Journal of Thrombosis and Haemostasis, **13**: 782–787 DOI: 10.1111/jth.12865

ORIGINAL ARTICLE

Antiphospholipid syndrome: antibodies to Domain 1 of β2-glycoprotein 1 correctly classify patients at risk

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To cite this article: V. Pengo, A. Ruffatti, M. Tonello, S. Cuffaro, A. Banzato, E. Bison, G. Denas, S. Padayattil Jose. Antiphospholipid syndrome: Antibodies to Domain 1 of β2-glycoprotein 1 correctly classify patients at risk. *J Thromb Haemost* 2015; **13**: 782–7.

Summary. *Background:* Determination of lupus anticoagulant (LA), anticardiolipin (aCL) and β2-Glycoprotein 1 (aβ2GP1) antibodies is mandatory to classify patients with antiphospholipid syndrome (APS) into risk categories. *Objectives:* To measure relevant antibodies, considered to be those of the IgG isotype directed towards β2GP1 and particularly those directed to Domain 1 (Dm1) of the molecule. *Patients/methods:* In this cross-sectional study we measured IgG aβ2GP1-Dm1 by a chemiluminescent immunoassay in a group of individuals initially positive for IgG aβ2GP1 and classified as triple (LAC+, IgG aCL+, IgG aβ2GP1+, $n = 32$), double (LAC–, IgG aCL+, IgG aβ2GP1+, $n = 23$) or single positive (LA-, IgG aCL-, IgG aβ2GP1+, $n = 10$). *Results and conclusion:* Geometric mean and standard deviation expressed as chemiluminescent units (CU) in triple, double and single positive groups were 273.0 ± 6.2, 18.2 ± 9.6 and 4.4 ± 2.2, respectively. The geometric mean obtained in 40 healthy subjects was 2.0 ± 2.0. Mean CU values were significantly different among groups and with respect to values found in 40 healthy subjects ($P < 0.0001$). Positive values of IgG aβ2GP1-Dm1 (above 14.2 CU) were found in 45 individuals while 20 individuals (20/65 = 30.8%) positive for IgG aβ2GP1 were negative for IgG aβ2GPI-Dm1. There was a significant association between positive IgG aβ2GP1-Dm1 and thromboembolic events ($P = 0.001$). Positive and negative values of IgG aβ2GP1-Dm1 were consistently confirmed after 12 weeks, with only three low positive values being negative after 12 weeks. In conclusion, IgG aβ2GP1-Dm1 seems a robust and reproducible test that in association with the classic tests may be useful in clinical practice in identifying individuals at high risk of developing thromboembolic events.

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Received 15 October 2014
Manuscript handled by: M. Levi
Final decision: F. R. Rosendaal, 24 January 2015

Keywords: antibodies; antiphospholipid syndrome; epitopes; glycoproteins; thrombosis.

Introduction

Antiphospholipid syndrome (APS) is characterized by the presence of antiphospholipid (aPL) antibodies in patients with thrombosis or pregnancy morbidity [1]. To categorize APS patients, three tests exploring the presence of aPL antibodies (lupus anticoagulant (LA), anticardiolipin (aCL) and anti-β2-Glycoprotein 1 (aβ2GP1) antibodies) are performed. In this way, APS patients are classified into category I when more than one test is positive, and IIa, IIb and IIc according to the presence of only one positive test [1]. When all three tests are positive (triple positivity), the association with thrombosis is very strong; it gets weaker in double-positive (with LA negative) and single-positive patients [2]. Antibodies determining positivity in all the three tests are those directed against β2GP1, a plasma protein without a defined function [3, 4]. Indeed, affinity-purified IgG aβ2GP1 spiked into normal plasma made all three tests positive [3]. Moreover, a subgroup of IgG aβ2GP1, those directed to Domain1 (Dm1) in the NH2 terminal region of the molecule, are able to interfere with the coagulation process and are associated with thrombosis [5]. The aim of this cross-sectional study was to measure both basal and after 12 weeks values of IgG aβ2GP1-Dm1 in individuals initially positive for IgG aβ2GP1.

Material and methods

Study population

Consecutive individuals referred to a rheumatology outpatient clinic dedicated to APS patients and initially

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positive for aPL antibodies were evaluated. The selection criteria for individuals were not based on the diagnosis of the patients, but rather on the presence of APS-related symptoms (thrombosis and pregnancy morbidity) and aPL antibody positivity. Because IgG aβ2GP1-Dm1 is a subgroup of IgG directed to β2GP1, the study focused on the group of individuals initially positive for IgG aβ2GP1. They were classified into three groups: triple positive (LA+, IgG aCL+, IgG aβ2GP1+), double positive (LA–, IgG aCL+, IgG aβ2GP1+) and single positive (LA-, IgG aCL-, IgG aβ2GPI1+). Two of us (VP and AR) consulted the available hospital records and diagnosed previous thromboembolic events only in the presence of objective criteria and if confirmed by positive compression ultrasonography or venography for deep vein thrombosis, and spiral tomography, ventilation-perfusion lung scan or pulmonary angiography for pulmonary embolism. Positive tomographic scanning, magnetic resonance imaging or angiography confirmed the diagnosis of arterial thromboembolism. The presence of pregnancy morbidity according to Miyakis [1] was assessed by individual interview. Hospital records were also consulted for the presence of associated autoimmune diseases (systemic lupus erythematosus [SLE] or other connective tissue disease). No subject was lost to follow-up after 12 weeks. We restricted this study to individuals positive for IgG aβ2GP1 but we have also tested IgG aß2GP1-Dm1 in individuals negative for IgG aβ2GP1 but positive for one or more tests exploring the presence of aPL.

Determination of aPL antibodies

Venous blood was collected in 3.8% sodium citrate (9 : 1) and centrifuged twice at 2000 $\times g$ for 15 min at 4 °C and obtained plasma was stored at −80 °C until used. Patients' plasmas were screened for the presence of aPL antibodies using home-made IgG and IgM aCL ELISA as previously described [6] and the recommendations proposed by a taskforce during the 13th International Congress on Antiphospholipid Antibodies (Galveston, TX, USA) [7]. LA was detected using dRVVT and SCT (Instrumentation Laboratory, Milan, Italy), according to the ISTH guidelines [8]. The presence of IgG aβ2GP1 antibodies was confirmed by a chemiluminescent immunoassay (QUANTA flash β2GPI IgG; Inova Diagnostics, San Diego, CA, USA). The cut-off value of tests exploring the presence of aPL antibodies was calculated by means of the 99th percentile of results obtained in 40 healthy subjects. The cut-off value for both dRVVT and SCT in mixing studies was 1.2; the cut-off values of IgG and IgM aCL and IgG and IgM aβ2GP1 were 10 GPL and 8 MPL and 13 and 7 home units, respectively. The IgG aβ2GP1 cut-off value in the chemiluminescent immunoassay was 19.4 CU.

Determination of IgG aß2GP1-Dm1 antibodies

Autoantibodies to β2GP1-Dm1 were measured by a chemiluminescent immunoassay (QUANTA Flash Domain 1 IgG; Inova Diagnostics) using the BIO-FLASH® technology (Inova Diagnostics). The plasma sample was diluted 1 : 10 by the instrument in a disposable plastic cuvette. The technician performing the assay was blinded to patient/individual classification categories. Small amounts of the diluted patient plasma and the assay buffer were all combined into a second cuvette containing purified recombinant β2GP1-Dm1 coated onto paramagnetic beads, mixed and incubated at 37 °C. Isoluminol-conjugated anti-human IgG antibody was then added to the cuvette and incubated at 37 °C. The light coming from the luminescent reaction produced by isoluminol conjugate was measured as Relative Light Units (RLU) by the BIO-FLASH® optical system. The RLU are proportional to the amount of bound isoluminol conjugate, which in turn is proportional to the amount of aβ2GP1-Dm1 antibodies bound to the β2GP1-Dm1 on the beads. Relative light units are converted to chemiluminescent units (CU) when the assay is assigned to a lot-specific master curve, and this curve is calibrated with QUANTA Flash calibrators to obtain the final CU values for results. The approximate cut-off value for IgG aβ2GP1-Dm1, calculated by means of the 99th percentile of results obtained in 40 healthy subjects, was 14.2 CU. Intra-assay and inter-assay coefficient of variation are 6.3% and 2.0%, respectively.

Statistics

Descriptive statistics are reported as appropriate: categorical data are expressed as frequencies (percentage); continuous data are reported as mean (standard deviation). Due to non-Gaussian distribution, data of IgG aβ2GPI-Dm1 (CU) were transformed into log10. In this way the distribution became Gaussian and the difference between groups was tested using one-way analysis of variance (ANOVA). The chi-squared test for independence was used to evaluate the association of thrombosis and pregnancy loss with positive values of IgG aβ2GPI-Dm1. Linear (Pearson) correlation was performed between IgG aβ2GPI-Dm1 (CU) initial data and data obtained after 12 weeks. Spearman correlation was used to compare Domain1 and the whole molecule titres. GRAPH-PAD INSTAT 3 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis (www.graphpad.com).

Results

During the period 2009–2013, 1120 individuals were tested for the presence of aPL antibodies (LAC, aCL IgG and IgM, aβ2GPI IgG and IgM). Reasons for testing

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were vascular thrombosis, previous pregnancy morbidity, SLE or other autoimmune diseases, familial aPL, oral contraceptive or hormone replacement therapy, accidental finding of prolonged activated partial thromboplastin time or false-positive test results for syphilis. The final study cohort was selected based on aPL antibody positivity and anti β2GP1 antibody positivity. Among the 106 individuals positive for one or more test, 65 were positive for the presence of IgG aβ2GP1. These individuals were grouped according to their aPL profile into triple positive (LAC+, IgG aCL+, IgG aβ2GP1+, $n = 32$), double positive (LAC–, IgG aCL+, IgG aβ2GP1+, $n = 23$) and single positive (LAC-, IgG aCL-, IgG aβ2GP1+, $n = 10$). DRVVT and SCT were both positive in triple-positive patients/carriers, and both were negative in the other two groups. Table 1 shows individuals' characteristics. Of note, only young females represented the single-positive group, while autoimmune disease was lower in the triple-positive group. The rate of clinical events related to APS (thromboembolism and pregnancy loss) was also different among groups; the rate of thromboembolic events was higher in triple-positive individuals, while the rate of pregnancy loss was more frequent in double-positive individuals. Figure 1 shows the values expressed as chemiluminescent units (CU) of IgG aß2GP1-Dm1 in the three groups. Geometric mean and standard deviation expressed as CU in triple, double and single-positive groups were 273.0 ± 6.2, 18.2 ± 9.6 and 4.4 ± 2.2, respectively. The geometric mean obtained in 40 healthy subjects was 2.0 ± 2.0. One-way analysis of variance (ANOVA) showed a statistically significant variation among columns in the mean values of IgG aβ2GP1-Dm1 antibodies ($P < 0.0001$).

The approximate cut-off value set at the 99th percentile obtained in 40 control subjects negative for the presence of IgG aβ2GP1 was 14.2 CU. Positive values of IgG aβ2GP1-Dm1 (above 14.2 CU) were found in 31 out of 32 triple-positive (97%), 13 out of 23 double-positive (43%) and in 1 out of 10 single-positive (10%) individuals. When comparing the mean values of IgG aβ2GP1-Dm1 between the groups, we found a significantly higher value in triple-positive individuals ($P < 0.0001$).

There is a significant correlation between the two tests (Spearman $r = 0.76$; 95% CI, 0.6–0.85; $P < 0.0001$) (Fig. 2). However, there were 20 individuals out of 65 (20/65 = 30.8%) positive for IgG aβ2GP1 and negative for IgG aβ2GPI-Dm1, all but one of them in the double and single-positive groups.

Thromboembolic events were present in 29 of 45 individuals (64%) with positive values of IgG aβ2GP1-Dm1 (above 14.2 CU) and in four of 20 (20%) individuals with negative values (Fig. 3).

Overall, there was a very significant association between positive IgG aβ2GP1-Dm1 and thromboembolic events ($P = 0.001$), with a sensitivity of 88% and a specificity of 50%. On the other hand, pregnancy loss was present in 16 out of 39 women (41%) with positive values of IgG aβ2GP1-Dm1 and in three out of 19 women (16%) with negative values ($P = 0.07$). All the individuals were tested after 12 weeks. As shown in Fig. 4, there was a consistent confirmation after 12 weeks of data obtained at initial screening (correlation coefficient [r] was 0.99; 95% CI, 0.98–0.99 [$P < 0.0001$]). Overall, of the 45 positive values at initial testing, only three low positive values of IgG aβ2GP1-Dm1 (two in double-positive and one in single-positive groups) were negative after 12 weeks. On the other hand, of 20 negative values at initial testing not one was positive after 12 weeks. The correlation coefficient between basal and 12-week values of 45 initially

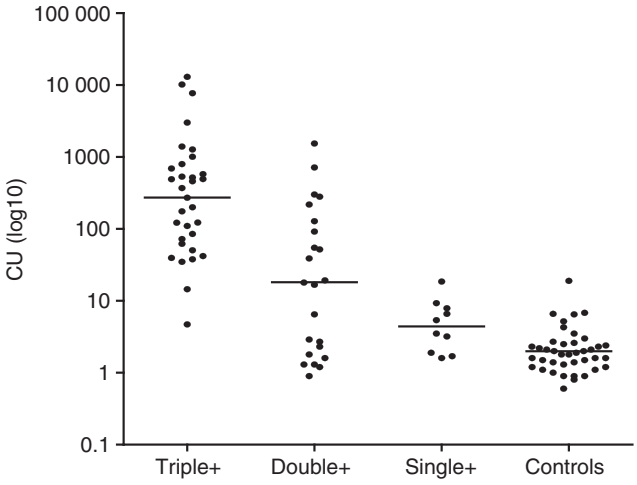

Fig. 1. Variations of geometric mean of IgG aβ2GP1-Dm1 values expressed as chemiluminescent units (CU) among groups are significantly greater than expected by chance ($P < 0.0001$). All the individuals in the three groups are positive for IgG aβ2GP1.

Table 1 Characteristics of individuals positive for IgG aβ2-glycoprotein 1 according to the aPL antibody profile

	LA+, IgG aCL+, IgG aβ2GP1+ $n = 32$	LA-, IgG aCL+, IgG aβ2GP1+ $n = 23$	LA-, IgG aCL-, IgG aβ2GP1+ $n = 10$
Age, years (mean ± SD)	41 ± 13	45 ± 12	32 ± 17
Male, no. (%)	6 (19)	2 (9)	0 (0)
Autoimmune disorders, no. (%)	2 (6)	7 (30)	3 (30)
Venous or arterial thrombosis, no. (%)	24 (75)	8 (35)	1 (10)
Pregnancy loss, no. (%)	7 (22)	10 (43)	0 (0)
Carriers, no. (%)	5 (16)	8 (35)	9 (90)

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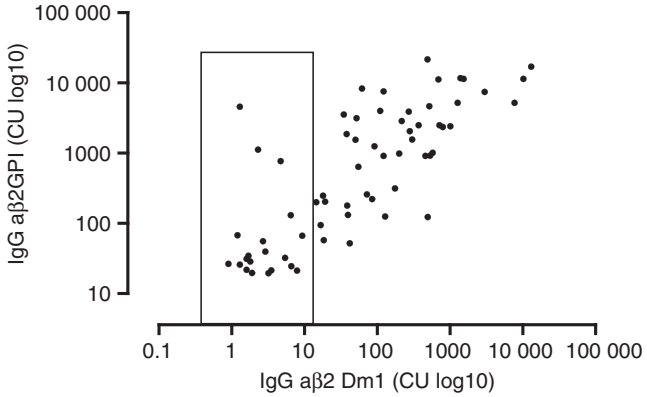

Fig. 2. Correlation between IgG aβ2GP1 and IgG aβ2GPI-Dm1 ($P < 0.0001$). Dots inside the rectangle denote individuals positive for IgG aβ2GPI and negative for IgG aβ2GP1-Dm1.

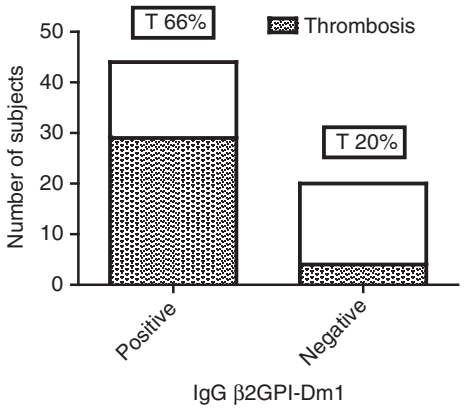

Fig. 3. Rate of thromboembolic events in individuals with values positive or negative for IgG anti-ß2GP1-Domain1.

positive IgG aβ2GP1-Dm1 values was extremely significant (Pearson $r = 0.94$, $P < 0.0001$), as was that of 20 initially negative IgG aβ2GP1-Dm1 values (Pearson $r = 0.89$, $P < 0.0001$).

Of the 106 initially positive individuals, 41 were negative for IgG aβ2GP1. In this group, 16 were triple positive ($n = 2$) and double positive ($n = 14$) for IgM aβ2GP1 and 25 were positive for only one test (8 IgM aβ2GP1, 8 IgG aCL, 7 IgM aCL, 2 LA). As shown in Fig. 5, no one was positive for IgG anti Dm1.

Discussion

In this study, we assessed individuals testing positive at initial screening for IgG aβ2GP1 alone or in combination with the other two tests required to classify APS patients. Of these, 69% were positive for antibodies directed against β2GP1-Dm1, a rate somewhat higher (55%) than previously reported [9]. Our results show that IgG aβ2GPI-Dm1 values are significantly higher in individuals with a triple-positive aPL profile, and that there is an association between the concentration of these antibodies

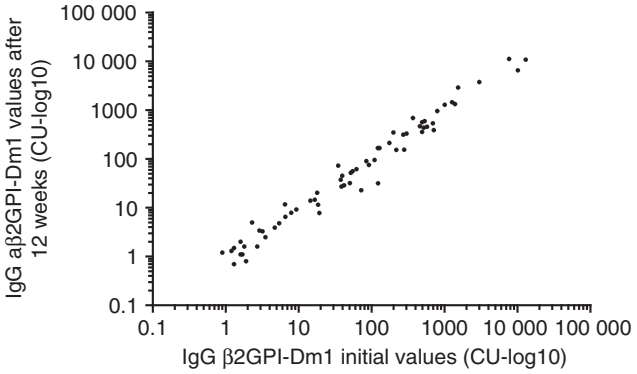

Fig 4. Linear correlation between data of IgG anti-ß2GP1-Domain1 (CU log10) obtained at initial testing and after 12 weeks.

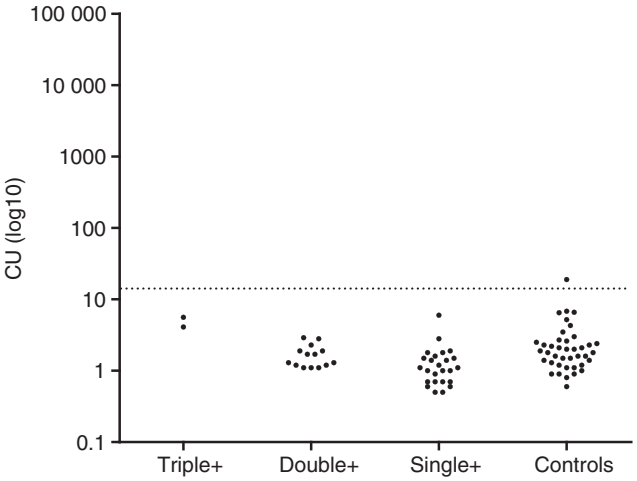

Fig 5. Values of IgG aβ2GP1-Dm1 expressed as relative light units (CU) in triple IgM isotype positive subjects ($n = 2$), double IgM isotype positive subjects ($n = 14$) and 25 individuals positive for only one test.

and thrombosis risk categories. All the triple-positive individuals, except one, had values well above the approximate cut-off calculated in 40 healthy subjects. Thus, both high titre IgG aβ2GPI-Dm1 and triple positivity identify patients with previous thromboembolic events at high risk of recurrence [10] and carriers at high risk of developing a first thromboembolic event [11]. At variance with other risk categories, triple-positive individuals have positive LA and this is a peculiar feature of the subgroup of IgG aβ2GP1 antibodies that are directed against Dm1 [5,12,13]. However, IgG aβ2GPI-Dm1 are also positive in some individuals without LA: one explanation might be that in this case, a low titre of antibodies is present, such that it is insufficient to determine LA activity; another reason might be that the detected subset of IgG aβ2GPI-Dm1 is not directed against the Gly40-Arg43 epitope. The main epitope of Dm1 possesses in fact fine specificity, and it is discontinuous in nature, involving arginine 39-arginine 43, aspartic

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acid 8-aspartic acid 9, and possibly the interlinking region between DI and DII [14–16]. Conversely, despite positivity in IgG aβ2GP1, many individuals in double and all but one in single-positive groups had negative values of IgG aβ2GP1-Dm1. In these individuals, IgG aβ2GP1 may be directed to other Domains of the molecule. For example, anti-Domain 4/5 antibodies have been detected in non-thrombotic conditions, such as atherosclerosis [17], leprosy [18] and in children with atopic dermatitis [19] or those born to mothers with systemic autoimmune diseases [20]. The presence of antibodies preferentially directed against Domain 4/5 is not associated with thromboembolic events [20]. Interestingly enough, lower levels of IgG aβ2GP1-Dm1, as in the double-positive group, might be relevant in pregnancy morbidity. Indeed, we have previously shown when analyzing data from retrospective studies, that low positive aCL and double positivity are more strongly associated with pregnancy morbidity than with thromboembolic events [2,21].

According to present guidelines, confirmation of aPL positivity is mandatory to exclude the presence of transient antibodies [1]. This is particularly true for individuals with single test positivity as confirmation is poor [22]. Results from the present study show that IgG aβ2GPI-Dm1 are not transient and confirmation values are very close to those initially detected. Positivity was confirmed after 12 weeks with a very high rate of concordance. As pointed out in a recent comprehensive review paper [23], we feel that this chemiluminescent immunoassay using the BIO-FLASH® technology is more sensitive and reproducible than the existing ELISA assays. The package insert for this CE marked assay contains ample testing of QUANTA Flash Domain 1 on a variety of disease controls, including IBD, infectious osteoarthritis, RA and systemic sclerosis, which shows a very high specificity (> 99%). However, prospective studies need to make clear their clinical utility. In conclusion, in addition to the other aPL tests, the finding of high levels of IgG aβ2GPI-Dm1 antibodies identifies individuals at high risk of thrombosis, and thus might become an additional useful tool in clinical and therapeutic decision-making in aPL-positive individuals and APS patients.

Addendum

V. Pengo and A. Ruffatti designed the research, analyzed and interpreted data and wrote the manuscript; M. Tonello and S. Cuffaro, collected samples and data; E. Bison, A. Banzato, G. Denas and S. P. Jose performed research and G. Denas performed statistical analysis.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

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