-β_2GPI IgG antibodies and presented with 13 thrombotic events (1 patient had 3 recurrences); the remaining patients did not carry anti-β_2GPI antibodies and presented with 4 new thrombotic events. Seven patients who were positive for anti-D1 antibodies and experienced an incident vascular event were also positive for anti-β_2GPI antibodies. In all but 1 patient, blood samples closer to the incident thrombosis were collected after the event, with the median time between thrombosis and blood sample collection of 64.5 days (IQR 28–115.5).

In our cohort, the rate of incident thrombosis was 1.30/100 person-years among anti-D1 antibody–positive patients and 2.63/100 person-years among anti-D1 antibody–negative patients. The rate of incident thrombosis was 1.62/100 person-years among anti-β_2GPI antibody–positive patients and 2.50/100 person-years among anti-β_2GPI antibody–negative patients.

Decrease in anti-D1 and anti-β_2GPI antibody titers at the time of vascular thrombosis. According to the multivariable model, incident vascular events were associated with 50% higher anti-D1 titers, approaching statistical significance (1.6-fold increase, 95% CI 1.0–2.5; P = 0.08) (Table 3 and Figure 2). Among patients who experienced thrombotic events during follow-up, those who were taking HCQ had 2.2-fold lower anti-D1 antibodies compared with patients not taking HCQ (95% CI −1.1 to −4.2; P = 0.020).

To elucidate the behavior of anti-D1 and anti-β_2GPI IgG titers at the time of the vascular event, fluctuations in antibody titers were further assessed in patients with incident thrombosis. In all patients but 1, a marked decrease in anti-D1 antibody titers was observed at the time of thrombosis (Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42459). However, anti-D1 antibodies were positive in all patients, even at the time of thrombosis, and titers increased after vascular events in the 4 of 5 patients with a follow-up sample.

Similarly, the multivariable model showed that patients with incident vascular events had 1.6-fold higher anti-β_2GPI titers, although this finding was not significant (95% CI 0.9–2.9; P = 0.138). Among patients who experienced thrombotic events

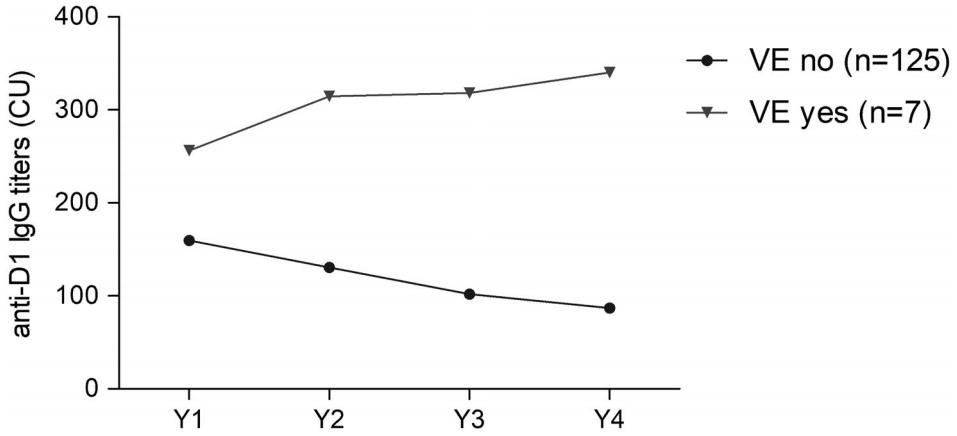

Figure 2. The fluctuation over time from year (Y) 1–4 in anti–domain 1 (anti-D1) antibody titers among patients with antiphospholipid syndrome with or without an incident vascular event (VE). Symbols represent the median chemiluminescent unit (CU) at each time point.

during follow-up, those who were taking HCQ had 2.9-fold lower anti-β_2GPI antibody titers than those who were not taking HCQ (95% CI −1.3 to −6.8; $P = 0.011$).

At the time of thrombosis, a marked decrease in anti-β_2GPI antibody titers was observed in 9 patients (Supplementary Figure 4, available on the *Arthritis & Rheumatology* website at https://onlinelibrary.wiley.com/doi/10.1002/art.42459), becoming negative in a single patient. Anti-β_2GPI IgG titers increased after the vascular event in 4 of 6 patients with a follow-up sample.

No time-dependent confounder variable was inserted in the case–crossover models, as the only modification over time was the introduction of antiplatelet agents after the incident thrombotic events.

A mean 1.6-fold decrease (35% decrease) in anti-D1 titers conferred an odds ratio (OR) for incident thrombosis of 6.0 (95% CI 0.62–59.3; Wald z test $P = 0.123$, likelihood ratio test [LRT] $P = 0.09$). A mean 2-fold decrease in anti-β_2GPI antibody titers (51% decrease) conferred an OR for incident thrombosis of 9.4 (95% CI 1.1–80.2; Wald z test $P = 0.041$, LRT $P = 0.01$).

DISCUSSION

This study offers several insights into the longitudinal titer fluctuation of antibodies targeting D1 of β_2GPI and those against the whole molecule, thus providing support to clinicians in the every-day interpretation of aPL tests. Indeed, physicians who are treating aPL-positive patients face several challenges to adequately decipher titer fluctuations in relation to the clinical scenario and the pharmacologic treatment of each patient. We showed for the first time, to our knowledge, that, among patients with persistent aPL positivity, the titers of both anti-D1 and anti-β_2GPI antibodies decrease over time, with a reduction of titers of 38% and 12%, respectively, at 3 years of follow-up. In the evaluation of the clinical impact of such decreases, it should be noted that the variations in antibody titers registered during patient follow-up are well above the coefficient of variation of 5% that has been reported for chemiluminescence, the closed and highly reproducible methodology used to test anti-β_2GPI and anti-D1 antibodies in our study (6). Anti-β_2GPI titers are more stable over time than anti-D1 antibodies, as evidenced by the minor percent decrement in antibody titers and the significantly lower rate of change in test results described for anti-β_2GPI antibodies.

This study provides a significant advancement over the available literature, which has addressed the issue of aPL profile stability in dichotomous terms, simply focusing on the rate of patients whose aPL tests turn negative or positive during follow-up. If there is unanimous consensus that the percentages of aPL positivity decrease during follow-up, then a wide heterogeneity exists in the rate of aPL negativization, which might be ascribed to the definition itself of seroconversion (namely, patients with formerly confirmed aPL positivity that turns negative at several subsequent determinations), the composition of the study cohort, concurrent treatment, length of follow-up, and study design. Overall, the so-called seroconversion has been reported in 4–59% of patients, occurring most frequently in those with a single aPL positivity (particularly isolated LAC), in those with lower aPL titers, and among asymptomatic aPL carriers (5,7–13). In cohorts composed exclusively of lupus patients, the same percentages of aPL negativization have been reported, ranging between 13.5% and 58% (14–17). Our data fall within this range, showing changes in test results in 19.3% of patients with anti-D1–positive samples and in 7.1% of patients with anti-β_2GPI–positive samples.

However, an accurate evaluation of aPL titer variation over time should go well beyond the mere assessment of the rate of aPL tests turning negative, and all of the variables that might impact antibody fluctuations should not be neglected. In this study, the longitudinal evaluation of aPL titers carefully accounted for demographic features, concomitant SLE diagnosis, thrombotic events, either previous or incident, and pharmacologic treatments. In our cohort, on-going HCQ treatment emerged as the

only variable to significantly affect both anti-β_2GPI and anti-D1 antibody titers, with comparable effects in both antibody subsets. In particular, patients taking HCQ at the time of blood sample collection had anti-D1 and anti-β_2GPI titers that were respectively 29% and 21% lower than patients not taking HCQ. Of note, this is the first description, to our knowledge, of the effect of HCQ on anti-D1 titers, while a greater burden of data is available for anti-β_2GPI antibodies.

After an early report that showed no difference in HCQ prescription between stable and unstable aPL profiles in a cohort of 204 aPL-positive patients (11), evidence has accumulated in support of the association between HCQ and decrementing aPL titers and positivity rates, both in patients with primary APS and in those with underlying SARDs (18–21). This finding might be ascribed to the well-known immunomodulatory properties of HCQ (22) and might lead to the postulation of HCQ having a thromboprotective effect. However, a decrease in antibody titers does not necessarily translate into a protection against thrombosis: patients in our cohort who experienced thrombotic events during follow-up while taking HCQ had 2.2-fold lower anti-D1 antibodies than those with incident vascular thrombosis who were not taking HCQ. Importantly, our study was not designed to assess the rate of incident thrombotic events among aPL-positive patients or to test the efficacy of thromboprophylaxis. Therefore, no definite statement can be formulated about the crude rates of incident thrombosis in our cohort, which, not appropriately accounting for treatment and prothrombotic risk factors, were higher among patients who were persistently negative for anti-D1 and/or anti-β_2GPI antibodies. Similarly, no conclusions can be drawn about the thromboprotection conferred by HCQ; firm answers can originate exclusively from multicenter international double-blind randomized controlled trials, despite available evidence suggesting a thromboprotective role for HCQ (22–30).

In our study cohort, concurrent immunosuppressive treatment did not affect anti-β_2GPI and anti-D1 antibody titers. This observation confirms the available data in studies of exclusively lupus patients (16,20,31), with only a single study identifying immunosuppressive agents as independent predictors of aPL negativization (15).

Biologic agents did not emerge as significant predictors of aPL fluctuation in this study, possibly because of the low rate of patients ever taking biologic disease-modifying antirheumatic drugs. Among these drugs, data are available on rituximab and belimumab. After a few case reports suggested aPL negativization or decrementing antibody titers following B cell depletive therapy, the effects of rituximab on aPL titers were assessed in heterogeneous populations, without any dramatic effect on aPL titers (18,32–38). Belimumab use in primary APS is limited to anecdotal cases (38–40), and data on the potential effects on aPL titers are available exclusively in aPL-positive lupus patients, all pointing toward a net beneficial effect on antibody reduction, although with some discrepancies (21,41–46).

It could be envisaged that a polypharmacologic approach might burst the effects on aPL titer reduction; however, data are presently too limited to draw any definite conclusion. In our study, no significant interaction could be identified between HCQ and immunosuppressive agents ($P = 0.259$), although previous studies related to the potential interaction of HCQ and belimumab have shown conflicting results (21,43).

In addition to HCQ treatment, our statistical model identified several other well-known predictors of anti-D1 and anti-β_2GPI titer fluctuations. Patients with a concomitant diagnosis of SLE had lower anti-β_2GPI but not anti-D1 antibody titers. Conversely, having multiple aPL positivities was associated with higher anti-D1 and anti-β_2GPI titers, with the number of criteria aPL tests being the most prominent predictor of antibody titers. In addition, patients with vascular thrombosis that were either previous or incident vascular events had higher baseline anti-D1 and anti-β_2GPI titers than those without any thrombosis. Notably, the relationship between antibody titers and incident vascular events was further explored by means of a case–crossover design, which allowed us to observe that, at the time of the thrombosis, both anti-D1 and anti-β_2GPI antibody titers are significantly lower, with a subsequent increase in titers in samples collected after the vascular event. To our knowledge, this is not only the first observation on the decrement of anti-D1 titers at the time of thrombosis but also the first on anti-β_2GPI titer fluctuations in a cohort of patients selected based on persistent aPL positivity. Previous data, all coming from the same group, indicated that aCL and anti-β_2GPI antibody titers were decreased in lupus patients who had experienced thrombovascular events (31,47,48). In the same study, anti-β_2GPI antibodies turned negative in 3 of 24 patients (31), a figure much lower than reported in the Hopkins Lupus Cohort (49).

Although we acknowledge that these results should be validated in larger prospective cohorts, our observations might still be highly relevant in terms of both pathogenic and clinical implications. Indeed, it might be envisaged that anti-D1 and anti-β_2GPI antibodies, the main pathogenic autoantibody subset, deposit at thrombotic sites. Interestingly, anti-D1 and anti-β_2GPI consumption might be paralleled by a net reduction in β_2GPI serum levels, which has been indeed shown to occur at the time of thrombosis in patients with APS and in those with non-APS thrombotic diseases (50). This observation warrants caution when interpreting aPL results tested shortly after the thrombotic event, which is extremely relevant in all patients without a previous APS diagnosis. Interestingly, the subsequent rise in antibody titers after thrombosis observed in our present cohort and in other cohorts strongly supports this hypothesis (31,47,49). Unfortunately, we were not able to raise mechanistic support to this intriguing scenario due to the clinical nature of this work.

Further limitations of this study include follow-up tests at only 4 time points; it would be interesting to evaluate aPL titer variations beyond this timeframe. Longitudinal data were available for

anti-D1 IgG, anti-β_2GPI, and aCL IgG/IgM/IgA but not for LAC, due to the peculiar nature of this functional assay, or for other noncriteria aPL tests such antiphosphatidylserine/prothrombin antibodies. Because of the longitudinal nature of the study, it might be claimed that our observations are affected by regression to the mean, a phenomenon that involves a tendency of high values to be lower on remeasurement in the absence of any intervention (51). The best approach to carefully account for the regression to the mean is to optimize the study design to include, as in this instance, control groups and observations taken from time points in which no interventions were implemented, since different groups should be equally affected by the phenomenon (51). Furthermore, this phenomenon can be partially ascribed to random measurement errors, although not a major concern in the present study due to the already cited high reproducibility of chemiluminescence (6).

Conversely, this study is strengthened by its multicentric nature, which allowed including a large and well-defined patient population. Furthermore, aPL were tested in highly experienced centralized laboratories using a very reproducible methodology. A longitudinal analysis that considered multiple potential confounding and time-varying effects was applied, leading to robust conclusions.

As a whole, our data suggest that both anti-β_2GPI and anti-D1 antibody titers decrease over time, with HCQ exerting a significant impact on such decrements. Patients with thrombosis, either previous or incident, display higher anti-β_2GPI and anti-D1 antibody titers; however, closer to the vascular event, the antibody titers are significantly lower than at other time points, unraveling a novel etiopathogenic scenario.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Chighizola had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Chighizola, Erkan, Bertolaccini.

Acquisition of data. Andrade, Tektonidou, Pengo, Ruiz-Irastorza, Belmont, Gerosa, Fortin, Branch, Andreoli, Petri, Cervera, Knight, Willis, Efthymiou, Cohen, Erkan, Bertolaccini.

Analysis and interpretation of data. Chighizola, Pregnolato, Willis, Erkan, Bertolaccini.

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APPENDIX A: MEMBERS OF THE APS ACTION GROUP

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