Avidity of anticardiolipin antibodies—A factor that could be important for their detection by ELISA methods

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Abstract

Avidity is an important feature of antibodies associated with their pathogenic effects. Anticardiolipin antibodies (aCLs) are the most commonly examined antiphospholipid antibodies, however, their avidity has been investigated only marginally. The aim of the study was to compare the avidity of the antibodies specifically bound to the cardiolipin-coated microtiter wells with those bound to the wells bearing no cardiolipin in various conditions. We analysed 22 serum samples with high, medium and low levels of aCL IgG. The avidity of aCL IgG was determined in serially diluted sera by the ELISA method in the presence of increasing urea concentrations (from 2 to 8 mol/L) as a chaotropic agent. The serum dilution 1:50 and 1:100 and the concentrations of urea 6 mol/L and 8 mol/L seemed to be suitable for the determination of aCL avidity. The avidity of antibodies specifically bound to the cardiolipin and those bound to the cardiolipin-free wells significantly differed in their avidity. The simultaneous determination of higher-avidity aCL and lower-avidity polyspecific antibodies, whose presence complicates the ELISA methods by an increase of the background signal, might limit the shortcomings of some ELISA methods.

Introduction

Antiphospholipid antibodies (aPLs) include a heterogeneous population of various types of antibodies directed against different phospholipids and protein cofactors. The examination of aPL is necessary for the diagnosis of the antiphospholipid syndrome (APS). Laboratory criteria comprise the determination of anticardiolipin (aCL) and anti-ß2-glycoprotein I (anti-ß2-GPI) autoantibody levels by enzyme-linked immunosorbent assays (ELISAs) and coagulation tests for the detection of lupus anticoagulant presence (Miyakis et al., 2006; Pengo et al., 2012). In addition to the levels of aPL, the determination of their avidity could be a valuable complementary characteristic of these autoantibodies. The avidity (or functional affinity) is an important feature of an antibody related to its specificity (Pohanka, 2009). It is expressed as the strength of interactions between an antigen and a corresponding antibody which is independent of mechanisms controlling antibody levels (Steward et al., 1979). The avidity of autoantibodies might play a critical role in certain organ-specific autoantibody-associated diseases (Gharavi and Reiber, 1996).
It seems that patients with APS synthesise aPL with higher avidity (Vlachoyiannopoulos et al., 1998; Cucnik et al., 2005). The risk for thrombosis especially correlated with the avidity of anti-β2-GPI antibodies (de Laat et al., 2006; Cucnik et al., 2011, 2012). Cucnik et al. (2005) assume that avidity of anti-β2-GPI antibodies may be a more reliable laboratory feature than aPL titre for the evaluation of long-term thrombotic risk.

In general, the antigen-binding capacity of different antibodies can range from high affinity, primarily monoreactive ones, to low affinity, broadly polyreactive immunoglobulins (Notkins, 2004). Polyreactivity is defined as the ability of the immunoglobulin molecule to bind a large spectrum of structurally unrelated antigens enabled by the flexibility of the antigen-binding pocket (Notkins, 2004; Dimitrov et al., 2008). Polyreactive antibodies are supposed to play a role in the natural defence and may protect against autoimmune diseases by contributing to immunological tolerance. It is known that ≤20% of B lymphocytes in peripheral blood produce polyreactive antibodies which represent an important portion of the immunoglobulin repertoire in normal individuals (Notkins, 2004).

The presence of low-avidity polyreactive antibodies in serum is supposed to constitute difficulties in the immuno-sorbent assays (Rupin et al., 1991; Szabo et al., 2010). The non-specific immunoglobulins can adhere to the surface of blocked microtitre wells and cause the increase of background binding in the wells not coated with antigen (Rupin et al., 1991; Xiao and Isaacs, 2012). Some studies reported significant binding of low-avidity antibodies to the microtitre wells not bearing any specific antigens (Rupin et al., 1991; Pellegrino and Caccavo, 2007; Szabo et al., 2010). This phenomenon may contribute to a considerable variability in the aPL results based on different ELISA assays kits (Wong et al., 2004).

The avidity of aCL seems to be an important factor that may influence the clinical manifestation of APS, but may also have an impact on the aCL determination by ELISA. Therefore, the aim of this study was (a) to compare the avidity of the specifically bound IgG antibodies to the cardiolipin (CL) with those bound to the wells bearing no cardiolipin; (b) to test the optimal conditions for the determination of aCL avidity by the ELISA method. The avidity of antibodies can be determined by the use of various chaotropic agents during the antibody binding in the ELISAs. Ammonium thiocyante, sodium chloride, guanidine hydrochloride or diethylamine facilitate the dissociation of immune complexes when they are applied in the process of the ELISA method as an extra step (Cucnik et al., 2011; Dauner et al., 2012; Almanzar et al., 2013). Urea represents another agent that acts as a mild denaturant capable of disrupting immune complexes (Vlachoyiannopoulos et al., 1998; Liaskos et al., 2005; Fialova et al., 2011, 2012). Previous studies described the use of urea as a chaotropic agent for the determination of aCL avidity. That is why we also applied urea for the dissociation of immune complexes in our experiments.

**Participants and methods**

**Participants**

We analysed 22 serum samples from patients (age: median, interquartile range 36, 31–40 years; sex: female 20, males 2) carried out at the Immunological Department of the Institute of Medical Biochemistry and Laboratory Diagnostics in Prague (Czech Republic). The following patients were included in our study: 13 sera from women with a history of obstetric disorders (predominantly infertility), four sera from patients with systemic autoimmune diseases (two with systemic lupus erythematosus, one with Sjögren’s syndrome, one with mixed connective tissue disease), three sera from patients with immunodeficiencies, one patient with coagulation disorder, and one serum from a patient with glomerulonephritis. APL IgG screening (ELISA Anti-phospholipid Screen IgG/IgM, Orgentec, Mainz, Germany) and aCL IgG levels (ELISA Anti-cardiolipin IgG/IgM, Orgentec, Mainz, Germany) had been determined in the sera as a part of the immunological follow-up of patients. aCL IgG levels were higher than 10 GPL in nine patients, the other patients had levels below 10 GPL in the screening of the antiphospholipid antibodies IgG. Basic clinical and laboratory data of the participants are shown in Table 1.

All subjects gave written informed consent regarding study participation. The Ethics Committee of the First Faculty of Medicine, Charles University, Prague approved the study.

**Methods**

IgG aCL were determined by ELISA according to a protocol for validated assay (Pierangeli and Harris, 2008) which is in fact a method to detect antカードiolipin antibodies dependent on β2-GPI being more specific for antiphospholipid syndrome. This ELISA procedure meets the requirements recommended by international consensus guidelines on anticardiolipin and anti-β2-glycoprotein I testing (Lakos et al., 2012). Use of 10% adult bovine serum (ABS) in phosphate buffered saline (PBS) as a blocking solution and to dilute patient samples appears to provide sufficient β2-GPI for a valid test.

At the beginning, we analysed sera serially diluted with 10% ABS in PBS (pH 7.2) 1:150, 1:100, 1:200, 1:400. In the extra step of the ELISA method (see below), the avidity of antibodies in each appropriately diluted serum was tested using increasing concentrations of urea solutions (2 mol/L, 4 mol/L, 6 mol/L and 8 mol/L). Our preliminary experiments showed that 6 mol/L and 8 mol/L urea solutions disrupted the interactions in immune complexes bound to the wells more effectively than those with the lower concentrations of urea. Some absorbances obtained from the more diluted sera (1:200 or 1:400) were too low and the results were less accurate. After the evaluation of the initial experiments, we continued with the sera diluted only 1:50 and 1:100 and the avidity was determined in the presence of urea 6 mol/L and 8 mol/L.

**Determination of aCL IgG avidity**

The avidity of IgG aCL was performed by the procedure described by Vlachoyiannopoulos et al. (1998) with our minor modifications. In brief, bovine cardiolipin (diphosphatidylglycerol) (Sigma-Aldrich Co., St. Louis, MO, USA) was used as the antigen for coating the microplate wells. One half of the individual wells of microtiter strips (Polyisorp, NUNC, Roskilde, Denmark) was coated with 50 μL of bovine cardiolipin solution in absolute ethanol (50 μg/mL), the second half of individual wells was coated only with 50 μL of ethanol (cardiolipin-free wells). The microtiter strips were allowed to incubate
overnight at 4 °C. After incubation, the wells were washed by PBS. Both wells coated with cardiolipin and the uncoated ones were blocked with 150 μL of 10% ABS in PBS for 1 h at room temperature and then the wells were washed. 100 μL of every appropriately diluted serum were added to two cardiolipin-coated wells as well as to two cardiolipin-free wells and incubated for 1 h at room temperature. After washings, 100 μL of urea solutions (2 mol/L, 4 mol/L, 6 mol/L or 8 mol/L) were added to the wells in which the avidity was examined and 100 μL of PBS were added to the other wells. After a 10-min incubation period at room temperature, the wells were washed. In the next step, 100 μL of horseradish peroxidase-conjugated goat anti-human IgG (SouthernBiotech, Birmingham, USA), diluted 1:5000 in 10% ABS in PBS, was added to all wells and the plates were washed. Colour was developed by adding 100 μL of substrate (tetramethylbenzidine with H2O2) (TEST-LINE, Brno, Czech Republic) for 20 min at room temperature. The enzymatic reaction was stopped by adding 2 mol/L H2SO4. The absorbance of each well was read at 450 nm using a microplate reader (LabSystems, Finland). All samples were analysed in duplicates.

Calculation of the avidity index
The avidity was expressed as the avidity index (AI) which represents the ratio of the residual autoantibodies bound in the wells in the presence of the urea solution to the total auto-antibodies bound in the absence of urea. It was calculated by dividing absorbance values obtained from the avidity ELISA assay (with urea addition) by absorbance values obtained using the standard ELISA method (without urea). The AI between 0.4 and 0.6 (40–60%) used to be classified as a moderate avidity and >0.6 (60%) as a high avidity (Almanzar et al., 2013).

Statistics
The Wilcoxon signed-rank test was used for the statistical analysis of differences in the paired measurements of aCL avidity in serum. The Spearman’s correlation coefficient was used for the study of the relationship between aCL IgG levels and their avidity. The significance level for all tests was 2α = 0.05. Statistical analysis was performed using MedCalc software (Ostend, Belgium).

Results
The results from the preliminary experiments of serially diluted sera in the condition of increasing urea concentration are shown in Fig. 1 which illustrates the comparison of antibodies bound to the wells coated with cardiolipin and cardiolipin-free ones in the individual patients with different aCL IgG levels. The avidities of antibodies bound to the cardiolipin-free wells were lower than those bound to the wells bearing cardiolipin. Similar patterns were seen when the sera were diluted 1:100, 1:200 and 1:400 (not shown).

Next, sera were analysed only in dilutions 1:50 and 1:100 using the urea concentrations of 6 mol/L and 8 mol/L. A statistical evaluation of all analysed sera confirmed that the specific aCL IgG antibodies were characterised by significantly higher avidities than the non-specific antibodies bound to the cardiolipin-free wells. The differences in the avidities were expressed for both sera diluted 1:50 and 1:100 after exposure of urea 6 mol/L or 8 mol/L (urea 6 mol/L: sera diluted 1:50, p < 0.005, sera diluted 1:100, p < 0.01; urea 8 mol/L: sera diluted 1:50, p = 0.0005, sera diluted 1:100, p = 0.014) (Fig. 2). The greatest difference was seen for the sera diluted 1:50 in the presence of urea 8 mol/L. Although the statistically significant

### Table 1 – Basic clinical and laboratory data of participants.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age</th>
<th>aCL IgG (GPL)</th>
<th>APL screen IgG</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>n.d.</td>
<td>2.6</td>
</tr>
<tr>
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<td>n.d.</td>
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<tr>
<td>3</td>
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<td>n.d.</td>
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<tr>
<td>4</td>
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<td>Female</td>
<td>35</td>
<td>n.d.</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>Obstetric disorder</td>
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<td>38</td>
<td>n.d.</td>
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</tr>
<tr>
<td>6</td>
<td>Obstetric disorder</td>
<td>Female</td>
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<td>n.d.</td>
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<tr>
<td>7</td>
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<td>n.d.</td>
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<tr>
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<td>n.d.</td>
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<tr>
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<td>n.d.</td>
<td>2.0</td>
</tr>
<tr>
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<td>SLE</td>
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</tr>
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<tr>
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<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<td>n.d.</td>
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<tr>
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<td>n.d.</td>
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</tr>
<tr>
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<td>Obstetric disorder</td>
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<td>31</td>
<td>n.d.</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Abbreviations: MCTD, mixed connective tissue disease; n.d., not done; SLE, systemic lupus erythematosus.
The concentration of urea especially affected the dissociation of immune complexes formed by non-specific antibodies. The more concentrated urea (8 mol/L) disrupted the immune complexes formed by non-specific antibodies significantly more easily than urea 6 mol/L (sera diluted 1:50, \( p < 0.01 \); sera diluted 1:100, \( p = 0.003 \)). The dissociation of immune complexes containing specific aCL IgG was also higher in the presence of urea concentration 8 mol/L but without a statistical significance (Fig. 3).

The dilution of sera also influenced the avidity index of aCL IgG. The dissociation of immune complexes both with aCL and polyreactive antibodies increased when sera were diluted 1:100 in comparison with those diluted 1:50 after exposure to urea 6 mol/L or 8 mol/L with the exception of the immune complexes formed by non-specific polyreactive antibodies in the presence of urea concentration 6 mol/L (cardiolipin wells: urea 6 mol/L, \( p = 0.03 \); urea 8 mol/L, \( p = 0.03 \); cardiolipin-free wells: urea 8 mol/L, \( p = 0.03 \); urea 6 mol/L, n.s.) (Fig. 4).

We also calculated the ratio of cardiolipin-coated well absorbances and cardiolipin-free ones. We did not observe any differences regardless of urea concentration or serum dilution (urea 6 mol/L vs. urea 8 mol/L, n.s.; serum 1:50 vs. serum 1:100, n.s.).

We correlated aCL IgG levels in the patients with positive values (\( \geq 10 \) GPL) and their avidity determined in various modifications described above. No correlation was observed with the exception when 8 mol/L concentration urea and sera diluted 1:100 were used (\( r = -0.78 \); \( p = 0.02 \)). With respect to the small number of patients (\( n = 9 \)), no conclusion should be drawn.

**Discussion**

We observed that the avidity of antibodies specifically bound to the ELISA wells coated with cardiolipin was significantly higher than those bound to the wells bearing no cardiolipin. Recent opinions supposed that the bindings of antibodies to the wells without specific antigens are attributed to the presence of polyreactive antibodies which are capable of reacting with multiple unrelated epitopes with low avidity (Szabo et al., 2010). Our findings agree with the results of Szabo et al. (2010). They tested the avidity of antibodies present in intravenous immunoglobulin (IVIG) preparations in the condition of the increasing concentration of chaotropic salt ammonium thiocyanate. The antibodies against β-amyloid oligomers had a greater avidity than those bound to the wells without specific antigen. In addition to the results of Szabo et al. (2010), we demonstrated that even sera of individual patients contained the antibodies reacting with specific antigens with a higher avidity while those bound to the wells without specific antigen were characterised by a lower avidity. The findings of Szabo et al. (2010) and those in the present study indicate that differences between avidity of the antibodies bound to the wells coated with the specific antigen and those non-specifically bound to the specific antigen-free wells may be a common phenomenon for the autoantibodies against various antigens.

Some procedures for the various autoantibody determination by ELISA methods include the recommendation to

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**Fig. 1** – (A–C) The avidity of specific aCL IgG antibodies bound to the cardiolipin-coated wells (solid lines and solid rhomb) and those bound to the cardiolipin-free wells (dashed lines and solid square) in three individual patients with different levels of aCL IgG (A serum with aCL IgG >100 GPL; B serum with aCL IgG 29 GPL and C serum with aCL 16 GPL). The serum of the same patient was analysed in four different dilutions (1:50, 1:100, 1:200 and 1:400; only dilution 1:50 is shown). The avidities of specific aCL IgG were higher than those of the non-specific antibodies bound to cardiolipin-free wells regardless of the concentration of urea and dilution of sera. **Abbreviations:** aCL, anticardiolipin antibodies; GPL, international standard units for IgG antibodies.
Fig. 2 – The comparison of the anticardiolipin antibody IgG avidities (solid lines) and non-specific antibodies (dashed lines) in sera using urea of 6 mol/L or 8 mol/L. Two different dilutions of sera were examined (1:50 and 1:100). The avidities of specific anticardiolipin antibodies were significantly higher than those bound to the cardiolipin-free wells independently of serum dilution and concentration of urea. The central box represents the values from the lower to upper quartile (25–75 percentile). The median is shown as the middle line. The vertical line extends from the minimum to the maximum value, excluding outside and far out values which are displayed as separate points. Abbreviations: CL, cardiolipin; M, mol/L.

Fig. 3 – The influence of urea concentration on the avidity indices of the specific anticardiolipin antibodies IgG and those bound non-specifically to the cardiolipin-free wells. The immune complexes formed by non-specific antibodies were significantly more intensively dissociated in the presence of urea concentration 8 mol/L than 6 mol/L. The central box represents the values from the lower to upper quartile (25–75 percentile). The median is shown as the middle line. The vertical line extends from the minimum to the maximum value, excluding outside and far out values which are displayed as separate points. Abbreviations: CL, cardiolipin; M, mol/L.
subtract the absorbances obtained in the specific antigen-free wells from those in the specific antigen coated wells (Rupin et al., 1991; Manfredini et al., 1995). Rupin et al. (1991) demonstrated that non-specific bindings to CL-free wells varied considerably with the IgG levels. However, it is supposed that binding to CL-free wells may at least in part reflect the presence of polyvalent antibodies that are capable of binding to both wells bearing no specific antigen and those coated with the certain antigen (Szabo et al., 2010). This assumption was corroborated by the experiment in which the passage of IVIG over polystyrene attempting to reduce background bindings yielded also in the significant loss of specific antibodies (Szabo et al., 2010). Therefore, it seems that a simple subtraction of the specific antigen-free well absorbance from those of the wells coated with antigen is not quite correct. The presence of low avidity non-specifically bound antibodies may influence the results of aCL levels determined by ELISA methods. Our findings confirmed that polyreactive antibodies are characterised by lower avidity in comparison with specific aCL. The presence of higher levels of low-avidity polyreactive antibodies may result in the false-positivity of aCL. This suggests that simultaneous measurement of levels as well as avidity of aCL may contribute to the explanation of some dubious results.

The determination of the immunoglobulin avidity by urea depended strongly on the concentration of antibodies (Dimitrov et al., 2011). At high levels of immunoglobulins, the avidity assays might be less sensitive to moderate decreases in the binding. Likewise, aCL IgG immune complexes dissociated easier in the more diluted sera while a similar relationship between concentration of antibodies and avidity index for the polyreactive antibodies was expressed only after exposure to a higher concentration of urea.

A reliable estimation of antibody avidity requires a 50% decrease in the antibody binding (Pullen et al., 1986). In our experiment, urea concentrations 6 mol/L or 8 mol/L resulted in the sufficient dissociation of immune complexes. However, urea concentration 8 mol/L seems to be more suitable for the discrimination between avidity of specific aCL and non-specific antibodies. The avidity indices and the number of outliers were significantly lower for non-specific antibodies in the presence of urea concentration 8 mol/L. Similar concentrations of urea for the avidity determination have also been described in other studies (Flori et al., 2004; Matheus et al., 2005). Zachou et al. (2006), who investigated the avidity of aCL in liver diseases, applied the lower concentration of urea for the disruption of immune complexes. However, the ELISA procedure applied
by Zachou et al. (2006) differed in some details compared with our avidity assay.

Although aCL are the most commonly examined aPL, their avidity had been investigated only marginally (Liaskos et al., 2005; Vlachoyiannopoulos et al., 1998; Zachou et al., 2006). High-avidity aCLs, comparable to those in APS patients, were reported in the patients with primary biliary cirrhosis, primary sclerosing cholangitis and type 1 autoimmune hepatitis (Liaskos et al., 2005; Zachou et al., 2006). Our study was designed as a methodological one rather than a clinical one. The patients with positive aCL comprised a heterogeneous group so that an association between aCL avidity and symptoms of participants should not be evaluated.

We used ELISA method with the presence of chaotropic agents for avidity determination. It represents the common technique in the clinical laboratories for the evaluation of avidity of different polyclonal antibodies in patients’ sera. The most accurate procedure is very sophisticated physical method of surface plasmon resonance (SPR). It analyses the biospecific interaction between epitopes and paratopes in real-time and without the use of labels and permits simultaneous measurement of antibody quantity and avidity. However, this technique is an expensive and its availability is limited.

Many attempts have been focused on the search for more appropriate solid phases such as a hydrophobic polyvinylidene-difluoride (PVDF). The porous structure of the hydrophobic membrane may hide the large hydrophobic part of phospholipids consisting mainly of the fatty acids moiety. This can lead to a denser presentation of the hydrophilic moiety of phospholipids on the membrane surface which interacts with cofactor thereof or specific autoantibodies. It may facilitate the binding of medium and low affinity aPL (Roggenbuck et al., 2012).

**Conclusion**

We demonstrated that the avidity of antibodies specifically bound to the cardiolipin and those bound non-specifically to the antigen-free wells significantly differ in their avidity.

Although the present study has not been focused to the clinical evaluation of aCL avidity, our results could have a potential clinical utility in certain patients. The presence of low-avidity polyreactive antibodies in higher concentration may false-positively increase the values of aCL antibody levels. The simultaneous avidity determination of antibodies bound to the CL coated wells and those bound to the CL-free wells might contribute to the explanation of the possible discrepancy between symptoms of the patient and laboratory findings of aCL. This approach of aCL testing might limit the shortcomings of the ELISA method caused by the presence of non-specifically bound antibodies which increase the background signal.

However, clinical studies will be necessary to establish the interpretation modus of aCL concentration and avidity values.

**Conflict of interest**

None.

**Acknowledgments**

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**References**


