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FLUCTUATION OF ANTI-DOMAIN 1 AND ANTI-β2 GLYCOPROTEIN I ANTIBODY TITERS OVER TIME IN PATIENTS WITH PERSISTENTLY POSITIVE ANTIPHOSPHOLIPID ANTIBODIES

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Running head: Longitudinal titer fluctuation of anti-D1 and anti-β2GPI antibodies

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Abstract

Objective

This work aims at evaluating longitudinally titers of antibodies against β2-glycoprotein I (β2GPI) and domain 1 (anti-D1), identifying predictors of the variation of anti-D1 and anti-β2GPI antibody titers and clarifying whether antibody titer fluctuations predict thrombosis in a large international cohort of patients persistently positive for antiphospholipid antibodies (aPL), the "APS ACTION Registry".

Methods

Patients with available blood samples from at least 4 time points were included. Anti-β2GPI and anti-D1 IgG were tested by chemiluminescence (BioFlash, INOVA Diagnostics).

Results

In a cohort of 230 patients, anti-D1 and anti-β2GPI titers decreased significantly over time ($p<0.0001$ and $p=0.010$, respectively). After adjustment for age, gender, and number of positive aPL tests, the fluctuation of anti-D1 and anti-β2GPI titers was associated with treatment with hydroxychloroquine (HCQ) at each time-point. Treatment with HCQ, but not immunosuppressors, was associated with 1.3-fold and 1.4-fold decrease in anti-D1 and anti-β2GPI titers, respectively. Incident vascular events were associated with 1.9-fold and 2.1-fold increase of anti-D1 and anti-β2GPI titers, respectively. Anti-D1 and anti-β2GPI titers at the time of thrombosis were lower compared to the other time-points: 1.6-fold decrease in anti-D1 titers and 2-fold

decrease in anti-β2GPI titers conferred an OR for incident thrombosis of 6.0 (95%CI 0.62-59.3) and 9.4 (95%CI 1.1-80.2), respectively.

Conclusions

Treatment with HCQ and incident vascular events significant predicted anti-D1 and anti-β2GPI titer fluctuation over time. Both anti-D1 and anti-β2GPI titers drop around the time of thrombosis, with potential clinical relevance.

Introduction

Anti-phospholipid antibodies (aPL) provide the main acquired risk-factor for both thrombosis and obstetric complications, the two clinical facets of anti-phospholipid syndrome (APS) [1]. The management of aPL-positive subjects dictates a careful evaluation of the risk of future clinical events, with important therapeutic implications in terms of both primary and secondary thromboprophylaxis. The aPL profile provides the main determinant of APS clinical manifestations: since each test conveys a characteristic specificity and sensitivity, clinicians consider the pattern of positive criteria aPL test(s) -namely anti-cardiolipin antibodies (aCL), anti-β2 glycoprotein I antibodies (anti-β2GPI) and/or lupus anticoagulant (LA)-, the number of positive aPL tests, the isotypes and antibody titers [1].

Notwithstanding this strategy, clinicians still deal with many difficulties in optimizing the management 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 while other subjects remain asymptomatic through the entire life-span. Research efforts have fostered the development of second-line testing tools, such as the characterization of domain reactivity of anti-β2GPI antibodies, which are regarded as the true pathogenic antibody subset. Following the ascertainment of their pathogenic role, antibodies against domain 1 of β2GPI (anti-D1) have catalysed much attention [2]. Testing for anti-D1 antibodies could be useful since positivity predicts aPL-associated manifestations: this antibody subset is highly prevalent in patients with

thrombotic APS and frequently positive among women with pure obstetric manifestations while is 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, those with triple aPL positivity [2].

Despite their consolidated prognostic role, no longitudinal data on anti-D1 antibody titers have been raised and no comparison between the longitudinal behaviour of titers of antibodies directed against D1 and those targeting β2GPI whole molecule is available. Thus, the aims of this large-scale prospective international study consist in i) assessing 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"); ii) evaluating the stability over time of anti-D1 and anti-β2GPI IgG antibodies; iii) identifying predictors of the longitudinal fluctuation of anti-D1 and anti-β2GPI antibody titers; and iv) clarifying whether the fluctuation of anti-D1 and anti-β2GPI antibody titers carries a clinical significance in predicting thrombosis.

Material and Methods

APS ACTION Registry.

The APS ACTION registry includes persistently aPL-positive patients based on the Updated Sapporo APS Classification Criteria [1], with or without systemic autoimmune rheumatic diseases (SARDs), followed every 12±3 months with clinical and laboratory data and blood collection.

Data collection.

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

Anti-phospholipid antibody assays.

Anti-D1 IgG, anti-β2GPI IgG/IgM/IgA and anti-aCL IgG/IgM/IgA were tested by a chemiluminescent immunoassay exploiting BIO-FLASH® technology (QUANTA Flash® β2GPI Domain 1 IgG, QUANTA Flash® β2GPI IgG/IgM/IgA and QUANTA Flash® cardiolipin IgG/IgM/IgA; Inova Diagnostics, San Diego, CA, USA) at Y1 and in 3 follow-up samples. Threshold values to define anti□□□□positivity were set upon manufacturer's cut-off at 20 chemiluminescent units (CU). This threshold was established by the 99% percentile of 250 donors. Each APS ACTION core laboratory has validated the manufacturers cut-off by testing 20 local healthy subjects [3].

Samples were tested in three APS ACTION core laboratories following validation.

At study inclusion, LA, anti-β2GPI IgG/IgM and aCL IgG/IgM were tested in core-lab laboratories as previously described [4, 5].

Patients enrolled in APS ACTION registry were considered eligible for inclusion in this study when serum samples from at least 4 different time-points were available for longitudinal anti-D1 and anti-β2GPI antibody testing.

Statistical analysis.

Descriptive statistics were generated for demographic, clinical and laboratory data. Due to the skewed distribution of anti-D1 and anti-β2GPI antibody titers, results are expressed as geometric mean. Associations between variables were assessed by chi-squared test and Mc Nemar's chi-squared test, as appropriate.

The rates of anti-D1, anti-β2GPI and aCL antibody positivity were calculated for each time point for the whole study cohort and for patients categorized upon clinical features. Four positivity categories for anti-D1 antibody titers were identified upon quartiles: low positivity (<57.6CU), median-low positivity (57.6-165.7CU), median-high positivity (165.7-680.3CU) and high positivity (<680.3CU).

Four positivity categories for anti-β2GPI antibody titers were identified upon quartiles: low positivity (<116.9CU), median-low positivity (116.9-702.7CU), median-high positivity (702.7-2254.6CU) and high positivity (>2254.6CU).

Anti-D1 IgG, anti-β2GPI IgG/IgM/IgA and aCL IgG/IgM/IgA antibody titers within the same subject were compared by Friedman's test.

Mixed linear models for repeated measurement nested within subject were built to identify predictors of anti-D1 and anti-β2GPI IgG antibody titer fluctuation.

To clarify whether fluctuation in anti-D1 and anti-β2GPI IgG antibody titers are associated with thrombotic events, a case-crossover design was applied.

A p-value <0.05 was considered statistically significant. Data were analyzed using R version 4.0.5.

Results

Approximately 60% of patients have at least one positive anti-D1 antibody test.

Out of the whole APS ACTION cohort, 1942 samples from 515 patients were tested for anti-D1 and anti-β2GPI IgG (**Supplementary Table 1**). Anti-D1 antibodies were tested at 4 time points in 230 patients, which were included in this longitudinal study. The clinical and laboratory details of patients are detailed in **Table 1**.

At least one positive anti-D1 antibody result was identified in 135 patients (58.7%) while the remaining 95 patients were persistently negative for anti-D1 antibodies both at baseline and during follow-up. Patients with at least one positive anti-D1 antibody result were significantly younger than those testing negative for anti-D1 antibodies at all time-points and had a significantly higher positivity rates and titers of criteria aPL tests at baseline. Subjects without anti-D1 antibodies were more frequently asymptomatic aPL carriers while there was no difference in the distribution of associated SARDs upon anti-D1 antibody positivity. Only 13 subjects (9.6%) displayed anti-D1 antibody positivity in a single occasion while most patients displayed persistent positivity for anti-D1 antibodies in all 4 tests (100, 74%) (**Supplementary Table 2**). There was no difference in the rate of thrombosis between these subjects and the remaining 52 patients with anti-D1 positivity not confirmed in all tests (χ^2=0.836, p=0.360).

Among criteria aPL tests, IgG isotype was the most prevalent: anti-β2GPI IgG antibodies tested positive at least once in 170 out of the 230 included subjects (74%) while aCL IgG in 151 subjects (65.6%) (**Supplementary Tables 3** and **6**). A positivity in IgM isotype emerged more frequently for aCL as compared to anti-β2GPI antibody test (83 [36.1%]

versus 73 [31.7%] (**Supplementary Tables 4** and **7**). Non criteria anti-β2GPI and aCL IgA were found positive in 76 (33.0%) and 94 (40.9%) of patients respectively (**Supplementary Tables 5** and **8**).

Anti-D1 and anti-β2GPI IgG antibody titers significantly fluctuate over time.

Among the 135 patients with at least one anti-D1 positive result, anti-D1 titers varied significantly over time (Friedman statistics: 508.5, p<0.0001; anti-D1 geometric mean at Y1 189.0 [95% CI 141.2 to 253.1]; Y2 132.3 [95%CI 97.4-179.7]; -15% versus Y1; Y3 113.8 [95% CI 83.8 to 154.4]; -17% versus Y2; Y4 109.2 [95%CI 80.3-148.5] -6% versus Y3, -38% versus Y1, **Figure 1A** and **Supplementary Figure 1A**). Anti-D1 titers at baseline were significantly higher compared to Y4 (p=0.029). The same fluctuation pattern was observed when patients were selected upon multiple anti-D1 positivities over time (**Supplementary Table 2**).

Anti-β2GPI titers correlated with anti-D1 titers at all time-points (Y1: r=0.804 [95%CI 0.750-0.847], p<0.0001; Y2: r=0.836 [95%CI 0.790-0.872], p<0.0001; Y3: r=0.831 [95%CI 0.784-0.869], p<0.0001; Y4: r=0.813 [95%CI 0.763-0.854], p<0.0001); among the 170 patients with at least one anti-β2GPI positive result, anti-β2GPI titers significantly reduced at Y4 compared to Y1 (Friedman statistics=11.32, p=0.010; anti-β2GPI geometric mean at Y1 187.1 [95%CI 14.5-1586.5]; Y2 150.8 [95%CI 11.1-1379.2]; -9% versus Y1; Y3 124.9 [95%CI 12.2-1304]; 0% Y3 versus Y2; Y4 117.6 [95%CI 8.7-1136.6]; -2% versus Y3; Y4 versus Y1 -12%; **Figure 1B** and **Supplementary Figure 1B**). When patients were selected upon multiple anti-β2GPI IgG positivities, a similar pattern of antibody titer variation over time emerged (**Supplementary Table 3**).

Among the other tested aPL tests, a similar longitudinal variation of antibody titers was noted exclusively for aCL IgG (**Supplementary Table 6**). For all the remaining assays, antibody titers significantly fluctuated over time but without a progressive decrease of titers (**Supplementary Tables 4, 5, 7** and **8**).

Anti-D1 and anti-β2GPI antibody positivity rates significantly decrease over time.

Anti-D1 titers over time significantly decreased in 79% (n=107) of patients (mean change 86.5CU [95%CI 62.3-120.0]), and increased in 19% (n=25) of samples (mean change 65.1CU [95%CI 32.2-131.5]. Any fluctuation of anti-D1 antibody titers was observed in 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, Mc Nemar's χ^2=6.5; p=0.011). When anti-D1 antibody titers were categorized upon quartiles, throughout follow-up 63 subjects (46.7%) remained in the same anti-D1 titer category, while 72 patients (53.3%) shifted titer categories, a change that was persistent in most cases (52, 72.2%). Shift in titer categories occurred more frequently in patients with high anti-D1 antibody positivity, while change from anti-D1 positivity to negative test result was less frequent among patients with previous thrombosis (**Table 2**).

Anti-β2GPI antibody titers over time significantly decreased in 61.5% (n=104) of patients (mean change 166.5CU [95%CI 112.6-246.2]), and increased in in 34.3% (n=58) of samples (mean change 218.9CU [95%CI 117.2-409.0]). Any fluctuation of anti-β2GPI IgG antibody titers was observed in 4.1% (n=7) of samples. In 7.1% of patients (n=12), anti-β2GPI IgG changed in test result: from positive to negative (n=11, 6.5%), or negative to positive (n=1, 0.6% Mc Nemar's χ^2=6.75; p=0.009). Shift in result category

was more frequently observed for anti-D1 compared to anti-β2GPI antibodies (χ^2=9.18, p=0.003). When anti-β2GPI antibody titers were categorized upon quartiles, throughout follow-up 105 subjects (61.8%) remained in the same titer category, while 65 patients (38.2%) shifted titer categories, a change that was usually persistent (41, 63%), although most samples (105, 61.8%) remained in the same titer category.

Patients with previous vascular events display higher anti-D1 and anti-β2GPI antibody titers.

Multivariable mixed linear models were drawn to identify predictors of the longitudinal fluctuation of anti-D1 and anti-β2GPI IgG titers. Patients with double/triple aPL positivity displayed 12.0-fold higher anti-D1 titers [95%CI 7.1-20.0] while concomitant systemic lupus erythematosus (SLE) did not affect anti-D1 titer fluctuation but was inserted in the model as confounder (p=0.531, **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 gender, anti-D1 antibody titers decreased significantly over time, with the most marked decrease at one year (21% decrease, -1.3 fold, 95%CI 1.2--1.4, p<0.0001). At Y4, adjusted anti-D1 antibody titers were 1.5-fold lower compared to Y1 (32% decrease, 95%CI -1.3--1.6, p<0.0001).

Patients with double/triple aPL positivity displayed 32.4-fold higher anti-β2GPI titers (95%CI 19.5-55.0, p<0.0001) while those with concomitant SLE had 1.7-fold lower anti-β2GPI antibody levels (95%CI 1.04-2.9, p=0.038, **Table 4**). Patients with previous thrombotic events had 2.1-fold higher anti-β2GPI antibody titers (114%, 95%CI 1.4-3.4). After adjustment for age and gender, anti-β2GPI antibody titers decreased significantly over time, with the most marked decrease at one year (16% decrease, -1.2 fold, 95%CI -

1.1--1.4, p<0.0001). At Y4, adjusted anti-β2GPI antibody titers were 1.3-fold lower compared to Y1 (21% decrease, 95%CI -1.1--1.4, p<0.001).

Hydroxychloroquine affects the fluctuation of anti-D1 and anti-β2GPI antibody titers.

The fluctuation of anti-D1 titers was associated with HCQ treatment at any given time-point (**Table 3** and **Supplementary Figure 2A**). In particular, HCQ was associated with a 21% decrease in anti-D1 titers (1.3-fold reduction, 95%CI 1.1-1.5). Treatment with immunosuppressors did not affect anti-D1 titer fluctuation but was inserted in the model as confounder (p=0.123), whereas treatment with biological agents was not inserted as did not contribute to its fit.

The fluctuation of anti-β2GPI titers was associated with HCQ treatment at any time-point (**Table 4** and **Supplementary Figure 2B**). In particular, treatment with HCQ was associated with a 29% decrease in anti-β2GPI titers (1.4-fold reduction, 95%CI 1.1-1.8). Treatment with immunosuppressors did not affect the fluctuation of anti-D1 titers but was inserted in the model as confounder (p=0.137). Treatment with biological agents was tested not inserted as did not contribute to its fit.

During follow-up, 17 incident thrombotic events occurred.

During follow-up 17 new thrombotic events occurred in 15 subjects (described in the **Supplementary Material**). Patients with triple aPL positivity and anti-D1 antibodies had a similar rate of thrombotic events compared to the remaining subjects. In all but one subject, blood samples closer to the incident thrombosis were collected after the event, and the median time between thrombosis and blood sampling was 61 days (IQR

25.75-93.5).

During follow-up, 11 anti-β2GPI IgG positive patients presented 13 thrombotic events (3 recurrences in the same subject), those not carrying anti-β2GPI antibodies had 4 new thrombosis. The 7 anti-D1+ patients who experienced an incident vascular event were also positive for anti-β2GPI antibodies. In all but one subject, blood samples closer to the incident thrombosis were collected after the event, and the median time between thrombosis and blood sampling was 64.5 days (IQR 28-115.5).

In our cohort, the rate of incident thrombosis was 1.30/100 person-years among anti-D1 positive subjects and 2.63/100 person-years among those negative for anti-D1 antibodies. The rate of incident thrombosis was 1.62/100 person-years among anti-β2GPI-positive subjects and 2.50/100 person-years among those negative for anti-β2GPI antibodies.

Anti-D1 and anti-β2GPI antibody titers drop 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 on HCQ had 2.2-fold lower anti-D1 antibodies compared to those not receiving HCQ (95%CI 1.1- 4.2, p=0.020).

To elucidate the behaviour of anti-D1 and anti-β2GPI IgG at the time of vascular event, the fluctuation of antibody titers was further assessed in patients with incident thrombosis. In all subjects but one, a marked decrease in anti-D1 antibody titers was

observed at the time of thrombosis (**Supplementary Figure 3**). However, anti-D1 antibodies were positive in all patients even at the time of thrombosis, and titers increased after vascular events in the 4 out of the 5 patients with a follow-up sample.

Similarly, multivariable model showed that patients with incident vascular events had 1.6-fold higher anti-β2GPI titers, which did not attain statistical significance (95%CI 0.9-2.9, p=0.138). Among patients who experienced thrombotic events during follow-up, those on HCQ had 2.9-fold lower anti-β2GPI antibody titers compared to those not receiving HCQ (95%CI 1.3-6.8, p=0.011).

At the time of thrombosis a marked decrease in anti-β2GPI antibody titers was observed in 9 subjects (**Supplementary Figure 4**), becoming negative in a single patient. Anti-β2GPI IgG titers increased after the vascular event in 4 out of the 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 anti-platelet 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, LRT p=0.09). A mean 2-fold decrease in anti-β2GPI 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 β2GPI and those against the whole molecule, thus providing support to

clinicians in the every-day interpretation of aPL tests. Indeed, physicians dealing with APS patients face several challenges to adequately decipher titer fluctuation in relation to the clinical scenario and the pharmacological treatment of each patient. We showed for the first time that among patients with persistent aPL positivity the titers of both anti-D1 and anti-β2GPI antibodies decrease over time, with a reduction of titers of 38% and 12% respectively, at 3 years of follow-up. When evaluating the clinical impact of such decrease, it should be considered that the variation in antibody titers registered in the course of follow-up are well above the coefficient of variation of 5% that has been reported for chemiluminescence, the closed and highly reproducible methodology employed to test anti-β2GPI and anti-D1 antibodies in this study [6]. Anti-β2GPI titers are more stable over time as compared to anti-D1 antibodies, as evidenced by the minor percentage decrement in antibody titers and the significantly lower rate of change in test results described for anti-β2GPI antibodies.

This study provides a significant advancement over 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, there exist a wide heterogeneity in the rate of aPL negativization, which might be ascribed to the definition itself of seroconversion (namely, patients with former confirmed aPL positivity who turn negative at several subsequent determinations), 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 case of single aPL positivity (particularly isolated LA), lower aPL titers and among asymptomatic aPL carriers [5, 7-13]. aPL negativization in

cohorts composed exclusively of lupus patients reflects the same figures, ranging between 13.5 and 58% [14-17]. Our data fall within this range, registering a change in test results in 19.3% of anti-D1 and 7.1% of anti-β2GPI 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 variables that might impact antibody fluctuations should not be neglected. In the present work, the longitudinal evaluation of aPL titers carefully accounted for demographic features, concomitant SLE diagnosis, thrombotic events, either previous or incident, and pharmacological treatments. In our cohort, on-going HCQ treatment emerged as the only variable to significantly affect both anti-β2GPI and anti-D1 antibody titers, with a comparable effect for the two antibody subsets. In particular, patients treated with HCQ at the time of blood sampling presented anti-D1 and anti-β2GPI titers that were respectively 29% and 21% lower than those not on HCQ. To note, this is the first description of the effect of HCQ on anti-D1 titers, while a greater burden of data is available for anti-β2GPI antibodies. Following an early report denying any difference in HCQ prescription between stable and unstable aPL profile in a cohort of 204 aPL positive subjects [11], evidence has accumulated in support of the association of HCQ with decrementing aPL titers and positivity rates, both in patients with primary APS and those with underlying SARDs [18-21]. This finding might be ascribed to the well-known immunomodulatory properties of HCQ [22], and might lead to postulate a thromboprotective effect for HCQ. However, a decrease in antibody titers does not necessarily translate into a protection against thrombosis: our data show that patients who experienced thrombotic events during follow-up while on HCQ had 2.2-fold lower anti-D1 antibodies compared to those with incident vascular thrombosis not receiving

HCQ. Importantly, this study was not designed to assess the rate of incident thrombotic events among aPL positive subjects nor to test the efficacy of thrombophrophylaxis. Therefore, no definite statement can be formulated about the crude rates of incident thrombosis in our cohort, which, not appropriately accounting for treatment and pro-thrombotic risk-factors, were higher among patients persistently negative for anti-D1 and/or anti-β2GPI 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, even though available evidence suggests a thromboprotective role for HCQ [22-30].

In our cohort, concurrent immunosuppressive treatment did not affect anti-β2GPI and anti-D1 antibody titers. This observation confirms available data all coming from studies recruiting exclusively lupus patients [16, 20, 31], with only a single study identifying immunosuppressors as independent predictors of aPL negativization [15].

Biological agents did not emerge as significant predictors of aPL fluctuation in this study, possibly due to the low rate of patients ever receiving a bDMARD. In literature, available data relate to rituximab and belimumab. Following few case reports suggestive of aPL negativization or decrementing antibody titers after B cell depletive therapy, the effects of rituximab on aPL titers have been assessed in heterogeneous populations, without any dramatic effect on aPL titers [18, 32-38]. Belimumab use in primary APS is limited to anecdotic cases [38, 39], and data about the potential effects on aPL titers are available exclusively in aPL-positive lupus patients, altogether pointing towards a net beneficial effect on antibody reduction even though with some discrepancies [21, 41-45].

It could be envisaged that a polypharmacological approach might burst the effects on aPL titer reduction, but data are currently too limited to draw any definite conclusion. In

our study, no significant interaction could be identified between HCQ and immunosuppressors (p=0.259), while previous observations related to the potential interaction of HCQ and belimumab, with conflicting results [21, 43].

Besides HCQ treatment, the statistical model identified several other well-known predictors of anti-D1 and anti-β2GPI titers. Patients with a concomitant diagnosis of SLE had lower anti-β2GPI -but not anti-D1- antibody titers. Conversely, multiple aPL positivity were associated with higher anti-D1 and anti-β2GPI titers, with the number of criteria aPL tests being the most prominent predictor of antibody titers. In addition, patients with vascular thrombosis, both in case of previous or incident event, had higher baseline anti-D1 and anti-β2GPI titers compared to those without any thrombosis. Notably, the relationship of antibody titers with incident vascular events was further explored by the means of a case-crossover design, which allowed us to observe that at the time of the thrombosis both anti-D1 and anti-β2GPI antibody titers are significantly lower, with a subsequent increase in titers in samples collected after the vascular accident. This is not only the first ever observation about the decrement of anti-D1 titers at the time of thrombosis, but also the first about anti-β2GPI in a cohort of patients selected upon aPL persistent positivity. Previous data, all coming from the same group, were available for a decrease in aCL and anti-β2GPI antibody titers in lupus patients who had experienced a thrombovascular event [31, 47, 48]. In the same study, anti-β2GPI antibodies turned negative in 3 out of 24 patients [31], a figure much lower than what reported in the Hopkins Lupus cohort [49].

We acknowledge that these data should be validated in larger prospective cohorts, but this observation might still be highly relevant in terms of both pathogenic and clinical implications. Indeed, it might be envisaged that anti-D1 and anti-β2GPI antibodies, the

main pathogenic autoantibody subset, deposit at thrombotic site. Interestingly, anti-D1 and anti-β2GPI consumption might be paralleled by a net reduction in β2GPI serum levels, which has been indeed shown to occur at the time of thrombosis in patients with APS as well as those with non-APS thrombotic diseases [50]. This observation warrants caution when interpreting aPL result tested shortly after the thrombotic event, extremely relevant in all patients without a previous APS diagnosis. Interestingly, the subsequent rise in antibody titers after thrombosis observed in the present and in all 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 a follow-up of 4 time points only, while it would be very interesting to evaluate aPL titer variations beyond this time-frame. Longitudinal data were available for anti-D1 IgG, anti-β2GPI and aCL IgG/IgM/IgA but not for LA, due to the peculiar nature of such functional assay, nor for other non criteria aPL tests such anti-phosphatidylserine/prothrombin antibodies. Due to the longitudinal nature of the study, it might be claimed that our observations are affected by regression to the mean, a phenomenon consisting in the tendency of high values to be lower on re-measurement in absence of any intervention [51]. The best approach to carefully account for the regression to the mean consists in the optimization of study design, which should envisage –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 error, 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 multi-centric nature, which allowed including a large and well-defined study patient population. Furthermore, aPL were tested in highly experienced centralized laboratories using a very reproducible methodology. A longitudinal analysis considering multiple potential confounding and time-varying effects was applied, leading to robust conclusions.

As a whole, our data suggests that both anti-β2GPI and anti-D1 antibody titers decrease over time, with HCQ exerting a significant impact on such decrement. Patients with thrombosis, either previous or incident, display higher anti-β2GPI and anti-D1 antibody titers, but closer to the vascular event antibody titers are significantly lower compared to other time points, unravelling a novel aetiopathogenic scenario.

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Data availability statement: Raw data can be made available upon request.

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FIGURE LEGENDS

Figure 1. Longitudinal fluctuation of anti-D1 and anti-β2GPI IgG antibody titers.

A: Bar Charts of log-transformed levels of anti-D1 antibodies (geometric mean [95% CI]) at Y1, Y2, Y3 and Y4. **B:** Bar charts of levels of log-transformed anti-β2GPI antibodies (geometric mean [95% CI]) at Y1, Y2, Y3 and Y4.

Figure 2. The fluctuation over time of titers of anti-domain 1 antibodies in patients with and without incident vascular event.

TABLES

Table 1. Demographic, baseline clinical and therapeutic details of 230 patients at study inclusion subgrouped upon anti-D1 antibody positivity in at least one determination.

Demographics	**Overall sample (n=230)**	**Anti-D1+ (n=135)**	**Anti-D1- (n=95)**	**p-value**
Age in years, *mean (SD)*	45.0 (12.7)	42.3 (11.8)	48.8 (13.0)	0.0001
Gender, *%F (n)*	69.1 (159)	71.9 (97)	65.3 (62)	0.358
Race, *% (n)*				
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)	
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
N of event(s) 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)	
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)	
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
N of pregnancy loss 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)	
Oligo-SLE*	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°				
aPL	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-β2GPI, IgG (SGU)	70.9 (161)	93.2 (124)	39.4 (37)	<0.0001
Anti-β2GPI, IgM (SMU)	29.1 (66)	34.6 (46)	21.3 (20)	0.043
LA (n=99)	N=173	n=99	n=74	
Positive	72.8 (126)	82.8 (82)	59.5 (44)	
Equivocal	12.1 (21)	5.1 (5)	21.6 (16)	
Not detected	6.9 (12)	3.0 (3)	12.2 (9)	<0.001
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				
Anti-platelets	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)	---
Other bDMARDs	0.4 (1)	0.7 (1)	0.0 (0)	---
Immunosoppressors				
AZA	6.1 (14)	5.2 (7)	7.4 (7)	0.688
CTX	0.4 (1)	0.7 (1)	0.0 (0)	---
MTX	5.7 (13)	5.2 (7)	6.3 (6)	0.940
MMF	4.8 (11)	4.4 (6)	5.3 (5)	0.765
PDN	14.8 (34)	18.5 (25)	9.5 (9)	0.087
CyA	1.7 (4)	2.2 (3)	1.1 (1)	0.644

* Each patient could have presented with multiple clinical events.

° At study inclusion, antibodies against cardiolipin and β2 glycoprotein I of IgG and IgM isotypes were tested by ELISA in APS ACTION core laboratories.

aPL: anti-phospholipid; PAPS: primary anti-phospholipid syndrome; SAPS: secondary anti-phospholipid syndrome; APS: anti-phospholipid syndrome; SARDs: systemic autoimmune rheumatic diseases; CAPS: catastrophic anti-phospholipid syndrome; gw: gestational week; SLE: systemic lupus erythematosus; UCTD: undifferentiated connective tissue disease; aCL: anti-cardiolipin antibodies; GPL: anti-cardiolipin

antibody IgG units; MPL: anti-cardiolipin antibody IgM units; anti-β2GPI: 49anti-β2 glycoprotein I antibodies; SGU: anti-β2 glycoprotein I antibody IgG units; SMU: anti-β2 glycoprotein I antibody IgM units; LA: lupus anticoagulant; VKA: vitamin K antagonists; LMWH: low molecular weight heparin; bDMARD: biologic disease-modifying anti-rheumatic drug; CTX: cyclophosphamide; MTX: methotrexate; MMF: mycophenolate mophetyl; PDN: prednisone; CyA: cyclosporine A.

Table 2. Rates of anti-D1 antibody positivity at different time points in the whole population of 230 subjects and in patients sub-grouped upon clinical and laboratory features.

Anti-D1 positivity, % (n)	**Y1**	**Y2**	**Y3**	**Y4**
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)
Median-low anti-D1 positivity	25.2 (34)	23.7 (32)	25.2 (34)	22.2 (30)
Median-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)

*Data about the number of positive aPL tests were missing for 3 patients.

Anti-D1: antibodies against domain 1; n: number; SLE: systemic lupus erythematosus; aPL: anti-phospholipid antibodies; Y1: year 1; Y2: year 2; Y3: year 3; Y4: year 4.

Table 3. The final multivariable mixed linear model of predictors of the fluctuation of anti-D1 antibody titers.

Variable	Estimate of coefficient (SE)	Degrees of freedom	t-value	p-value
Intercept	1.58 (0.23)	662	6.88	<0.0001
Y2 vs. Y1	-0.11 (0.02)	662	-4.93	<0.0001
Y3 vs .Y1	-0.16 (0.02)	662	-7.35	<0.0001
Y4 vs .Y1	-0.17 (0.02)	662	-7.56	<0.0001
Age at enrolment (years)	-0.02 (0.004)	217	-4.68	<0.0001
Gender	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
Immunosuppressors	-0.11 (0.07)	662	-1.55	0.123
Double/triple positivity	1.08 (0.11)	217	9.41	<0.0001
Incident VE*HCQ	-0.24 (0.14)	662	-1.75	0.081

Y1: year 1; Y2: year 2; Y3: year 3; Y4: year 4; M: male; F: female; SLE: systemic lupus erythematosus; VE: vascular event; HCQ: hydroxychloroquine; VE*HCQ: interaction between vascular event and hydroxychloroquine.

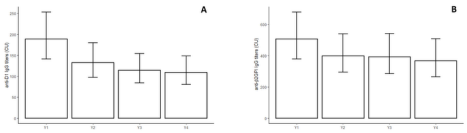
Estimates of the regression coefficients are expressed as log10 of anti-D1 titers.

Table 4. The final multivariable mixed linear model of predictors of the fluctuation of anti-β2 glycoprotein I antibody titers.

Variable	Estimate of coefficient (SE)	Degrees of freedom	t-value	p-value
Intercept	1.85 (0.23)	659	7.98	<0.0001
Y2 vs .Y1	-0.08 (0.03)	659	-2.83	0.0048
Y3 vs. Y1	-0.07 (0.03)	659	-2.38	0.0174
Y4 vs .Y1	-0.10 (0.03)	659	-3.61	<0.001
Age at enrolment (years)	-0.02 (0.004)	217	-4.15	<0.0001
Gender	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
Immunosuppressors	-0.12 (0.08)	659	-1.49	0.137
Double/triple positivity	1.52 (0.11)	217	13.25	<0.0001
Incident VE*HCQ	-0.32 (0.18)	659	-1.81	0.070

Y1: year 1; Y2: year 2; Y3: year 3; Y4: year 4; M: male; F: female; SLE: systemic lupus erythematosus; VE: vascular event; HCQ: hydroxychloroquine. VE*HCQ: interaction between vascular event and hydroxychloroquine.

Estimates of the regression coefficients are expressed as log10 of anti-D1 titers.



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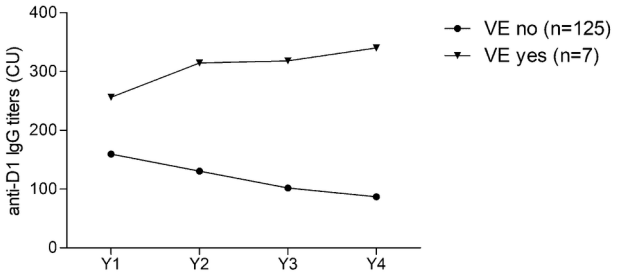

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