Evaluation of Some Immunological Serodiagnostic Markeres in Systemic Lupus Erythmatosus

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Abstract

Background: Antiphospholipid syndrome (APS) is a systemic autoimmune disease, associated with a hypercoaguable state and thrombocytopenia with persistence of antiphospholipid antibodies (APA) such as anticardiolipin (ACL) & lupus articoagulant, which is dependant on the presence of Anti-β_2 glycoprotein (anti–β_2-GPI) antibodies.

Objectives: Determination of the level of anti–β_2-GPI level (IgG and IgM isotypes) and ACL antibodies as inflammatory markers in children with Systemic Lupus Erythmatosus (SLE)

Patients and methods: The study included fifteen cheldern with SLE, their ages range between 9 – 17 years (5 males and 10 females), in addition to fifteen apparently healthy children of comparable age, sex and nutritional status as a control group. All patients and controls were subjected to determination of rheumatoid factor, antinuclear antibodies (ANA), IgG and IgM isotypes of both ACL and anti–β_2-GPI.

Results: Anti–β_2-GPI (IgG & IgM) isotypes were found to be positive in 4 (26.6) & 3 (20%) of patients respectively. The mean leveles of both IgG and IgM isotypes of Anti–β_2-GPI were found to be significantely higher in patients in comparison to controls (P < 0.001). ACL (IgG & IgM) isotypes were found to be positive in 3 (20%) and 2 (13.3%) of patients respectively. A statistically significant positive correlations were found between IgM and IgG isotypes of both markers.

Conclusions: both IgG and IgM isotypes of anti-β_2 glycoprotein and ACL could be used as a useful serodiagnonstic immunological marker in SLE patients.

Keywords: Anti-β_2 glycoprotein type I, Anticardiolipin Antibody, Systemic Lupus Erythematosus

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease that can involve any organ system with a wide range of disease manifestations, and can lead to significant morbidity and even Mortality (Deborah and Sylvia; 2012).

Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) can be associated with significant morbidity in children and adolescents (Lee et al; 2001).

Antiphospholipid syndrome (APS) is a systemic autoimmune disease, associated with a hypercoaguable state (venous and arterial thrombosis), recurrent abortion and thrombocytopenia with presence of persistence of APA such as anticardiolipin – lupus articoagulant (Miri; 2003).

APS is characterized by the presence of pathogenic autoantibodies against B_2 GPI which have molecular mimicry related synthetic peptides and bacterial or cytomegalovirus membrane (Iverson et al; 2002).

Anticardiolipin antibodies (aCL) need a circulating plasma protein as a cofactor, which binds to the cardiolipin antigen in solid-phase ELISA assays. This cofactor has been identified as β_2-glycoprotein I (β_2GPI). It has been shown subsequently that β_2GPI is able to bind to activated polystyrene plates as an antigen and can be used to facilitate the detection of specific anti-β_2GPI antibodies in patient sera. This has led to a number of studies in which these autoantibodies have been reported to occur in up to 82% of SLE and APS patients (Loizou et al; 2000).

Aim of the study: Determination of the level of IgG and IgM isotypes of anti-β_2 glycoprotein type β and anticardiolipin antibodies as inflammatory markers in children with SLE.

Patients and Methods: This study was conducted at Al-Azhar and Beni Suef University Hospitals and included fifteen children [10 (66.7%) females and 5 (33.3%) males] in addition to fifteen apparently healthy children [9 (60%) females and 6 (40%) males] of comparable age, sex and nutritional status as a control group. The mean age for patients and control group was 15.9 ± 4.2 years and 16.1 ± 4.8 years respectively. All SLE children patients had four or more of the revised American Colleague of Rheumatology (ACR) criteria for the diagnosis of SLE (Felson et al., 2004) and they were not under definite drug regimen. Our study excluded patients older than 17 years and younger than 9 years, patients with inactive stage of disease, and patients with associated chronic illnesses such as tuberculosis.

All patients and controls were subjected to complete clinical history, examination including complete joint examination and radiology of the affected joints. Electrocardiography (ECG) and echocardiography were performed in suspected cases as well as abdominal ultrasound to evaluate the condition of the kidneys, liver and spleen.

In addition both patients and controls were investigated for routine laboratory investigations used in diagnosis of SLE. These included complete urine analysis (to detect urinary proteins, red cells and casts), complete blood count (CBC), erythrocytes sedimentation rate by Westergreen's method (ESR), and renal and liver function tests. Also, the immunological investigations used in SLE diagnosis were evaluated in both patients and controls. These included C-reactive protein (CRP), serum complements C3 and C4, antinuclear antibodies (ANA), rheumatoid factor (RF), and anti-native DNA antibodies (Anti-nDNA). Serum levels of anticardiolipin (ACL) and anti- β2 glycoprotein-I (anti-β2GPI) IgM and IgG antibodies were measured by ELISA, in SLE patients and controls.

Blood samples from SLE patients and controls were collected by venipuncture under aseptic conditions, one millilitre of blood was collected on 1.5mg/ml dipotassium ethylene diamine tetra-acetic acid (EDTA potassium) for CBC, 0.8 ml of blood on 0.2 ml (32g/L) trisodium citrate for ESR. Three millilitres of blood were collected in a sterile glass tube with no additive, left to clot and serum was separated by centrifugation and stored in small aliquots at -20 oC until evaluated for the defined serological parameters and measured for serum levels of ACL (IgG and IgM) and anti-β2GPI (IgM and IgG) antibodies.

Semi-quantitative determination of serum CRP was made using a latex agglutination test (Quick slide agglutination test) (Herbert, 1978). Quantitative determination of serum C3 and C4 by turbidimeter (Behring turbitimer, Behringwerk, Germany) was done on the basis of an immunochemical reaction, where the proteins contained in human serum from immune complexes react with specific antibodies in the reagent. The turbidity generated in the reaction was measured photometrically.ANA were detected in sera by an indirect fluorescent antibody technique using Hep-2 Substrate slides which contain wells of human epithelial cells (Kallested Quantafluor Hep-2 Kit, BioRad, France).It was viewed for fluorescent patterns with a fluorescent microscope (Nakamura et al., 1984). Anti-nDNA antibodies were semi-quantitatively measured by immunofluorescence using Crithidia luciliae as a substrate (The Binding Site, Birmingham, U.K.) (Nakamura et al., 1984). Semi-quantitative determination of RF (IgM) was made using a latex agglutination slide test (Latex Quick slide agglutination test) (Singer and Plotz; 1956, and Signer; 1957).

*Determination of serum levels of both IgM and IgG anti-β2GPI antibodies:*The serum levels of anti- β2GPI IgG and IgM antibodies in SLE children patients and controls were evaluated by enzyme-linked immunosorbent assay (ELISA) technique using IMUCLONE Anti- β2GPI IgG ELISA and IMUCLONE Anti- β2GPI IgM ELISA kits according to instructions of kits manufacturer (Sekisui Diagnostics, LLC, Germany).

Measurement of anti-β2GPI IgG antibodies: The imuclone Anti- β2GPI IgG ELISA kit is a solid phase enzyme immunoassay for measurement of IgG autoantibodies against β2GPI. The antibodies recognize molecules of β2GPI at high density attached to a gamma-irradiated solid phase (Mutsuura et al., 1994; Rouby et al., 1995). The kit contained 2 plates; each plate had six strips of control blank wells and six strips of β2GPI-coated wells. So each unit consisted of a pair of wells (blank and β2GPI). During incubation, the antibody present in the test sample was bound to the solid phase.

Determination of absorbance values of anti-β2GPI IgG: The absorbance of each well was measured at 450 nm using the microwell plate reader (Microplate Autoreader Bio-TEK Instruments). The net absorbance for   each sample was calculated by subtracting the absorbance value of the control (blank) well from the absorbance value of the antigen coated well (β2GPI well) i.e. each specimen had a separate blank which was subtracted to obtain net absorbance. The presence of antibodies was detected by a colour change read by an ELISA reader.

Calculation of anti-β2GPI IgG: The anti- β2GPI IgG activity in the specimen was calculated using the following equation: Activity in specimen = Activity in Calibrator/Net absorbance of Calibrator X Net Absorbance of specimen.

Where the performance of both calibrators and controls was provided in the included data sheet.

The units used to express anti- β2GPI antibody are the same as those used for anticardiolipin antibody (Anti-Phospholipid Standardization Laboratory, Atlanta, Gorgia).The same standards have been used to calibrate the anti- β2GPI antibody units. For each isotype, 1 U of anticardiolipin is considered equal to 1 U of anti- β2GPI. Specimens were considered in the area of the positive range when the anti- β2GPI IgG is 25-40 units/ml. High level was considered to be greater than 40 of Gβ2GPI units/ml.

Measurement of anti-β2GPI IgM antibodies: imuclone Anti-ß2GPI IgM ELISA Kit is performed as an indirect enzyme-linked immunosorbent assay (ELISA) intended for the detection and semi-quantitative determination of IgM anti-Beta2 Glycoprotein l (β2GPI) antibodies in human serum or citrated plasma. It has been shown that the binding of β2GPI to the microwell surface may produce a neoepitope similar to that when combined with a phospholipid and the results with this system showed a good correlation with the anti-phospholipid syndrome (Matsuura, et al., 1994; Keil et al., 1995; Amengual et al., 1996 and Tsutsumi et al., 1996).

The diluted sera, calibrator sera, and controls were incubated in microwells coated with purified and stabilized human-β2GPI allowing the anti-β2GPI antibodies present in the samples to

react with the immobilized antigen. The intensity of color developed in the wells was proportional to the serum concentration of anti-β2GPI antibodies.

Determination of absorbance values of anti-β2GPI IgM: Results were obtained by reading the O.D. (optical density or absorbance) of each well in a spectrophotometer with the IgM anti-β2GPI antibody concentrations expressed in M units in calibrator sera. The plate reader was blanked against single water blank well, and the O.D.s of the wells were read at 450 nm. The mean O.D. values of the calibrators, controls and patient samples were calculated.

A multipoint calibration curve using linear regression analysis with four calibrator values against their mean O.D.s was performed, and the calibrator curve was plotted manually with graph paper. A zero intercept was used when generating the regression line to avoid negative values and to best fit the line drawn through the plotted points. The control and patient sample values were determined from the calibrator curve.

Determination of serum levels of ACL IgM and IgG antibodies: Serum levels of ACL IgM and IgG antibodies were measured by The APL ELISA™ IgM Kit and the APL ELISA™ IgG Kit according to instructions of the kits manufacturer (LOUISVILLE APL DIAGNOSTICS, INC., USA). The assay is a standard indirect semi-quantitative enzyme linked immunoassay (ELISA) technique which enables measurement of ACL antibody levels of the IgM and IgG isotypes in human serum.

The APL ELISA™ IgM and IgG Calibrators, APL ELISA™ IgM and IgG Positive Controls (with defined ranges) and APL ELISA™ Negative Controls were included as in-house controls (Pierangeli and Harris, 2008; Harris,1990; Harris et al., 1986; Harris, 1990; Pierangeli et al., 1998; Harris and Pierangeli, 2000; Pierangeli et al., 2001; Harris and Pierangeli, 2002). The technique allows IgM and IgG ACL antibodies in calibrators, controls and patients sera to react with the APL ELISA™ Cardiolipin Antigen coating the polystyrene microwell strips.

All test samples, calibrators and controls were run in duplicate.

The O.D. readings of the plates in the spectrophotometer previously mentioned, were checked until calibrator 1 reached an O.D. reading of 1.1-1.2 (at 405 nm). After stopping the color reaction, the plates were read once again at 405 nm. And the data obtained was used to establish a calibration curve.

A measurable color change in the presence or absence of cardiolipin antibody is determined by comparing the sample optical density with that of a five point calibration curve. The APL ELISA™ IgM and IgG Kits have been calibrated using standard ACL units (MPL units and GPL units) and the results are reported in MPL and GPL units. One MPL unit is the anticardiolipin binding activity of 1μg/ml of an affinity purified IgM antibody and one GPL unit is the

anticardiolipin binding activity of 1μg/ml of an affinity purified IgG antibody (Pierangeli and Harris, 2008 and Harris; 1990b).

Elaboration of the calibration curves: For construction of calibration curves the mean optical density (O.D.) reading of the calibrators (C1 to C5), positive control and reagent blank was determined. The mean O.D. readings of reagent blank were subtracted from all mean readings. The mean O.D. readings of C1 – C5 against appropriate concentrations were plotted using a log log calibration plot. The initial concentration of the calibrator was provided on the calibrator label.

Values lower than 25 MPL for IgM and lower than 19 GPL for IgG and above the cut-off points for IgM and IgG respectively were considered "indeterminate. Samples falling in these categories were retested to confirm positivity at a later date (Pierangeli and Harris, 2008, and Budd et al., 2006).

Patient sample with a higher O.D. reading than calibrator C1 was serially diluted and tested again. The values obtained in MPL and GPL units were multiplied by the appropriate dilution factor.

Statistical methods: Statistical Package for Social Science (SPSS) program version 16 was used for analysis of data. Data were presented as number, (percent) and mean + SD. Chi-square (x2) test was used to compare qualitative data.Significant correlation between different variables was determined using Pearson's correlation coefficient.

Results

Table (1): Age and sex distribution of the studied groups

	Patient group (n = 15)	**Control group (n = 15)**
Age in years (mean ± SD)	**15.9 ± 4.2**	**16.1± 4.8**
Sex n. (%)		
• **Females**	**10 (66.7 %)**	**9 (60%)**
• **Males**	**5 (33.3 %)**	**6 (40%)**

No significant differences between both groups **(P > 0.05)**

Regarding laboratory parameters in the present study normochromic normocytic anemia Leucopenia, thrombocytopenia, prolonged prothrombin time (PT), decreased prothrombin

concentration (PC), increased erythrocytes sedimentation rate (ESR), positive C-reactive protein (CRP) and elevated liver enzymes were reported in all patients.

Table (2): percentage of positive immunological markers for SLE in patient group

Immunological marker*	Patient group (n = 15)
• Rheumatoid factor (RF)	3 (20%)
• Antinuclear antibodies (ANA)	12 (80%)
• Anticardiolipin antibodies (ACL)	5 (33.3%)

*Values are given as n.%

Table (3): percentage of positive IgG & IgM isotypes of ACL& anti–β_2-GPI in patients

Immunological markers		IgG isotype	IgM iostype
ACL	• Cut off level (mg/dl)	>13.7	> 7.5
	• Positive cases n. (%)	3 (20%)	2 (13.3%)
anti–β_2-GPI	• Cut off level (u/l)	>40	>10
	• Positive cases n. (%)	4 (26.6%)	3 (20%)

Table (4): Man ± SD of IgG & IgM isotypes of ACL& anti–β_2-GPI in studied groups

Immunological markers		patients	controls	P value
ACL (mg/dl)	• IgG isotype	25.8 ± 9.3	6.3± 0.8	
	• IgM isotype	19.7± 7.9	3.7 ±.2	P < 0.001
Anti – β_2-GPI (u/l)	• IgG isotype	12.6± 9.7	1.83±1.6	
	• IgM isotype	9.87±5.38	0.39±0.3	

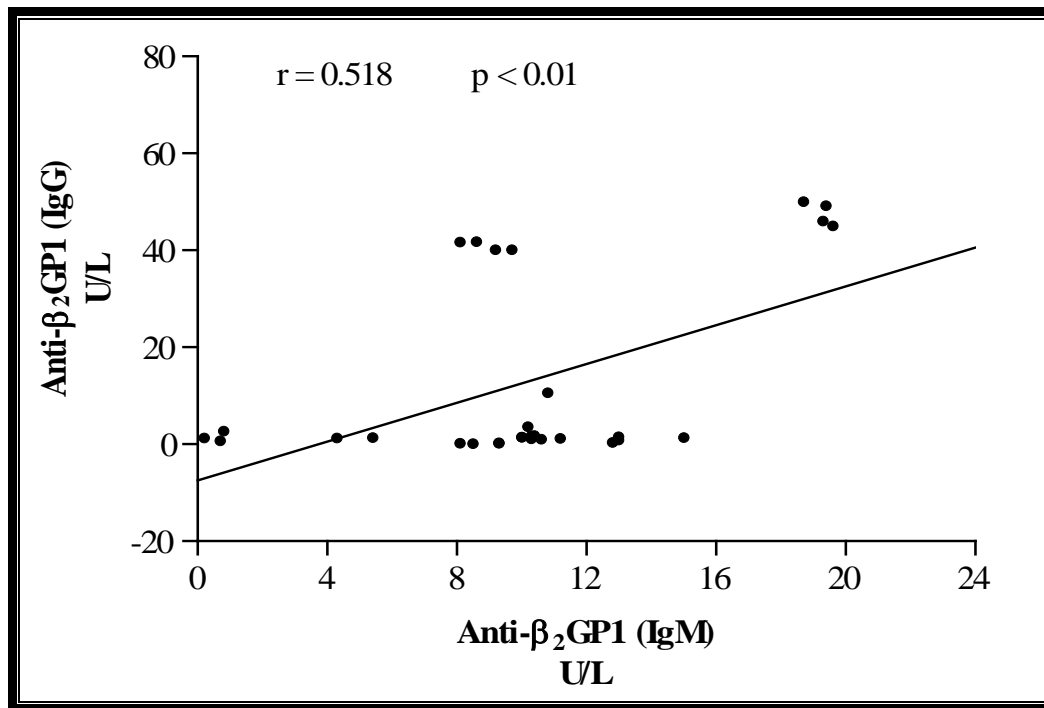$r = 0.518$ $p < 0.01$

Figure (1): Correlation between IgM and IgG isotypes of Anti – β2-GPI in patients

Discussion

In the present study the mean age of patients at onset of diagnosis of SLE was found to be 15.9. However Fukumi et al; 2000 reported an increase in the mean age of onset of SLE to 10.4 years.

In the present study 10/15 (66.7%) of our patients were female (table 1). This is in agreement with Tincani et al; 2003 who reported predominance of female sex in patients with systemic lupus erythematosus.

Regarding laboratory parameters in the present study normochromic normocytic anemia Leucopenia and thrombocytopenia were found in our patients compared to control. These results were in accordance with Fukumi et al; 2000 and Spiegel et al; 2000 who attributed these findings to decreased production of erythropoietin, bone marrow hypoactivity, red cell destruction by active reticuloendothelial system and decrease iron release from tissue to the plasma.

In the current study prolonged prothrombin time (PT), decreased prothrombin concentration (PC) and elevated liver enzymes were reported in all patient compared to controls, these results were comparable to Shin; 2000 who attributed similar findings to the presence of inhibitors of prothrombin activator complex called (PAC), while rise in the level of AST and ALT might be due to autoimmune hepatitis.

In the present study, increased erythrocytes sedimentation rate (ESR) and positive C-reactive protein (CRP) were reported in all patients compared to controls. These findings were similalr to Cassidy; 2003 and Klemp; 2000, who reported that ESR & CRP are usually elevated in SLE and significantly decreased in remission stage.

In the present study 3 out of 15 (20%) and 12 out of 15 (80%) of our patients were positive for RF & ANA respectively (table2). These findings are in agreement with Michael; 2008, who reported positive rheumatoid factor in 21% of patients with SLE, in addition he stated that antinuclear antibodies is an excellent screening test for lupus and is almost always positive in high titre but positive test alone do not confirm the diagnosis of lupus.

In the current study 5 out of 15 (33.3%) of patients with systemic lupus erythematosus showed elevated titre of ACL (table 4). This is in agreement with the finding of Hasegawa and Fuller; 2009 who reported that ACL is positive in 22.8% of patients with SLE. The results of the present study may be explained by Fort; 2000 who stated that a significant relation was found between Anticardiolipin antibodies level and type of treatment in patients with SLE as all patients on steroid therapy have normal anticardiolipin antibodies level (ACL) when compared to NSAID who showed high anticardiolipin level in 60% of patients.

In the present study, both anticardiolipin and anti-β_2 GPI were elevated in SLE with no definite relation to clinical manifestations. Campos et al; 2003 reported that the presence of anticardiolipin in the studied children and adolescents with SLE fluctuated during the course of illness with no association between clinical manifestations and either ACL or anti B_2GPI.

Cucurull et al; 2005 reported that antibodies against anti-B_2GPI is present in the absence of ACL in patients with SLE, they also reported that the assay of anti-B_2 GPI may improve the specificity for anti-phospholipid syndrome on follow up of the patients positive to anti-B_2-GPI.

In contrary to our results Hasegawa and Fuller; 2009 reported that patient's positive to IgG or IgM ACL were significantly more likely to have haemolytic anaemia or a positive serologic test for syphilis and less likely to have Raynaud's phenomenon. However, no associations were found between ACL positivity and thrombocytopenia, seizures, renal insuffency presence of positive antinuclear antibodies or rheumatoid factor, subcutaneous nodules or digital ulcers. However a significant positive correlation was found between the elevation of anti-B_2 GPI (IgG) and thrombocytopenia.

Chang et al; 2001 reported that autoimmune haemolytic anaemia occurring in SLE frequently associated with the concomitant presence of thrombocytopenia (Evans syndrome) and with the presence of ACL and anti-B_2 GPI.

In our stydy we found that both IgM and IgG isotypes of anti-B_2 GPI were positive in 20%

& 26.6% of patients with SLE respectively table (3). This is in agreement with Jimene et al; 2008 who reported prevelance of IgM and IgG anti-B$_2$ GPI isotypes among 27% & 25% of SLE respectively.

In the present study the mean leveles of both IgG and IgM isotypes of anti-B$_2$GPI were found to be significantely increased in patients with SLE in comparison to controls (P < 0.001) table(4).

In the present study a significant positive correlation was found between IgM and IgG isotypes of anti-B$_2$ GPI and with their corresponding class of anticardiolipin SLE. This is in agreement with Voss; 2001 who stated that patients of systemic lupus erythematosus with thrombocytopenia or thrombotic events had positive IgG isotype of anticardiolin in 29 & 13% respectively while IgM isotype of anticardiolipin was 38 & 58% respectively. He also reported that the presence of anti-β_2 glycoprotein I isotypes (IgM & IgG) always found in association with corresponding class of anticardiolipin as anti-β_2 glycoprotein. IgG isotype was present in 15 & 14% of SLE patients with thrombocytopenia or thrombotic events respectively while anti-β_2 GPI IgM isotype was present in 8 & 10% respectively. There is also high association of IgM isotype of anti-β_2 GPI with thrombocytopenia while IgG isotype of anti-β_2 glycoprotein I with arterial/venous thrombosis. Tsutsumi et al; 2006 reported that 24.5% of patients with SLE without manifestations of antiphospholipid syndrome had a positive titre of ACL, but only 4.1% of them had positive anti-B$_2$GPI.

Cuchacovich et al; 2001 reported that IgG was the most prevelant for both ACL anti-B$_2$GPI (16% and 6% respectively) in patients with SLE. This study supports our findings where IgG isotypes for ACL and anti-β_2 GPI were positive in 20% & 26.6% of SLE patients respectively.

 In Conclusions: A significant positive correlation was found between IgM and IgG isotypes of Anti β_2 glycoprotein-1 and with their corresponding class of anticardiolipin SLE patients. A significant positive correlation was found between elevated anti-B$_2$ GPI (IgG) levels and thrombocytopenia in SLE.

Recommendations: Both IgG and IgM isotypes of anti-β_2 glycoprotein and ACL should be used as a serodiagnonstic immunological marker in SLE patients

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