Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12762

Antibodies to age-$\beta_2$glycoprotein I in patients with anti-phospholipid antibody syndrome

M. Sorice,*[1] B. Buttari,†[1]
A. Capozzi,* E. Profumo,†
F. Facchiano,‡ S. Truglia,§
S. Recalchi,* C. Alessandri,§
F. Conti,§ R. Misasi,* G. Valesini§
and R. Riganò†

*Department of Experimental Medicine, 'Sapienza' University of Rome, †Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore Di Sanità, ‡Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore Di Sanità, and §Department of Internal Medicine and Medical Specialties, 'Sapienza' University of Rome, Italy

Accepted for publication 20 December 2015
Correspondence: Professor M. Sorice, Department of Experimental Medicine 'Sapienza' University of Rome, viale Regina Elena 324, 00161 Rome, Italy.
E-mail: maurizio.sorice@uniroma1.it

[1]Maurizio Sorice and Brigitta Buttari contributed equally to this work.

Summary

Anti-phospholipid antibody syndrome (APS) is a systemic autoimmune disease characterized clinically by arterial and/or venous thromboses, recurrent abortions or fetal loss and serologically by the presence of 'anti-phospholipid antibodies' (aPL). The main target antigen of the antibodies is $\beta_2$glycoprotein I (β_2GPI). Post-translational oxidative modifications of the protein have been widely described. In this study we aimed to analyse sera reactivity to glucose-modified β_2GPI (G-β_2GPI). Sera collected from 43 patients with APS [15 primary APS (PAPS) and 28 APS associated with systemic lupus erythematosus (SLE) (SAPS)], 30 with SLE, 30 with rheumatoid arthritis (RA) and 40 healthy subjects were analysed by an enzyme-linked immunosorbent assay (ELISA) using a G-β_2GPI. Nine of 15 consecutive PAPS out-patients (60%) and 16 of 28 SAPS (57.1%) showed serum antibodies [immunoglobulin (Ig)G class] against G-β_2GPI (anti-G-β_2GPI) by ELISA. The occurrence of anti-G-β_2GPI was significantly higher in APS patients compared to patients suffering from SLE. No RA patients or control healthy subjects resulted positive for anti-G-β_2GPI. Of note, aG-β_2GPI prompted to identify some APS patients (four PAPS and seven SAPS), who were negative in the classical anti-β_2GPI test. Moreover, in APS patients, anti-G-β_2GPI titre was associated significantly with venous thrombosis and seizure in APS patients. This study demonstrates that G-β_2GPI is a target antigen of humoral immune response in patients with APS, suggesting that β_2GPI glycation products may contain additional epitopes for anti-β_2GPI reactivity. Searching for these antibodies may be useful for evaluating the risk of clinical manifestations.

Keywords: $\beta_2$glycoprotein I, anti-phospholipid antibody syndrome, cryptic or neoepitopes, glycation, systemic lupus erythematosus

Introduction

Anti-phospholipid antibody syndrome (APS) is a systemic autoimmune disease characterized clinically by arterial and/or venous thromboses, recurrent abortions or fetal loss and serologically by the presence of anti-phospholipid antibodies (aPL) [1,2]. Diagnosis of APS requires the combination of at least one clinical and one laboratory criterion [3]. Anti-cardiolipin antibodies (aCL) and anti-β_2-glycoprotein I antibodies (aβ_2GPI) detected by enzyme linked immunosorbent assay (ELISA), and lupus anti-coagulant (LA) detected by clotting assays, are the recommended tests for detection of aPL [4,5]. However, aPL represent a heterogeneous family of antibodies reacting with phospholipid-binding proteins, including not only β_2GPI [6,7], but also different anionic phospholipids, proteins or phospholipid–protein complexes, such as prothrombin [8], protein S [9,10], protein C [11], annexin V [12], annexin II [13], vimentin [14], oxidized low-density lipoproteins, lyso-bis-phosphatidic acid (LBPA) and sulphatides, etc. [15–17]. β_2GPI, an abundant plasma glycoprotein involved in clotting mechanisms and lipid pathways [18], is the most common target for aPL associated frequently with vascular cell dysfunction [19], thrombotic events and proatherogenic mechanisms [20–22]. The aPL action mechanism forms the basis of APS pathophysiology. Moreover, only autoimmune-type aCL are usually dependent upon the

presence of β_2GPI and anti-β_2GPI have been involved in the expression of LA activity, an *in-vitro* phenomenon associated with an increased risk of arterial and/or venous thromboembolic events. Understanding the mechanisms of how this abundant self-plasma protein becomes a target of pathogenic autoantibodies will improve the knowledge of APS pathophysiology.

The molecular structure and location of the major epitopic region(s) of the β_2GPI molecule are controversial, although they were recently characterized fully [23]. Several studies have investigated whether the immune response is directed to native β_2GPI [22–25] or to cryptic or neoepitopes [26,27]. Decisive events generating cryptic or neoepitopes include β_2GPI binding to anionic surfaces, such as phospholipids, and oxidative modifications that alter phospholipid binding [27–30]. Many mechanisms may be responsible for generating neoepitopes, and multiple mechanisms receive support from the heterogeneous antigenic specificities in β_2GPI-specific antibodies. One candidate mechanism is non-enzymatic glycosylation (glycation), a process that leads to the formation of early, intermediate and advanced glycation end products (AGEs), able to modify self-molecule structures and functions. Even though glycation is present physiologically and is modulated by several factors, diet, ageing as well as disorders of glucose metabolism and systemic autoimmune diseases associated with inflammation and oxidative stress may favour the formation and accumulation of these products [31–33]. AGEs represent new epitopes and contain new antigenic structures, thereby possibly contributing to the generation of autoimmune responses. Recently, we showed that several potential glycation sites are present within the β_2GPI primary structure and that, after *in-vitro* exposure to glucose, β_2GPI was sugar-modified and this modification probably consisted of an AGE formation. Our results on the ability of AGE-β_2GPI to activate human monocyte-derived immature dendritic cells suggest a possible role for glycation in the increase of β_2GPI immunogenicity [34]. Although there is accumulating evidence that AGEs are involved in the progression of inflammatory and immune-mediated diseases [35], further investigations are needed to clarify the role of glycation in generating new antigenic epitopes in β_2GPI possibly contributing to the heterogeneous specificity of aPL.

This study was designed to investigate whether β_2GPI *in vitro* treated with glucose (G-β_2GPI) is a target of humoral response in APS patients and whether anti-G-β_2GPI may be associated with clinical features.

Materials and methods

Patients

This study included 73 consecutive out-patients attending the Lupus Clinic of the Sapienza University of Rome. Forty-three patients had APS, diagnosed according to the Sydney Classification Criteria [3], primary (PAPS, $n = 15$) or APS associated with systemic lupus erythematosus (SLE) (SAPS, $n = 28$); 30 patients had SLE fulfilling the ACR revised criteria for the classification of SLE [36]. Sera were collected at several times and stored at -20°C until use. Finally, 30 patients with RA and 40 healthy subjects (normal blood donors) matched for age and sex were also studied as controls. This study was approved by the local ethic committees and participants gave written informed consent in accordance with the Declaration of Helsinki.

Bioinformatic analysis of β_2GPI

The primary structure of β_2GPI (Accession no. P02749) was analysed by means of tools available online at http://sysbio.unl.edu/SVMTriP/index.php, to predict the presence of flexible and functional sites, including antigenic epitopes [37], related closely or structurally to potential glycation sites [34]. The most recommended epitope(s) within β_2GPI sequence were calculated using 20 amino acids as the default epitope length search for putative epitopes on the web server, as reported [38].

Preparation of glucose-modified β_2GPI

Human native β_2GPI was purchased from Calbiochem (La Jolla, CA, USA). Purity of β_2GPI $>98\%$ was checked by mass spectrometry, as reported by the manufacturer.

To prepare glucose-modified β_2GPI (G-β_2GPI), human β_2GPI was dissolved in glycation buffer solution (GB) (0·144 g/l KH_2PO_4, 0·426 g/l Na_2HPO_4) pH 7·4, at 10 μg/ml final concentration and frozen immediately at -80°C under sterile conditions. Then, β_2GPI aliquots were incubated in the presence of 250 mM glucose or mannitol (Sigma-Aldrich, Milan, Italy) in the dark, at 37°C for increasing intervals of time (0, 12 h, 10 and 22 days) (in sealed vials), as described previously [34,39]. As glycation control, a highly purified preparation of human albumin (Sigma-Aldrich) was treated with D-glucose under the same conditions used for β_2GPI.

Characterization of glucose-modified β_2GPI

Size exclusion chromatography was performed by fast protein liquid chromatography (FPLC; Pharmacia, Uppsala, Sweden) interfaced to an ultraviolet (UV) monochromator detector (ProteomeLab PF2D Protein Fractionation System; Beckman Coulter, Brea, CA). Twenty micrograms of native or G-β_2GPI resuspended into 50 μl of phosphate-buffered saline (PBS) without calcium and magnesium ($PBS^{-/-}$) were injected onto a Superose S12 Pharmacia column equilibrated in $PBS^{-/-}$, pH 7·4. Elution was carried out with a 0·4 ml/min flow rate at 22°C, $PBS^{-/-}$ as elution buffer, and protein peaks were detected under UV recording (optical density at 214 nm). Purity of β_2GPI and

its molecular weight under denatured conditions was checked by electrophoretic analysis [34].

UV spectrophotometry

The UV absorption characteristics of G-β_2GPI and native-β_2GPI were recorded on a UV/visible spectrophotometer (dual beam Uvikon 860 Instrument; Kontron, Zurich, CH) between 200 and 400 nm, using 1 cm optical length UV quartz cuvettes, as described previously [40], with some modifications: G-β_2GPI was analysed onto the 'sample' quartz cuvette, while native-β_2GPI was analysed onto the 'reference' quartz cuvette. In this manner, the differential absorption spectrum indicates the spectral regions of β_2GPI affected by glucose-induced modifications.

Non-tryptophan fluorescence studies

Fluorescence studies were carried out as reported previously [34,39]. Steady-state fluorescence emission spectra of β_2GPI preparations were collected with a FluoroMax-2 spectrofluorometer (Jobin Yvon-Spex, Edison, NJ, USA) using an excitation wavelength of 370 nm, equal bandwidths for excitation and emission (5/5). Emission fluorescence at 450 nm was measured by subtracting contributions of sugar solutions from the fluorescence of the sugar-β_2GPI mixture.

ELISA for aCL and anti-β_2GPI antibodies

aCL and anti-β_2GPI ELISA kits were obtained from Inova Diagnostics Inc. (San Diego, CA, USA). ELISA was performed for all the patients' and healthy subjects' sera according to the manufacturer's instructions. aβ_2GPI were also tested by ELISA, as reported below.

LA assay

LA for all the patients' and healthy subjects' sera was studied in two coagulation systems, a dilute sensitized activated partial thromboplastin time (aPTT) and a dilute Russell's viper venom time (dRVVT), followed by confirmatory test, using reagents and instrumentation by Hemoliance Instrumentation Laboratory (Lexington, MA, USA).

ELISA for glucose-modified β_2GPI

Ninety-six-well polystyrene plates were coated and incubated overnight at 4°C with 1 μg/well of native-β_2GPI or G-β_2GPI in 0·05 μM $NaHCO_3$ buffer, pH 9·5. Coated plates were incubated overnight at 4°C and then washed three times with PBS containing 0·05% Tween 20 (PBS-T). Plates were blocked for 2 h at room temperature with 100 μl of 1% bovine serum albumin (BSA) in PBS. After washing three times with PBS-T, the wells were incubated for 1 h at room temperature with 100 μl of patient sera diluted 1 : 100 in the blocking buffer. Each serum was analysed in triplicate. Goat polyclonal anti-β_2GPI (Affinity Biologicals Inc., Ancaster, ON, Canada) was used as positive control. After three washes with PBS-T, the plates were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated antibodies, anti-human immunoglobulin (Ig)G (Sigma-Aldrich) or anti-goat IgG (Dako Italia S.p.A., Cernusco sul Naviglio, Italy) diluted in the blocking buffer. The plates were washed three times with PBS-T; the bound peroxidase was then revealed with 100 μl of *O*-phenylenediamine dihydrochloride buffer and colour development was stopped with H_2SO_4 0·2 M for 5 min. Absorbance was measured at 492 nm in a microplate reader. Data were presented as the mean optical density (OD) corrected for background (wells without coated antigen). Forty sera from healthy subjects were also tested and a cut-off value was established at the mean of OD ± 3 standard deviations (s.d.) of normal human sera. Parallel experiments were performed with an identical procedure but without coated G-β_2GPI.

Absorption tests

Sera of patients with APS, positive for both aβ_2GPI and anti-G-β_2GPI, and of healthy subjects, were diluted 1 : 100 in the blocking buffer and incubated with native β_2GPI (100 μg/ml) for 1 h at 37°C and then overnight at 4°C. The mixture was centrifuged for 15 min at 27 000 ***g*** at 4°C, according to Alessandri *et al.* [16]; the supernatant fraction was kept as absorbed serum and tested for anti-G-β_2GPI and anti-native-β_2GPI antibodies by ELISA, as reported above.

Statistical analysis

All the statistical procedures were performed by GraphPad Prism software Inc. (San Diego, CA, USA). Normally distributed variables were summarized using the mean ± s.d. and non-normally distributed variables by the median and range. Differences between numerical variables were tested with the Wilcoxon test. Correlation was tested with Spearman's rank order or Pearson's correlation coefficient. For comparison of categorical variables or percentages, Fisher's exact and χ^2 tests were used when appropriate. *P*-values less than 0·05 were considered significant.

Results

Clinical and serological characteristics of APS and SLE patients

All patients enrolled into this study were Caucasian. APS patients were 39 females and four males with a median age of 45 years (range = 17–75), and a median disease duration of 7 years (range = 0·5–24). SLE patients were 28 females and two males, with a median age of 42 years (range = 25–64) and a median disease duration of 6 years (range = 1–22). The clinical characteristics of APS and SLE patients

Table 1. Clinical characteristics of patients.

Characteristics *n* (%)	PAPS *n* = 15	SAPS *n* = 28	SLE *n* = 30
Arterial thrombosis	9 (60)	11 (39·3)	0
Venous thrombosis	4 (26·7)	16 (57·1)	0
Pregnancy morbidity	4 (26·7)	5 (17·9)	3 (10)
Livedo reticularis	6 (40)	12 (42·9)	5 (16·6)
Thrombocytopenia	4 (26·7)	8 (28·6)	1 (3·3)
Migraine	3 (20)	2 (7·1)	2 (6·6)
Seizures	2 (13·3)	2 (7·1)	1 (3·3)

PAPS = primary anti-phospholipid antibody syndrome; SLE = systemic lupus erythematosus; SAPS = APS associated with SLE.

are reported in Table 1. All patients were screened for aCL, anti-β_2GPI (tested by both ELISA methods with overlapping results) and LA; the prevalence of the antibodies is reported in Table 2.

Bioinformatic analysis of β2GPI

Analysis of functional and immunological sites within β_2GPI sequence showed that the most recommended epitope corresponds to the residues 51–70. Of note, this region contains the residue with the highest potentiality to be glycated (#63: score = 0.967, as calculated by Netglycate-1·0 prediction), as published previously [34] (Supporting information, Table S1).

Characterization of glucose-modified β_2GPI

Purified β_2GPI preparation was incubated for increasing times in the presence of D- glucose and characterized as described in the Methods section.

In order to verify whether G-β_2GPI might undergo a significant molecular size modification, a size exclusion chromatographic separation was carried out. UV recording at 214 nm of eluate showed the formation of a molecular size increase due probably to the formation of glucose-β_2GPI adducts (G-β_2GPI) (see arrow in Fig. 1a). Analysis of the chromatographic profile showed a molecular size increase in the 22-day glycated β_2GPI corresponding to approximately 18% of total protein.

UV absorption spectrum indicated that three main regions of β_2GPI were affected by glucose treatment. The change of absorbance observed at region corresponding to 310–340 nm suggests the formation of non-enzymatic glycosylation adducts, as reported by published studies [40], whereas the changes of absorbance observed at 260–280 and 215–240 nm suggest structural modifications occurring on β_2GPI protein folding (Fig. 1b).

Table 2. Prevalence of antibodies of patients.

Autoantibodies *n* (%)	PAPS *n* = 15	SAPS *n* = 28	SLE *n* = 30
aCL	12 (80)	22 (78·6)	3 (10)
aβ_2GPI	**5 (33·3)**	**9 (32·1)**	1 (3·3)
LA	8 (53·3)	16 (57·1)	1 (3·3)
aG-β_2GPI	**9 (60)**	**16 (57·1)**	8 (26·6)

PAPS = primary anti-phospholipid antibody syndrome; SLE = systemic lupus erythematosus; SAPS = APS associated with SLE; aCL = anti-cardiolipin antibodies; LA = lupus anti-coagulant.

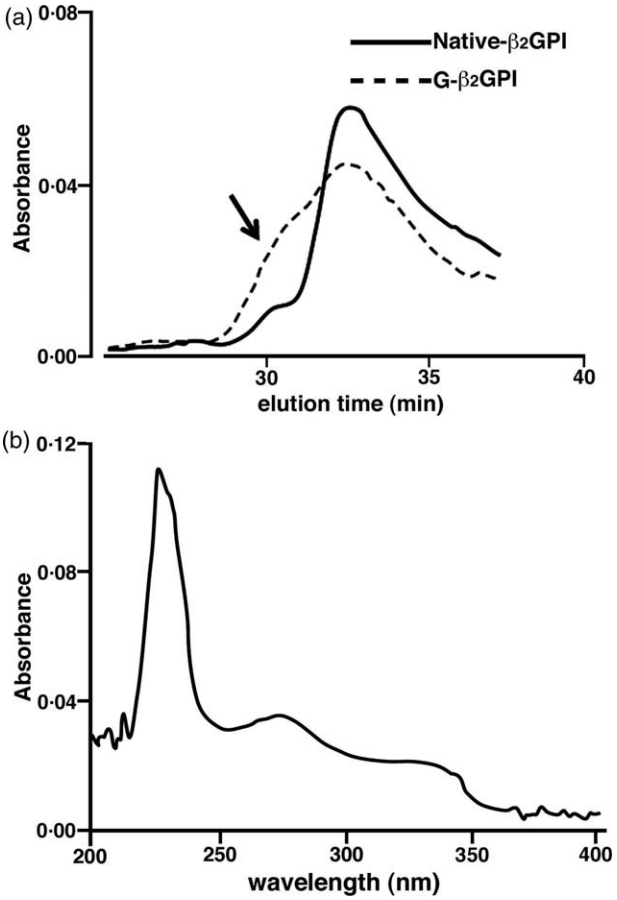

Fig. 1. (a) Chromatographic elution of native and glucose-modified $\beta_2$glycoprotein I (G-β_2GPI). Twenty micrograms of highly purified β_2GPI incubated previously with sugar were injected onto Superose S12 fast protein liquid chromatography (FPLC) column as described in the Methods section and protein peaks were detected under ultraviolet (UV) recording (optical density at 214 nm). The dashed line indicates that G-β_2GPI elutes as a complex protein population involving higher molecular weight complexes (highlighted by the arrow). A semiquantitative evaluation of such polymeric G-β_2GPI, corresponding to the left-side shoulder of the dashed line, indicates that under these non-denaturating chromatographic conditions, such complexes may represent the 15–20% of total G-β_2GPI proteins; (b) differential absorption spectrum between native β_2GPI and G-β_2GPI. Using a dual-beam UV spectrophotometer, absorbance of G-β_2GPI and native-β_2GPI were analysed. The absorption spectrum shows the different spectral regions of β_2GPI affected by glucose-induced modification. The analysis indicates that three main regions are affected: the change of absorbance observed at region corresponding to 310–340 nm suggests the formation of non-enzymatic glycosylation adducts [34], whereas the changes of absorbance observed at 260–280 and 215–240 suggest structural modifications occurring on the β_2GPI protein folding.

Fluorescence at 450 nm, specific for AGE formation, was measured and confirmed the creation of time-dependent AGE products (Supporting information, Table S2).

Detection of antibodies to glucose modified-β_2GPI by ELISA

Nine of 15 consecutive PAPS out-patients (60%) and 16 of 28 SAPS (57·1%) showed serum antibodies (IgG class) against G-β_2GPI (anti-G-β_2GPI). No significant difference was found between PAPS and SAPS patients (Table 2). Of note, four sera from patients with PAPS and seven with SAPS were positive for anti-G-β_2GPI but negative for anti-native-β_2GPI (Fig. 2, arrows).

The occurrence of anti-G-β_2GPI was significantly higher in APS patients (25 of 43, 58·1%) compared to patients suffering from SLE (26·6%) ($P = 0{\cdot}037$). No RA patients or control healthy subjects resulted positive for aG-β_2GPI (Fig. 3).

Sera of patients with APS, positive for both anti-β_2GPI and anti-G-β_2GPI, were absorbed with native β_2GPI and then tested for anti-G-β_2GPI antibodies, or alternatively for anti-native-β_2GPI, by ELISA. Reactivity with G-β_2GPI showed significant inhibition (63% inhibition, $P < 0{\cdot}001$)

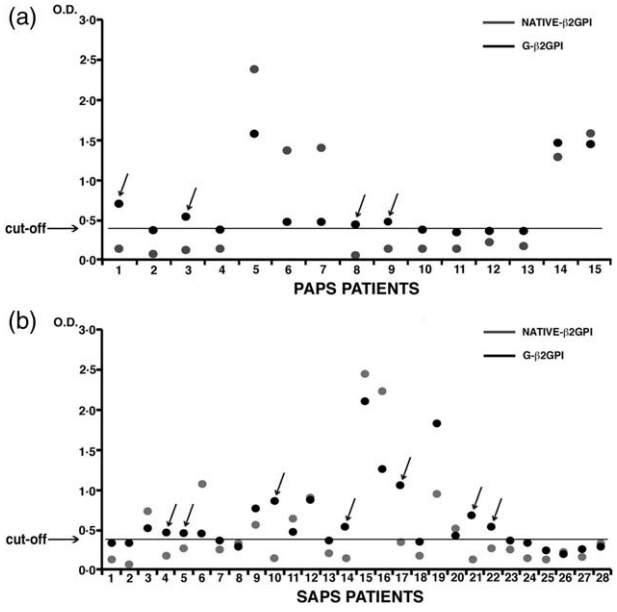

Fig. 2. Relationship between anti-native $\beta_2$glycoprotein I (β_2GPI) and anti-glucose-modified GPI (G-β_2GPI) antibodies in primary anti-phospholipid antibody syndrome (PAPS) and APS associated with systemic lupus erythematosus (SLE) (SAPS) patients. Sera from patients with PAPS and SAPS were analysed by enzyme-linked immunosorbent assay (ELISA) for the detection of anti-native-β_2GPI and anti-G-β_2GPI immunoglobulin (Ig)G. The arrows indicate the sera from patients (PAPS or SAPS) positive for anti-G-β_2GPI but negative for anti-native-β_2GPI. The horizontal line shows the cut-off level calculated as mean optical density (OD) value in control healthy subjects' sera ± 3 standard deviations (s.d.).

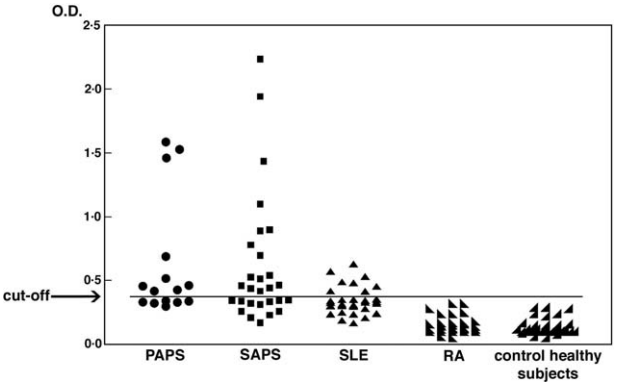

Fig. 3. Anti-glucose-modified $\beta_2$glycoprotein I (G-β_2GPI) antibodies in patients and healthy subjects. Sera from patients with anti-phospholipid antibody syndrome (PAPS) and APS associated with systemic lupus erythematosus (SLE) (SAPS), SLE, rheumatoid arthritis (RA) and of healthy subjects were analysed by enzyme-linked immunosorbent assay (ELISA) for the detection of anti-G-β_2GPI immunoglobulin (Ig)G. All the sera from RA patients and healthy subjects were negative for anti-G-β_2GPI IgG antibodies. The horizontal line shows the cut-off level calculated as mean optical density (OD) value in control healthy subjects' sera ± 3 standard deviations (s.d.).

by first absorbing sera with native β_2GPI (Fig. 4a). As expected, reactivity with native-β_2GPI showed almost complete inhibition (83·8% inhibition, $P < 0{\cdot}001$) (Fig. 4b). These findings suggest that anti-G-β_2GPI recognize specific glycation-related epitopes.

Associations of antibodies to glucose-modified β_2GPI with classical aPL and clinical features

Analysis of the correlations between anti-G-β_2GPI OD and classical aPL in APS patients revealed a significant association with aCL ($P = 0{\cdot}013$) and anti-native β_2GPI ($P = 0{\cdot}009$). Furthermore, a significant correlation was found between anti-G-β_2GPI with venous thrombosis ($P = 0{\cdot}017$) and seizure ($P = 0{\cdot}027$) in these patients.

Discussion

This study provides new findings showing that G-β_2GPI is a target antigen of humoral immune response in patients with APS and suggests a possible usefulness of anti-G-β_2GPI to improve the diagnosis of this disease.

Several studies have suggested that post-translational oxidative modifications of β_2GPI may affect antigenic properties of the molecule. Among these, glycosylation processes may play a relevant role [41]. In this study we analysed the presence of antibodies to glucose-modified β_2GPI by glycation (non-enzymatic glycosylation). Glycation is the non-enzymatic addition or insertion of saccharide derivatives to proteins, lipids or nucleic acids, leading to the formation of intermediary Schiff bases and Amadori products and,

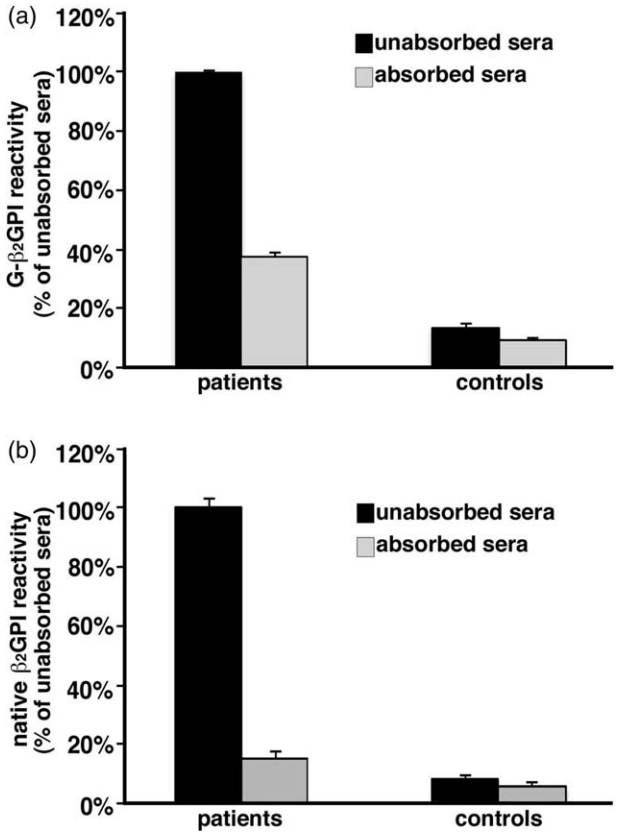

Fig. 4. Absorption tests for detection of anti-glucose-modified $\beta_2$glycoprotein I (G-β_2GPI) specificity. Sera of patients with anti-phospholipid antibody syndrome (APS), positive for both anti-native β_2GPI and anti-G-β_2GPI, and of healthy subjects (controls), unabsorbed or absorbed with native β_2GPI, were analysed by enzyme-linked immunosorbent assay (ELISA) for the detection of: (a) anti-G-β_2GPI IgG, mean absorbance of unabsorbed sera [optical density (OD) 2·264] was set to 100%. Absorbed patients' sera *versus* unabsorbed patients' sera: $P < 0{\cdot}001$; absorbed controls' sera *versus* unabsorbed controls' sera: $P > 0{\cdot}05$. (b) anti-β_2GPI IgG, mean absorbance of unabsorbed sera (OD 1·116) was set to 100%. Absorbed patients' sera *versus* unabsorbed patients' sera: $P < 0{\cdot}001$; absorbed controls' sera *versus* unabsorbed controls' sera: $P > 0{\cdot}05$.

finally, to irreversible AGEs [42]. Glycation reaction, similarly to other decisive events, including β_2GPI binding to anionic surfaces, such as phospholipids, and oxidative modifications [29,30,43,44], may induce a significant misfolding effect on the β_2GPI structure, contributing to the expression of cryptic or neoepitopes recognized by the immune system. In a previous investigation we demonstrated, by bioinformatic analyses of the β_2GPI primary structure, that several potential glycation sites are present within the molecule [34]. The intriguing co-localization of high glycation sites with potential epitopes found in the present study suggests that sugar-induced modifications occurring on β_2GPI may be able to affect its antigenic behaviour.

In our previous investigation we also showed that a 10-day β_2GPI treatment with glucose induces a protein modification probably consisting of AGE-β_2GPI formation, and that this glucose-modified protein is able to activate human monocyte-derived immature dendritic cells [34]. In the present study, we investigated the effects of a longer incubation (22 days) with glucose on β_2GPI structure. G-β_2GPI was studied under non-denaturing conditions, in order to evaluate the formation of glycation end products and/or adducts with sugar characterized by non-covalent bonds. The chromatographic analysis of G-β_2GPI indicated the presence of a protein population with shorter elution time, i.e. with larger molecular size. Peak integration of the chromatogram suggested that this molecular size shift could involve a significant portion of β_2GPI protein.

Therefore, we verified that glucose treatment may induce a significant misfolding effect on the β_2GPI structure, probably leading to expression of cryptic or neoepitopes recognized by the immune system. Upon modification with glucose, increase in UV absorbance was recorded. This finding provided a useful insight into the structural perturbation of β_2GPI protein probably inducing the formation of glycation adducts. The fluorescence spectrophotometric analysis of G-β_2GPI confirmed the creation of time-dependent advanced glycation end products (AGEs).

In this study we observed that anti-G-β_2GPI were present in a significant percentage of patients with APS. Interestingly, ELISA for anti-G-β_2GPI prompted to identify some APS patients (four PAPS and seven SAPS), who were negative in the 'classical' anti-β_2GPI test.

The mechanisms by which G-β_2GPI accumulates and may trigger a humoral immune response in APS patients are not completely known. A possible explanation is that G-β_2GPI accumulation occurs in patients with APS driven by oxidative stress and inflammation, thereby initiating a local autoimmune process and becoming the target of autoimmune responses. The role of oxidative post-translational modification of β_2GPI has been described widely [45,46]. In particular, it can produce new antigens that are not represented in the thymus, so that autoreactive T cells can escape negative selection and move to the periphery. Alternatively, the way by which antigen-presenting cells process oxidized β_2GPI may be different from that processing the reduced form. In both cases an autoimmune response can be triggered. Thus, as epitope dominance is influenced by protein structure, glycation events may change the molecular context of β_2GPI epitopes (by altering secondary or tertiary structure), thus permitting the efficient presentation of cryptic and neodeterminants [45]. According to our previous results [34,44], we can hypothesize that a pro-oxidant and proinflammatory microenvironment predisposes local β_2GPI to glycation and/or oxidation, thereby initiating a local autoimmune process.

Another interesting finding of this study is the observation that anti-G-β_2GPI were correlated significantly with several clinical manifestations of APS, including venous

thrombosis and seizure. To date, the most commonly investigated antigenic target in APS patients is β_2GPI, and anti-β_2GPI antibodies represent a highly specific test for diagnosis of the syndrome. In particular, IgG anti-β_2GPI antibodies comprise a family of antibodies which recognize different epitopes of the protein. In recent years, several studies showed that antibodies to domain I (DI), that specifically recognize the glycine40-arginine43 epitope, showed a good correlation with thrombosis and pregnancy morbidity [47,48]. Moreover, anti-β_2GPI antibodies with DI specificity were found in the majority of APS patients and were associated significantly with LA and venous thrombosis [49]. Our findings extend these data, strongly suggesting a relationship between the presence of anti-G-β_2GPI and thrombosis. This observation is not surprising. Indeed, since 1985, Vlassara *et al.* showed that AGE-modified proteins can be bound to a special receptor receptor for advanced glycation end-product (RAGE) [50], and the same binding has been shown for AGE-β_2GPI [34]. Engagement of RAGE results in intracellular signalling, which leads to activation of the proinflammatory transcription factor nuclear factor kappa B (NF-κB), with consequent production of cytokines, adhesion molecules, prothrombotic and vasoconstrictive gene products [34,51]. For example, the endothelial surface is changed in a way that coagulation events are favoured; indeed, AGE-bound RAGE on the endothelium may result in alteration of the cell surface structure, inducing a procoagulant endothelium, via reduced thrombomodulin activity [52] concomitant with increased tissue factor expression [53].

Taken together, our data demonstrate the existence of anti-G-β_2GPI in APS patients, suggesting that β_2GPI glycation products may represent additional epitopes for anti-β_2GPI reactivity, possibly contributing to the heterogeneous specificity of aPL [54]. Searching for these antibodies may be useful for evaluating the risk of clinical manifestations in APS patients.

Acknowledgements

The technological support from the Facility for Complex Protein Mixture (CPM) Analysis at ISS (Rome) is kindly acknowledged. All authors read and approved the final manuscript.

Disclosure

The authors declare that they have no disclosures.

Author contributions

M.S. designed the study and drafted the manuscript, B.B. designed the study and carried out the experiments, A.C. carried out the experiments, E.P. designed the study and carried out the experiments, F.F. carried out the experiments (preparation of G-β_2GPI and chromatographic analyses), C.A. and S.T. collected the sera samples, characterized the patients clinically and performed statistical analysis, R.M. drafted the manuscript and revised the manuscript critically, F.C. and G.V. revised the manuscript critically, R.R. designed the study and drafted the manuscript.

References

1 Hughes GR, Harris NN, Gharavi AE. The anticardiolipin syndrome. J Rheumatol 1986; **13**:486–9.
2 Levine JS, Branch DW, Rauch J. The antiphospholipid syndrome. N Engl J Med 2002; **346**:752–63.
3 Miyakis S, Lockshin MD, Atsumi T *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006; **4**: 295–306.
4 Tincani A, Balestrieri G, Spatola L, Cinquini M, Meroni PL, Roubey RA. Anticardiolipin and anti-beta 2glycoprotein I immunoassays in the diagnosis of antiphospholipid syndrome. Clin Exp Rheumatol 1998; **16**:396–402.
5 Bertolaccini ML, Gomez S, Pareja JF *et al.* Antiphospholipid antibody tests: spreading the net. Ann Rheum Dis 2005; **64**: 1639–43.
6 Galli M, Barbui T, Zwaal RF, Comfurius P, Bevers EM. Antiphospholipid antibodies: involvement of protein cofactors. Haematologica 1993; **78**:1–4.
7 Bas de Laat H, Derksen RH, de Groot PG. Beta2-glycoprotein I, the playmaker of the antiphospholipid syndrome. Clin Immunol 2004; **112**:161–8.
8 Arvieux J, Darnige L, Caron C, Reber G, Bensa JC, Colomb MG. Development of an ELISA for autoantibodies to prothrombin showing their prevalence in patients with lupus anticoagulants. Thromb Haemost 1995; **74**:1120–5.
9 Sorice M, Griggi T, Circella A *et al.* Protein S antibodies in acquired protein S deficiencies. Blood 1994; **83**:2383–4.
10 Sorice M, Arcieri P, Griggi T *et al.* Inhibition of protein S by autoantibodies in patients with acquired protein S deficiency. Thromb Haemost 1996; **75**:555–9.
11 Oosting JD, Derksen RH, Bobbink IW, Hackeng TM, Bouma BN, de Groot PG. Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? Blood 1993; **81**:2618–25.
12 Kaburaki J, Kuwana M, Yamamoto M, Kawai S, Ikeda Y. Clinical significance of anti-annexin V antibodies in patients with systemic lupus erythematosus. Am J Hematol 1997; **54**:209–13.
13 Salle V, Maziere JC, Smail A *et al.* Anti-annexin II antibodies in systemic autoimmune diseases and antiphospholipid syndrome. J Clin Immunol 2008; **28**:291–7.
14 Ortona E, Capozzi A, Colasanti T *et al.* Vimentin/cardiolipin complex as a new antigenic target of the antiphospholipid syndrome. Blood 2010; **116**:2960–7.
15 Kobayashi T, Stang E, Fang KS, de Moerloose P, Parton RG, Gruenberg J. A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature 1998; **392**:193–7.
16 Alessandri C, Bombardieri M, Di Prospero L, Conigliaro P, Conti F, Labbadia G. Anti-lysobisphosphatidic acid antibodies

in patients with antiphospholipid syndrome and systemic lupus erythematosus. Clin Exp Immunol 2005; **140**:173–80.
17 Valesini G, Alessandri C. New facet of antiphospholipid antibodies. Ann NY Acad Sci 2005; **1051**:487–97.
18 Muller G, Biering A, Lux E, Richter V. Beta 2 glycoprotein I analysis in patients with hyperlipoproteinemia, arteriosclerotic occlusive disease and diabetes mellitus. Z Gesamte Inn Med 1983; **38**:282–4.
19 Hattori N, Kuwana M, Kaburaki J, Mimori T, Ikeda Y, Kawakami Y. T cells that are autoreactive to beta2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. Arthritis Rheum 2000; **43**:65–75.
20 Ferro D, Pittoni V, Quintarelli C *et al.* Coexistence of antiphospholipid antibodies and endothelial perturbation in systemic lupus erythematosus patients with ongoing prothrombotic state. Circulation 1997; **95**:1425–32.
21 George J, Shoenfeld Y, Harats D. The involvement of beta2-glycoprotein I (beta2-GPI) in human and murine atherosclerosis. J Autoimmun 1999; **13**:57–60.
22 Garcia CO, Kanbour-Shakir A, Tang H, Molina JF, Espinoza LR, Gharavi AE. Induction of experimental antiphospholipid antibody syndrome in PL/J mice following immunization with beta 2 GPI. Am J Reprod Immunol 1997; **37**:118–24.
23 Zager U, Kveder T, Cučnik S, Božič B, Lunder M. Anti-β2-glycoprotein I paratopes and β2-glycoprotein I epitopes characterization using random peptide libraries. Autoimmunity 2014; **47**: 438–44.
24 Tincani A, Spatola L, Prati E *et al.* The anti-beta2-glycoprotein I activity in human anti-phospholipid syndrome sera is due to monoreactive low-affinity autoantibodies directed to epitopes located on native beta2-glycoprotein I and preserved during species' evolution. J Immunol 1996; **157**:5732–8.
25 Roubey RA, Eisenberg RA, Harper MF, Winfield JB. 'Anticardiolipin' autoantibodies recognize beta 2-glycoprotein I in the absence of phospholipid. Importance of Ag density and bivalent binding. J Immunol 1995; **154**:954–60.
26 Aron AL, Cuellar ML, Brey RL *et al.* Early onset of autoimmunity in MRL/++ mice following immunization with beta 2 glycoprotein I. Clin Exp Immunol 1995; **101**:78–81.
27 Artenjak A, Locatelli I, Brelih H *et al.* Immunoreactivity and avidity of IgG anti-β2-glycoprotein I antibodies from patients with autoimmune diseases to different peptide clusters of β2-glycoprotein I. Immunol Res 2015; **61**:35–44.
28 Wang MX, Kandiah DA, Ichikawa K *et al.* Epitope specificity of monoclonal anti-beta 2-glycoprotein I antibodies derived from patients with the antiphospholipid syndrome. J Immunol 1995; **155**:1629–36.
29 Horkko S, Miller E, Branch DW, Palinski W, Witztum JL. The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and beta2 glycoprotein 1 (and other proteins). Proc Natl Acad Sci USA 1997; **94**:10356–61.
30 Arvieux J, Regnault V, Hachulla E, Darnige L, Berthou F, Youinou P. Oxidation of beta2-glycoprotein I (beta2GPI) by the hydroxyl radical alters phospholipid binding and modulates recognition by anti-beta2GPI autoantibodies. Thromb Haemost 2001; **86**:1070–6.
31 Ramasamy R, Yan SF, Schmidt AM. RAGE: therapeutic target and biomarker of the inflammatory response-the evidence mounts. J Leukoc Biol 2009; **86**:505–12.
32 Shanmugam N, Kim YS, Lanting L, Natarajan R. Regulation of cyclooxygenase-2 expression in monocytes by ligation of the receptor for advanced glycation end products. J Biol Chem 2003; **278**:34834–44.
33 Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP. AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus. I. The AGE concept. Cardiovasc Res 1998; **37**: 586–600.
34 Buttari B, Profumo E, Capozzi A *et al.* Advanced glycation end products of human beta(2) glycoprotein I modulate the maturation and function of DCs. Blood 2011; **117**:6152–61.
35 Nienhuis HL, Westra J, Smit AJ, Limburg PC, Kallenberg CG, Bijl M. AGE and their receptor RAGE in systemic autoimmune diseases: an inflammation propagating factor contributing to accelerated atherosclerosis. Autoimmunity 2009; **42**:302–4.
36 Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997; **40**:1725.
37 Facchiano AM, Facchiano A, Facchiano F. Active Sequences Collection (ASC) database: a new tool to assign functions to protein sequences. Nucleic Acids Res 2003; **31**:379–82.
38 Yao B, Zhang L, Liang S, Zhang C. SVMTriP: a method to predict antigenic epitopes using support vector machine to integrate tri-peptide similarity and propensity. PLOS ONE 2012; **7**: e45152.
39 Facchiano F, D'Arcangelo D, Russo K *et al.* Glycated fibroblast growth factor-2 is quickly produced *in vitro* upon low-millimolar glucose treatment and detected *in vivo* in diabetic mice. Mol Endocrinol 2006; **20**:2806–18.
40 Ansari NA, Dash D. Biochemical studies on methylglyoxal-mediated glycated histones: implications for presence of serum antibodies against the glycated histones in patients with type 1 diabetes mellitus. ISRN Biochem 2013; **7**:198065.
41 Kondo A, Miyamoto T, Yonekawa O, Giessing AM, Osterlund EC, Jensen ON. Glycopeptide profiling of beta-2-glycoprotein I by mass spectrometry reveals attenuated sialylation in patients with antiphospholipid syndrome. J Proteomics 2009; **73**:123–33.
42 Smit AJ, Lutgers HL. The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceutics to reduce AGE accumulation. Curr Med Chem 2004; **11**: 2767–84.
43 Matsuura E, Igarashi Y, Fujimoto M *et al.* Heterogeneity of anticardiolipin antibodies defined by the anticardiolipin cofactor. J Immunol 1992; **148**:3885–91.
44 Buttari B, Profumo E, Mattei V *et al.* Oxidized beta2-glycoprotein I induces human dendritic cell maturation and promotes a T helper type 1 response. Blood 2005; **106**:3880–7.
45 Passam FH, Giannakopoulos B, Mirarabshahi P, Krilis SA. Molecular pathophysiology of the antiphospholipid syndrome: the role of oxidative post-translational modification of beta 2 glycoprotein I. J Thromb Haemost 2011; **9**:275–82.
46 Mirarabshahi P, Abdelatti M, Krilis S. Post-translational oxidative modification of beta2-glycoprotein I and its role in the pathophysiology of the antiphospholipid syndrome. Autoimmun Rev 2012; **11**:779–80.
47 Sebire NJ, Fox H, Backos M, Rai R, Paterson C, Regan L. Defective endovascular trophoblast invasion in primary antiphospholipid antibody syndrome-associated early pregnancy failure. Hum Reprod 2002; **17**:1067–71.

48 Iverson GM, Victoria EJ, Marquis DM. Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. Proc Natl Acad Sci USA 1998; **95**:15542–6.
49 de Laat B, de Groot. PG. Autoantibodies directed against domain I of beta2-glycoprotein I. Curr Rheumatol Rep 2011; **13**:70–6.
50 Vlassara H, Brownlee M, Cerami A. High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. Proc Natl Acad Sci USA 1985; **82**:5588–92.
51 Bierhaus A, Humpert PM, Morcos M *et al*. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med (Berl) 2005; **83**:876–86.
52 Esposito C, Gerlach H, Brett J, Stern D, Vlassara H. Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. J Exp Med 1989; **170**: 1387–407.
53 Bierhaus A, Illmer T, Kasper M *et al*. Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. Circulation 1997; **96**: 2262–71.
54 Gharavi AE, Sammaritano LR, Wen J, Elkon KB. Induction of antiphospholipid autoantibodies by immunization with beta 2 glycoprotein I (apolipoprotein H). J Clin Invest 1992; **90**: 1105–9.

Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Bioinformatic analysis of functional and immunological sites within β2GPI sequence.
Table S2. Time-dependent non-tryptophan fluorescence of sugar-treated β2GPI.