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PAPER

Association of β_2-glycoprotein I IgG and IgM antibodies with thrombosis and thrombocytopenia

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Antiphospholipid antibodies (APA) have been known for decades. Their relation to clinical manifestations, primarily thromboses and thrombocytopenia, was recognised in the 1980s. In this clinical study two cohorts of patients, a population-based (84 patients with systemic lupus erythematosus (SLE)) and a hospital-based (87 patients with SLE and 53 with other connective tissue diseases) were investigated for APA and associated clinical manifestations. Anticardiolipin antibodies (ACA) of IgG and IgM classes were found in 13 and 38% of the population-based patients and in 29 and 58% of the hospital-based patients, respectively. The corresponding figures for antibodies against β_2-glycoprotein I (anti-β_2GPI) were 15 and 10% in the population-based patients and 14 and 8% in the hospital-based cohort. Anti-β_2GPI antibodies were always found in association with the corresponding immunoglobulin class of ACA.

In both cohorts anti-β_2GPI of the IgG class were associated with arterial/venous occlusion, a result concordant with other studies. A novel finding in both cohorts, however, was an association between thrombocytopenia and IgM anti-β_2GPI. *Lupus* (2001) **10,** 533–538.

Keywords: antiphospholipid antibodies; β_2-glycoprotein I; antiphospholipid syndrome; thrombosis; thrombocytopenia

Introduction

Antiphospholipid antibodies (APA) were noted as early as in the 1940s[1] in patients with systemic lupus erythematosus (SLE). The clinical significance of APA was first recognised in the 1980s through descriptions of their association with thromboembolic events, miscarriage and thrombocytopenia, but possibly also with livedo reticularis, migraine and chronic leg ulcers.[2,3] Accordingly, the antiphospholipid syndrome (APS) was designated.[4–6] Several aspects of the pathogenesis of APA have still not been elucidated. However, a cofactor, β_2-glycoprotein I (β_2GPI, apolipoprotein H), has been described in studies of ACA reactivity as being necessary for the binding of ACA to phospholipids.[7] Other possible antibody specificities associated with immune-mediated thrombosis include protein C, annexin V and prothrombin.[8] Antibodies against β_2GPI (anti-β_2GPI) have been shown to be more specific for clinical manifestations of the APS than ACA[9] and in some patients with clinical manifestations of APS the presence of anti-β_2GPI has even been described without the presence of ACA.[10] Anti-β_2 GPI have been found in 5–31% of unselected lupus patients.[11] The diverging prevalences probably illustrate differences in populations studied and techniques used. In healthy Danish individuals ACA were found in 1% and anti-β_2GPI in less than 0.5% using the techniques described in this study (personal communication, Dr Heegaard).

The purpose of the present study is to investigate the prevalence of ACA and anti-β_2 GPI, and to evaluate their association with thromboembolic events and thrombocytopenia in two independent groups of Danish patients: a population-based cohort of 84 SLE patients and a hospital-based cohort of 140 patients with autoimmune diseases including SLE.[12]

Methods

Patient material and study design

Cohort 1. A population-based cohort of 84 SLE patients was identified using several retrieval sources

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in the Danish County of Funen.[13] The diagnosis of SLE was validated according to the 1982 ACR criteria.[14] Manifestations of APS in the past were registered from the patients' medical records and examinations. The patients were then followed prospectively for 2 y with registration of incident manifestations. Blood samples for APA analyses were drawn at least twice during follow-up and one or more positive tests accounted for a positive result. The cohort consisted of 78 (93%) women and six men. Mean age at the end of follow-up was 45 y (range 17–77).

Cohort 2. Another cohort, which was hospital-based (Hvidovre Hospital), consisted of 140 patients with various connective tissue diseases. In this cohort, there were 128 (91%) women and 12 men. Most of these patients ($n = 87$) either had or evolved into SLE during follow-up after initial admission to hospital, but 20 had systemic sclerosis, 11 rheumatoid arthritis, eight primary antiphospholipid syndrome (PAPS), three Sjögrens syndrome, three various vasculitides and eight persisted with a diagnosis of undifferentiated connective tissue disease. This cohort represents 1824 patient-years at risk of developing an outcome event after initial admission. Blood samples were drawn at the patient's first admission to hospital; mean age at the time of blood sampling was 40 y (range 5–88).

Clinical outcomes

Vascular thrombosis included episodes of arterial, venous and small vessel occlusion confirmed by imaging, Doppler studies or histopathology.[15] Thrombocytopenia was defined as thrombocyte counts below 100×10^9/l confirmed on at least two separate occasions.

Antibody assays

Antibodies were determined in serum samples diluted 1:100. Quantitation of ACA immunoglobulin G, IgG and immunoglobulin M, IgM, was performed by means of an enzyme-linked immunosorbent assay (ELISA) on polystyrene microtiter plates using purified cardiolipin (Sigma, St Louis, USA) and bovine serum for blocking as previously described.[16,17] Cut-off values for APA were 30 units/ml for IgM and 35 units/ml for IgG antibodies.[16] The units have not been correlated with GPL or MPL standards.

IgG and IgM anti-β_2GPI were also measured by ELISA using human β_2GPI purified from outdated human plasma by perchloric acid precipitation,[18] heparin-sepharose affinity chromatography, anion exchange chromatography, and size-exclusion chromatography (personal communication, I Schousboe). Purity was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and crossed immunoelectrophoresis using rabbit antibodies against total human serum proteins (Dako Corp., Glostrup, Denmark). The purified β_2GPI was coated onto chemically activated covalently binding microtiter plates (Xenobind from Xenopore Corp., Saddle Brook, NJ, USA).[19] During the development of the test different microtiter plate types were evaluated and we found that the background response and number of positives in infectious disease control samples was less on covalently binding plates in comparison with high-binding plates such as MaxiSorp. The purified protein was coated at 1.5 g/well in phosphate-buffered saline at pH 7.4 for 2 h at 37°C. Plates were then blocked with 4% bovine serum albumin (BSA) for 1 h and sera were subsequently applied for 30 min at room temperature. The use of delipidised BSA as a blocking agent or treating the β_2GPI with delipidising agents did not affect levels of positivity in various sera. Bound IgG and IgM antibodies were detected using alkaline phosphatase conjugated goat anti-human reagents from Sigma (St Louis, MO, USA) at 1:4000 dilutions for 1 h. Plates were read at 405 nm with subtraction of signal at 650 nm after staining for alkaline phosphatase activity. Results are expressed in arbitrary units based on a calibration curve made from dilutions of a positive serum included on each plate. Based on the response of 100 normal donors and disease and infectious disease controls the cut-off value (mean + 3 × standard deviation) for positivity of both IgG and IgM antibodies were set at 15 U/ml. The assay response range was 0–100 U and the cut-off value of 15 U was well within the dynamic range of the assay. In the development of the anti-β_2GPI ELISA no binding to uncoated wells was observed. Using covalently bound β_2GPI as the solid phase and the cut-off values described we found no IgG or IgM positive samples among connective tissue disease controls, among infectious disease controls or among donors or elderly persons (data not shown).

Statistical methods

ELISA results are the means of duplicate incubations. Groups were compared using non-parametric statistics including the χ^2 test with Yates' correction. Associations between APA and clinical manifestations of APS were examined by calculating odds ratios in the population-based cohort 1 and relative risks in the hospital-based cohort 2.

Results

Cohort 1

ACA and/or anti-β_2GPI were found in 37 (44%) patients from the population-based cohort. Most positive results were confirmed at least twice; however, in two patients only one sample was drawn and in seven patients single tests were positive only once. In two patients low titre anti-β_2GPI were found in association with persistently negative tests for ACA. In all other cases, a positive anti-β_2GPI test was associated with a positive ACA test. The IgM class of ACA was found more frequently than IgG (38 *vs* 13%) whereas the immunoglobulin classes were equally frequent when investigating anti-β_2GPI (15 *vs* 10%; Table 1). The presence of the IgG class of anti-β_2 GPI was associated with the presence of both IgG ACA and IgM ACA (Table 2). The presence of IgM anti-β_2 GPI was only associated with the presence of IgM ACA (Table 2).

Thrombocytopenia had been experienced by 18%, vascular thrombosis by 25% and one or both of these APS manifestations by 36%. Most events had appeared before the measurement of APA, but in eight patients one or more thrombotic events occurred during the prospective follow-up: four of these patients had APA. The association between APA and clinical events is shown in Figure 1. Thrombosis was significantly associated with the presence of IgG anti-β_2 GPI (OR = 7.1, 95% confidence interval 2.0–25.4), whereas thrombocytopenia was associated with

Table 1 The presence of anticardiolipin antibodies (ACA) and anti-$\beta_2$glycoprotein I (anti-β_2GPI) in two patient populations: cohort 1, a population-based cohort, and cohort 2, a hospital-based cohort

	Cohort 1 (n = *84*)		*Cohort 2* (n = *140*)		P[a]
ACA IgG antibody	11	13%	41	29%	0.01
ACA IgM antibody	32	38%	81	58%	0.01
Anti-β_2GPI IgG antibody	13	15%	20	14%	0.9
Anti-β_2GPI IgM antibody	8	10%	11	8%	0.85

[a]*P*-value from comparison of the two cohorts by means of χ^2 test.

Table 2 The presence of anticardiolipin antibodies (ACA) in patients with and without anti-$\beta_2$glycoprotein I (anti-β_2GPI) in the population-based cohort 1

	IgG anti-β_2GPI				*IgM anti-β_2GPI*			
	Positive (n = *13*)		*Negative* (n = *71*)		*Positive* (n = *8*)		*Negative* (n = *76*)	
ACA IgG antibody	6	46%*	5	7%	3	38%	8	11%
ACA IgM antibody	9	70%*	23	32%	8	100%*	24	32%

*Significant ($P < 0.05$) association between anti-β_2GPI and ACA class.

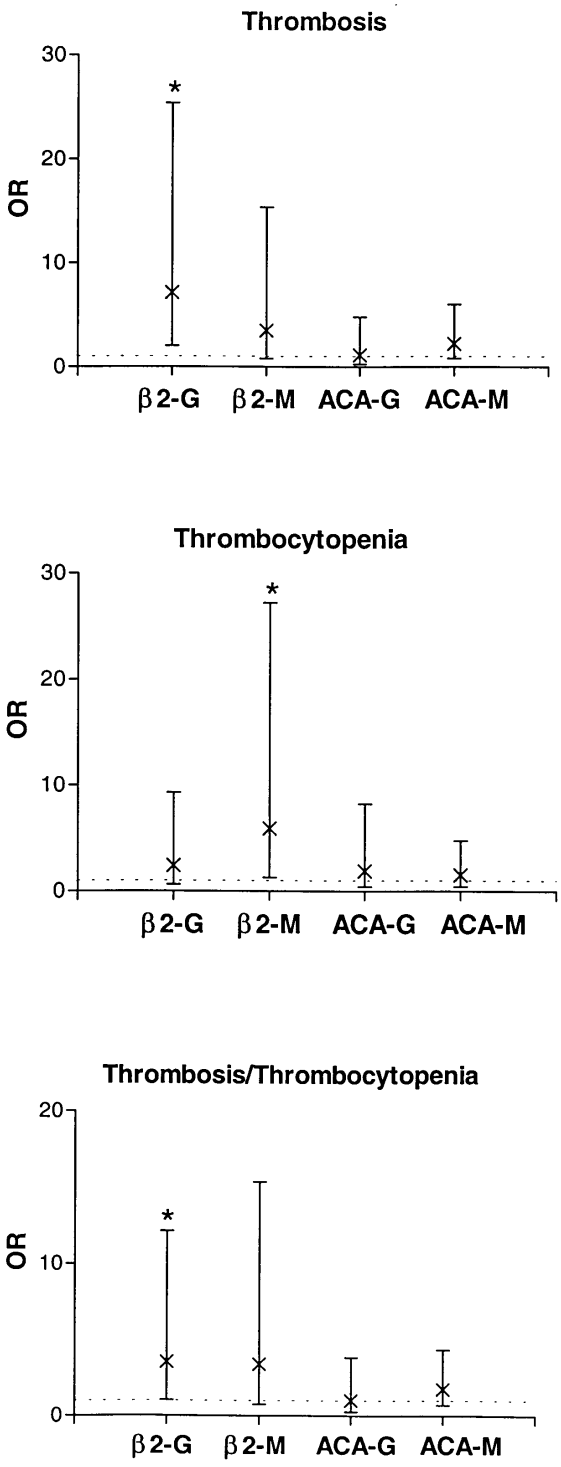

Figure 1 Antiphospholipid antibodies and clinical manifestations in the population-based cohort. Association between the presence of antiphospholipid antibodies and thrombosis (upper graph), thrombocytopenia (middle graph), and thrombocytopenia and/or thrombosis (lower graph) in the population-based cohort. Odds ratios (OR) and their 95% confidence intervals are given. OR = 1 is marked with a dotted line, confidence intervals that do not include this level indicate significant associations and are marked with an asterisk. β2-G, anti-β_2GPI of IgG class; β2-M, anti-β_2GPI of IgM class; ACA-G, IgG anticardiolipin antibodies; ACA-M, IgM anticardiolipin antibodies.

IgM anti-β_2 GPI (OR = 5.9, 95% confidence interval 1.3–27.2). There was no correlation between the degree of thrombocytopenia and level of anti-β_2 GPI IgM (data not shown). The combination of thrombocytopenia and/or thrombosis was significantly associated with anti-β_2 GPI IgG (OR = 3.6, 95% confidence interval 1.0–12.1, Figure 1).

Cohort 2

In the hospital-based cohort, ACA and/or anti-β_2 GPI were found in 98 (70%) patients. The IgM class of ACA was more frequent than the IgG class (58 *vs* 29%) and both were seen more frequently than in the population-based cohort (Table 1). Anti-β_2GPI classes were approximately distributed as in the population-based cohort. The presence of IgG anti-β_2GPI was associated with the presence of IgG ACA, and the presence of IgM anti-β_2GPI with IgM ACA (Table 3).

During the mean follow-up period of 13 y from initial admission 13% of the patients developed thromboses, 28% thrombocytopenia and 37% developed one or both manifestations. Also in this cohort thrombosis was associated with IgG anti-β_2GPI (RR = 2.7, 95% confidence interval 1.6–4.3) and thrombocytopenia with IgM anti-β_2GPI (RR = 5.9, 95% confidence interval 2.7–12.6, Figure 2). The combination of thrombosis and/or thrombocytopenia was significantly associated with IgG (RR = 2.4, 95% confidence interval 1.7–3.5) and IgM anti-β_2GPI (RR = 2.5, 95% confidence interval 1.7–3.6 Figure 2).

Discussion

In our population-based cohort of SLE patients IgG and IgM ACA were found in 13% and 38% of the patients, respectively. Both classes of ACA were found in more patients from our hospital-based cohort of mainly SLE patients: 29 and 58%, respectively. These figures are in accordance with other

Table 3 The presence of anticardiolipin antibodies (ACA) in patients with and without anti-$\beta_2$glycoprotein I (anti-β_2GPI) in the hospital-based cohort 2

	IgG anti-β_2GPI				*IgM anti-β_2GPI*			
	Positive (n = 20)		*Negative (n = 120)*		*Positive (n = 11)*		*Negative (n = 129)*	
ACA IgG antibody	15	75%*	26	22%	6	55%	35	27%
ACA IgM antibody	14	70%	67	56%	11	100%*	70	54%

*Significant ($P < 0.05$) association between anti-β_2GPI and ACA class.

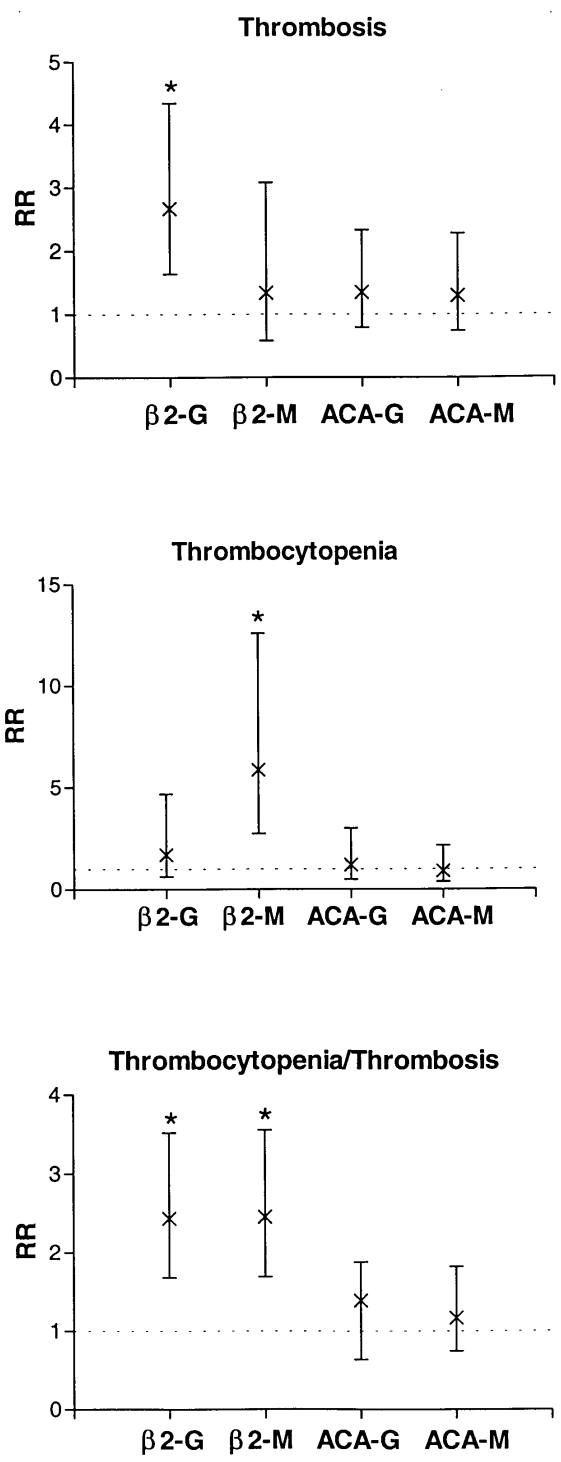

Figure 2 Antiphospholipid antibodies and clinical manifestations in the hospital-based cohort. Association between the presence antiphospholipid antibodies and thrombosis (upper graph), thrombocytopenia (middle graph), and thrombocytopenia and/or thrombosis (lower graph) in the hospital-based cohort. Relative risks (RR) and their 95% confidence intervals are given. RR = 1 is marked with a dotted line, confidence intervals above this level indicate significant associations and are marked with an asterisk. β2-G, anti-β_2GPI of IgG class; β2-M, anti-β_2GPI of IgM class; ACA-G, IgG anticardiolipin antibodies; ACA-M, IgM anticardiolipin antibodies.

studies of ACA in which prevalences of 21–63% have been reported.[11,20–22] In a Swedish population-based cohort of SLE patients 54% had ACA[21] while Alarcòn-Segovia reported 52% with low level and 30% with high-level APA.[4] When comparing these different studies, the use of varying laboratory techniques and cut-off values should be considered. The different prevalence of ACA in our two cohorts may, however, also reflect important aspects of patient recruitment such as identification sources (ie diagnosis registers and laboratory registers among others), referral patterns and referral bias.

IgG and IgM anti-β_2GPI antibodies were found in 15 and 10%, respectively, of the patients in the population-based cohort and in 14 and 8% of the patients in the hospital-based cohort. This is comparable to the outcome of other studies that also showed anti-β_2GPI antibodies in only some ACA-positive patients.[23]

In the present study, only few patients had anti-β_2GPI without ACA, which corroborates the idea that the anti-β_2GPI test identifies a subclass of patients with ACA. On the other hand it should be kept in mind that antibodies detected in anticardiolipin ELISAs and/or anti-β_2GPI ELISA are not necessarily distinct. The identification by anti-β_2GPI may be of clinical relevance to the APS: clinical manifestations of APS in terms of arterial or venous occlusion were diagnosed in 25–28% of the patients while thrombocytopenia was verified in 13–18%. Each manifestation was registered with a comparable prevalence in the two populations studied. An increased risk of arterial/venous occlusion was associated with the presence of IgG anti-β_2GPI antibodies in both populations. IgG anti-β_2GPI has previously been associated with APS manifestations in general[9] and with thrombosis in particular.[24]

Thrombocytopenia on the other hand appeared to be associated with the presence of IgM anti-β_2GPI antibodies in both populations investigated. This association was also found in a study of 79 Spanish SLE patients: IgM anti-β_2GPI antibodies were significantly associated with thrombocytopenia and the specificity was higher using anti-β_2GPI than ACA as a serological marker.[23] In a study by Day *et al* thrombocytopenia was also found to be related to the presence of IgG and/or IgM anti-β_2GPI antibodies; however, the classes were not described separately.[25] Day *et al* found that the anti-β_2GPI antibodies test had a high specificity but low sensitivity and positive predictive value in detecting thrombocytopenia in that study.[25] On the other hand, in another comprehensive analysis of APA and APS manifestations, thrombocytopenia was not associated with any of the anti-β_2GPI immunoglobulin classes.[24]

It is remarkable that the pattern of autoantibodies and clinical associations are similar in our two independent cohorts. Figure 1 and 2 clearly show that the differences in odds ratios and relative risks between the two types of ELISAs are parallel in our two cohorts. The fact that cohort 2 includes patients with other connective tissue diseases than SLE and still shows the same clinical and serological associations as in cohort 1 corroborates the findings and indicates that the associations may not only be valid in patients with SLE.

We did not measure whether other autoantibodies of possible importance for thrombocytopenia were present in our material. Nevertheless, antibodies against platelet glycoproteins have been proposed to be of pathogenetic importance for the thrombocytopenia seen in APS and SLE.[26]

Recently, the presence of IgG β_2GPI containing immune-complexes was found to be associated with thrombocytopenia but not with thrombosis in 38 lupus patients.[27] An interesting observation in that study was that most patients with high levels of immune complexes did not have any detectable anti-β_2GPI antibodies. It would have been of great interest if also IgM and IgA classes had been studied as it might be speculated that similar findings could be made in these classes too. The authors speculate that circulating β_2GPI-containing immune complexes may be deposited in situ exerting their clinical effect[27] and this may indicate why some anti-β_2GPI Ig classes with probable effects may not be demonstrated in serum samples.

In conclusion, our study corroborates the superior role of anti-β_2GPI antibodies in delineating patients with cardinal manifestations of the APS compared with ACA. Why IgG anti-β_2GPI antibodies are associated with thrombosis and IgM anti-β_2GPI antibodies are associated with thrombocytopenia has yet to be revealed but these associations seem to be consistent with recently described findings from other workers.

Measurement of anti-β_2GPI in patients with APA seems to offer some advantages compared to measurement of ACA. However, several problems of technical character and standardisation still need to be solved.

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