

**Antiphospholipid Syndrome: Determination of Antibodies to
Beta 2 Glycoprotein I (anti-b2GPI) by ELISA**

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Introduction:

Antiphospholipid antibodies are a heterogeneous group of immunoglobulins (autoantibodies) with specificity toward negatively-charged phospholipids, including cardiolipin (diphosphatidylglycerol), phosphatidylserine and phospholipid-protein complexes. The association of elevated serum levels of “autoimmune” antiphospholipid antibodies with recurrent venous or arterial thrombotic events, thrombocytopenia and fetal loss (antiphospholipid syndrome) has been well established and is now widely recognized (1).

These antiphospholipid antibodies frequently require the presence of a serum protein as cofactor for optimal *in vitro* binding activity. Cofactors show a high binding affinity for phospholipids forming phospholipid-protein complexes. The most studied cofactor is beta 2 glycoprotein I (B2GPI), a natural anticoagulant protein found in normal serum (2). Experimental evidence suggests that “autoimmune” antiphospholipid antibodies, may in fact, recognize a newly formed antigenic site located on the cofactor molecule (i.e. B2GPI) when bound to phospholipids (3). Continuing research on the role of protein cofactors has increased our understanding of the heterogeneous nature of antiphospholipid antibodies and their possible *in vivo* pathogenic role in thrombosis (4).

It has been shown that the attachment of purified B2GPI to certain plastic surfaces of microwell plates (in the absence of phospholipids) can be used to detect antibodies to this cofactor. The binding of B2GPI to the plastic surface would produce antigenic sites similar to those produced when bound to phospholipids (3). Anti-B2GPI antibodies detected by these systems are thought to be more specific for thrombosis than antibodies detected by classic antiphospholipid ELISAs (phospholipids bound to plastic, with exogenous source of cofactor, i.e. anti-cardiolipin and anti-phosphatidylserine assays) (5,6). Testing for anti-B2GPI antibodies in the clinical laboratory by ELISA is becoming increasingly valuable and provides additional clinically relevant results to assess patients for the antiphospholipid syndrome and/or the risk of thrombosis.

A new ELISA test kit for the semi-quantitative determination of antibodies specific for human B2GPI in serum has been developed by Corgenix, Inc. The technical and clinical performance of the assay are discussed below.

Material and methods: (Assay description)

Corgenix's anti-B2GPI test kit is performed as an indirect ELISA for the detection of IgG, IgM or IgA antibody isotypes in human serum. One hundred microliters of diluted (1:50) patient samples, calibrator and control sera are incubated in 96-well microplates coated with purified human B2GPI (purity > 95% SDS-PAGE). B2GPI is stabilized and non-coated areas are blocked to prevent non-specific binding. Incubation at room temperature for 15 minutes allows anti-B2GPI antibodies present in the samples to react with the immobilized antigen. After the removal of unbound serum proteins by washing with PBS-Tween-20 solution, antibodies specific for human IgG, IgM or IgA, labeled with horseradish peroxidase (HRP), are added to form complexes with the human antibodies bound to B2GPI. Following another washing step, a single solution containing tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂) is added as a chromogenic substrate. The bound enzyme-antibody conjugate develops a colored signal proportional to the serum concentration (activity) of anti-B2GPI antibodies in the sample. Color development is stopped with the addition of 0.36N sulfuric acid solution.

Results are obtained by reading the O.D. (optical density or absorbance) at 450nm of each well in a spectrophotometer. Calibrator and control sera are provided, with the anti-B2GPI antibody concentrations expressed in units for IgG, IgM and IgA. The O.D. values of the samples are multiplied by the conversion factor to obtain anti-B2GPI antibody concentrations in units. These units are traceable to available reference preparations.

The kit contains sufficient materials and reagents for 96 determinations including a calibrator, positive and negative control for IgG, IgM or IgA anti-B2GPI antibodies. The assay features microplates with breakaway wells and color-coded liquid reagents for user convenience. Total incubation time is 40 minutes at room temperature (15', 15', 10').

Technical performance :Normal range

120 serum samples from healthy blood bank donors were tested for anti-B2GPI antibodies to confirm the cut-off of the assay which was established at 20 units for all three antibody isotypes. The specificity of the assay was 100% for IgG, 93% for IgM and 96% for IgA. The assay measures antibody activity up to 200 units. Samples with anti-B2GPI values greater than 200 units should be further diluted and re-tested for more accurate measurement.

Precision

Three selected serum samples with known anti-B2GPI unit values (one low, one moderate, and one high) were assayed in 23 replicates on three different occasions. The mean intra-assay and inter-assay coefficient of variation (%CVs) of the assay are shown below in Table #1:

Clinical performance :

When serum samples from 120 healthy blood donors were assayed for the presence of anti-B2GPI antibodies, a mean value of 2.1 +/- 1.1 G units was obtained for IgG; 7.7 +/- 9.5 M units for IgM; and 6.9 +/-5.7 A units for IgA antibodies (mean +/- 1 standard deviation).

Sensitivity for SLE

Serum samples from 40 consecutive (unselected) patients with SLE were tested for anti-B2GPI antibodies. Nine of the samples (sensitivity of 22.5%) were positive for IgG with a mean value of 24.5 +/- 49.6 G units; 8 samples (sensitivity of 20%) were positive for IgM with a mean value of 13.9 +/- 18.1 M units; and 10 samples (sensitivity of 25%) for IgA with a mean value of 42.6 +/- 72.5 A units. Anti-B2GPI antibody levels of this SLE population were statistically different when compared to the healthy controls (single factor ANOVA, $p < 0.001$ for all 3 isotypes). The value distribution for IgG anti-B2GPI antibodies is summarized in Graph # 1.

A good positive correlation was found between IgG anti-B2GPI with both IgG anti-phosphatidylserine ($r = 0.928$) and IgG anti-cardiolipin ($r = 0.864$) antibody levels in this group

of patients. IgM and IgA anti-B2GPI antibodies showed a lower degree of correlation than IgG ($r =$ from 0.4 to 0.5).

Sensitivity for thrombosis in SLE

Serum samples from 12 selected female patients with SLE who had a clinical history of thrombosis were evaluated for anti-B2GPI antibodies. Seven of the samples (sensitivity of 58%) were positive for IgG with a mean value of 69 +/- 66.2 G units; 5 samples (sensitivity of 42%) were positive for IgM with a mean value of 24.2 +/- 25.8 M units; and 8 samples (sensitivity of 67%) were positive for IgA with a mean value of 105.6 +/- 97.5 A units for this group of selected SLE patients.

Many of these SLE patients also had a history of thrombocytopenia, and when their obstetric history was reviewed, there were 24 fetal losses out of 46 pregnancies recorded (55% abortion rate). The clinical history, along with anti-B2GPI results, indicate that these patients showed the clinical manifestation of the antiphospholipid syndrome.

Serum samples from 6 selected female patients with SLE who had a history of thrombocytopenia (no thrombosis) were tested for anti-B2GPI antibodies. Only one sample (17%) tested weak positive for IgG with a mean value of 12.7 +/- 12.2 G units; none tested positive for IgM with a mean value of 6 +/- 6.5 M units; and none tested positive for IgA with a mean value of 11.1 +/- 5 A units for this group. No abortions out of 11 pregnancies were recorded in this group.

Serum samples from 10 selected female patients with SLE who were known not to have had thrombotic episodes, nor any other feature of the antiphospholipid syndrome (disease control), were tested for anti-B2GPI antibodies. Two of the samples (20%) were positive for IgG with a mean value of 8.5 +/- 13.5 G units; one sample (11%) was positive for IgM with a mean value of 9.2 +/- 12.1 M units; and one sample (11%) was positive for IgA with a mean value of 22.2 +/- 49.4 A units. One sample in this group was moderate to strong positive for all

immunoglobulin isotypes and was the only patient that had an abortion of 18 pregnancies recorded.

Mean anti-B2GPI antibody levels from SLE patients with history of thrombosis were significantly higher than the disease control groups : single factor ANOVA, $p < 0.002$ for IgG; $p = 0.038$ for IgM and $p = 0.005$ for IgA. The value distribution for IgG anti-B2GPI antibodies is summarized in Graph # 2.

Discussion:

Our results support previous reports that purified human B2GPI bound to microplates forms an epitope similar to that produced when B2GPI binds to phospholipids. Since most of the clinically relevant antiphospholipid antibodies have specificity toward the newly formed epitope, the laboratory determination and measurement of anti-B2GPI antibodies provided more specific information for thrombosis (antiphospholipid syndrome) in SLE patients.

Compared to the healthy controls, our results show significantly higher anti-B2GPI antibody levels in SLE patients where elevated levels of antiphospholipid antibodies are most likely to be found. As previously described for anti-cardiolipin and anti-phosphatidylserine antibodies (1), some healthy individuals may present elevated serum levels of anti-B2GPI antibodies (mostly IgM and IgA), however, the exact clinical significance of these antibodies is not yet well understood. They may be relevant as risk factors for the development of thrombosis (antiphospholipid syndrome). The clinical sensitivity of anti-B2GPI antibodies in our unselected SLE population (20 to 25%) is in agreement with previous studies and with the expected prevalence of other antiphospholipid antibodies in SLE patients (1,2,6). Our results with selected SLE patients show a strong association between elevated serum levels of anti-B2GPI antibodies with the history of thrombosis, thrombocytopenia and fetal loss (antiphospholipid syndrome).

The laboratory determination of anti-B2GPI antibodies offers the following advantages: 1) anti-B2GPI testing provides additional relevant information, along with classic antiphospholipid

assays for assessing the antiphospholipid syndrome; 2) because phospholipids are not used in this assay system, it is expected to improve the inter-lot and inter-lab variability which has been observed with classic antiphospholipid assays; 3) significantly reduce or eliminate the number of false positive results with antiphospholipid antibodies not associated with thrombosis which are occasionally produced by infections, i.e. syphilis; and 4) the use of purified human B2GPI favors the detection of those “autoimmune” antibodies (increased sensitivity) that require this protein for optimal binding and are considered pathogenic for thrombosis.

References:

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Table # 1 : Summary of anti-B2GPI assay precision

Serum Sample	Unit range	Intra-assay Precision			Inter-assay Precision		
		IgG (%CV)	IgM (%CV)	IgA (%CV)	IgG (%CV)	IgM (%CV)	IgA (%CV)
High +	>90	3.4	2.4	3.5	1.7	3.4	3.7
Mod. +	60-70	4.7	3.5	4.4	3.5	4.5	3.7
Low +	<50	4.3	3.8	4.7	4.0	6.1	4.7

Graph captions:

Graph # 1 : Distribution of IgG anti-B2GPI antibodies in healthy controls and SLE patients measured by Corgenix ELISA test kit (*broken line* = normal cut-off).

Graph # 2 : Distribution of IgG anti-B2GPI antibodies in selected SLE patients with history of : group I - thrombosis; group II - thrombocytopenia; and group III – control, measured by Corgenix ELISA test kit (*broken line* = normal cut-off).



