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## The outcome of ELISA for antiphosphatidylethanolamine antibodies is dependent on the composition of phosphatidylethanolamine

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### Abstract

**Objective**—The presence of circulating autoantibodies against phosphatidylethanolamine (PE) has been shown to be positively associated with symptoms of antiphospholipid syndromes (APS). However, the current ELISA-based tests for antiphosphatidylethanolamine (aPE) antibodies remain inconsistent and controversial. The term PE refers to a collection of phospholipids that have phosphorylethanolamine head group as a common structural feature, but can vary in fatty acids with diverse physicochemical properties. The present study was to investigate, using synthetic positionally symmetrical PE species as a model system, the impact of PE structural variations on aPE ELISA.

**Methods**—Single and combinations of synthetic PE species, including 16:0 (fatty acid length:degree of unsaturation), 18:0, 18:1, 20:4 and 22:6, were screened with ELISA using serum samples from aPE patients and compared with chicken egg PE. There were a total of 37 aPE patient serum samples, including 11 cofactor-independent IgM, 14 ABP-independent IgG and 12 ABP-dependent aPE serum samples (3 IgM, 8 IgG and 1 IgA). The ELISA conditions were investigated for different isotypes and cofactor dependence. Based on the initial screening, adjustments in phospholipid compositions were made for achieving optimal OD readings. Finally, we isolated total IgG from aPE sera to validate different antigenic preferences.

**Results**—The antigenic preference was different among immunoglobulin isotypes and between cofactor-dependent versus cofactor-independent aPE antibodies. More specifically, 18:1 PE was a preferred antigen for cofactor-dependent aPE, whereas 20:4 PE was the preferred antigen for cofactor-independent IgG aPE. In contrast, cofactor-independent IgM aPE appeared to have a

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general preference for a more complex PE combination with longer fatty acids and a higher degree of unsaturation.

**Conclusion**—The present data indicated that the outcome of aPE ELISA was dependent on the composition and physicochemical properties of PE antigens. The discovery that aPE antibodies may have different antigenic preferences could shed light on the nature of their binding interactions.

### Keywords

phosphatidylethanolamine; antiphosphatidylethanolamine antibodies; antiphospholipid syndrome

## Introduction

The term antiphospholipid syndrome (APS) refers to a collection of autoimmune disorders with thrombosis and/or recurrent pregnancy loss as main clinical manifestations (1–3). Clinically established tests for APS currently include anticardiolipin, anti- $\beta$ 2-glycoprotein I, and lupus anticoagulant (1–3). There are so called “non-criteria APS”, where antiphospholipid antibodies have clinical symptoms consistent with APS but fall outside current standard tests (4, 5). These autoantibodies, including antiphosphatidylethanolamine (aPE), are being actively pursued in order to establish a complete diagnostic panel for APS patients. In a number of clinical studies, aPE antibodies are shown to positively correlate with symptoms of APS (6–21). While different antiphospholipid autoantibodies can coexist in the same patient, aPE antibodies are sometimes the sole antibodies in patients with symptoms of APS, suggesting aPE may be an independent etiological entity (16, 17).

Enzyme-linked immunosorbent assays (ELISA) are widely practiced in the diagnosis for APS. However, there lacks standardized ELISA protocols for aPE (22). In the current aPE literature, various assay conditions have been documented from different laboratories. Variations in aPE ELISA conditions include the source of PE (from bovine brain, egg yolk, and bacteria), buffer compositions, blocking conditions and the presence of cofactors, where it has been shown that different assay conditions may have a significant effect on the outcome of ELISA tests (6, 23).

The physicochemical properties of the antigen are an important determinant in ELISA. In this respect, PE refers to a heterogeneous group of phospholipids that share phosphorylethanolamine head group as a common structural feature, but differ in fatty acid compositions (24). It has been well documented that the fatty acids of PE species can affect the surface topology, fluidity and structural organization of PE membranes (25–27). The fact that PE extracted from different natural sources produce variable ELISA readout is indicative of a potential impact of the physical composition of the antigen on the reactivity of aPE antibodies (28–30). Whether specific PE species may contribute to the interactions with aPE antibodies remain largely unexplored and undefined.

The goal of this study was to examine the impact of PE compositions on the performance of aPE ELISA using single and mixtures of synthetic PE species, based on a prior report which demonstrated the feasibility using a simple two-component system (31). The present study

involved positionally symmetrical PE species, with variable fatty acid length and the degree of unsaturation. Our data indicated that the ELISA reactivity of aPE is dependent on the composition of PE species and there is a preference of specific PE species based on the antibody isotype and cofactor dependence.

## Methods

### Patient sera

The study was approved by the Northwestern University Institutional Review Board and was conducted under NIH guidelines. The patient serum samples were kind gifts from Dawn R. Wagenknecht, M.S., and Nathalie Bardin, M.D., and the selected samples were positive for aPE antibodies when tested by the HLA-Vascular Biology Laboratory at Franciscan St. Francis Health, Indianapolis and Service d'Immunologie du Pr. Mège, Hôpital de la Conception, Marseille, France, respectively. There were a total of 37 patient serum samples included in this paper, which included 11 cofactor-independent IgM aPE sera, 14 ABP-independent IgG aPE sera and 12 ABP-dependent aPE serum samples (3 IgM, 8 IgG and 1 IgA).

### Chemicals and reagents

All of the phospholipids used in this paper were purchased from Avanti polar lipids, which included 16:0 PE (cat# 850705X); 18:0 PE (cat# 850715X); 18:1 PE (cat# 850725C), 20:4 PE (cat# 850800C); 22:6 PE (cat# 850797C); and egg PE (cat# 840021C).

Pooled normal human sera, BSA and phosphatidylserine (PS) were purchased from Sigma-Aldrich. ABP was a kind of gift from Dr. Dawn R. Wagenknecht. Both BSA and ABP were dissolved in 1X PBS and filtered with a 0.22 micron syringe filter before use. Anti-human IgG (Cat# A9544), IgM (Cat# A3275) and IgA (cat# A9669) alkaline phosphatase and p-nitrophenyl phosphate liquid substrate system (cat# N7656) were obtained from Sigma-Aldrich. NAb™ protein A/G spin kit (Cat# 89950) was obtained from Thermo Fisher Scientific.

### aPE ELISA

Each of the different PE preparations that were in chloroform was diluted in ethanol and loaded in 96-well plate (Thermo Fisher Scientific, Cat# 12565138). The plate was dried in a desiccator overnight and was blocked with 10% BSA for one hour on the platform shaker. The blocking buffer was removed by aspiration and the plate was left in liquid nitrogen vapor for 10 minutes. Each of the patient serum samples was diluted 100 times in 10% BSA for cofactor-independent samples or in 10% ABP for cofactor-dependent samples. Fifty µl of the dilute was loaded in each well of 96 well plate, and the plate was left on the platform shaker for one hour. The plate was washed three times with PBS and 50 µl of alkaline phosphatase-conjugated anti-IgG, IgM or IgA was added. After incubated for 1 hour and washing three times with PBS, the plate was reacted with p-nitrophenyl phosphate substrate. The optical density at 405 was measured when the OD value of the positive samples reached 1.0. The ELISA for each sample was performed in triplicates.

## Total IgG purification

Total IgG was isolated from patient serum by using NAb™ protein A/G spin kit. Briefly, NAb™ column was equilibrated with IgG binding buffer (100 mM phosphate, 150 mM sodium chloride, pH 7.2) and incubated with normal control or patient serum for 15 minutes. The total IgG was eluted with low pH elution buffer, and was immediately mixed with neutralization buffer (1M Tris-HCl, pH8.5). The total IgG was dialyzed against PBS and stored at -80°C for later use.

## Result

In the present study, we investigated the impact on aPE ELISA using a series of synthetic positionally symmetrical PE species. These PE species, including 16:0 PE, 18:0 PE, 18:1 PE, 20:4 PE and 22:6 PE, are commercially available as individual chemically defined products.

### The effect and optimization of synthetic phospholipid compositions on the ELISA performance of cofactor independent IgM aPE

ELISA data using single and combinations of synthetic PE to detect cofactor-independent aPE IgM reactivity in human patient serum samples is shown in Figure 1. The results indicated that PE species with longer fatty acid tails with a greater degree of unsaturation (for example 20:4 PE and 22:6 PE) tend to have a better performance (Figure 1, formulations 2-6). Comparatively, single PE species with shorter fatty acid tails and a lower degree of unsaturation had inferior performance than egg PE (Figure 1, formulations 2-4); however, single PE species with longer fatty acid tails and a greater degree of unsaturation could approach or exceed the ELISA OD readings obtained with egg PE as antigen (Figure 1, formulations 5 and 6). Additionally, the combinations consisting of one saturated and one unsaturated PE species can have a comparable or a higher OD reading than either one of the two individually (Figure 1, formulation 7 versus 2 and 3; formulation 8 versus 2 and 5; formulation 9 versus 2 and 6; formulation 10 versus 3 and 4; formulation 11 versus 3 and 5, formulation 12 versus 3 and 6). The compositions consisting of two PE species, with one saturated and one unsaturated, can have a higher OD reading than egg PE when the unsaturated PE species contains longer fatty acid tails with a greater degrees of unsaturation (Figure 1, formulations 8, 9, 11 and 12). Furthermore, a combination of a greater variety of PE species, four or five species, tends to have an even higher OD readings (Figure 1, formulations 13-16), being better than single PE species and combinations of two PE species, and significantly higher than egg PE.

Next, we examined the ELISA performance of different PE compositions by varying the contents of saturated PE species from 0 to 80%. We determined that a higher ELISA performance was achieved when the content of saturated PE species was relatively low, between 0 to 40% (Figure 2, formulation 2-6, 9-13), where these formulations significantly and consistently had higher OD readings than egg PE. When the percentage of saturated PE species was too high (Figure 2, formulations 7-8, 14-15), it was accompanied with diminished OD readings.

Based on the data in Figure 2, we fixed the saturated PE species in the formulation at 20% or 10%, and investigated the impact of variations among the remaining unsaturated PE species (Figure 3). In these studies, the content of unsaturated PE species, which accounted for 80 or 90% of the formulations, were varied so that the ratios among 18:1 PE, 20:4 PE and 22:6 PE were 1:1:1, 1:3:0, 1:0:3, 1:5:0, 1:0:5, 1:2:3, and 1:3:2 (Figure 3, formulations 2-8 and 9-15 for 20% or 10% of saturated PE species, respectively). For these studies, we determined that overall, the formulations with 20% saturated PE species were generally superior to 10%, and that variations in the ratios among the unsaturated PE species did not have a dramatic impact on ELISA performance.

Based on the results in Figure 3, after selecting a formulation (20% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE, and 26.6% 22:6 PE) among the candidates that gave similarly optimized ELISA performance, we tested the impact of the amount of coating material on ELISA performance (Figure 4). As shown in Figure 4B, the optimal amount of coating was in the range from 0.25 to 1 µg per well in a 96 well ELISA plate. When the coating material was too little (0.125 µg per well) or too much (2 µg per well or greater), it tended to adversely affect the OD readings.

After selecting an optimal synthetic PE composition as a kit formulation, we conducted a head-to-head comparison on the ELISA performance with egg PE using 11 cofactor-independent aPE IgM patient serum samples. As shown in Figure 5, the current kit formulation consistently and significantly led to higher OD readings than egg PE in ELISA ( $P = 0.006$ ,  $n = 11$ ). It was noteworthy that in multiple cases, the OD values of the ELISA was improved by 100% to more than 400%.

### **The effect and optimization of synthetic phospholipid compositions on the ELISA performance of cofactor independent IgG aPE**

Figure 6 demonstrated the relative ELISA performance against egg PE from single and combinations of synthetic PE species for detecting cofactor-independent aPE IgG reactivity. The results indicated, somewhat unexpectedly, that for a representative aPE serum sample, the single 20:4 PE (Figure 6, formulation 5) had the highest OD value, which was nearly 14 folds greater than egg PE on a representative patient serum sample. A number of other formulations of synthetic PE (Figure 6, formulations 4, 6, 7, 11, 14, 15 and 16) also had significantly higher OD values than egg PE. It appeared that when the combination contains a higher percentage of 20:4 PE, it had a greater tendency to result in better sensitivity than egg PE, which indicated 20:4 PE may be an important component in the PE formulations. The inclusion of PE species with longer fatty acid tails with a greater degree of unsaturation (for example 22:6 PE) also appeared to result in improved ELISA results compared to egg PE.

Based on these results, we conducted a head-to-head study between the 20:4 PE and egg PE for detecting the aPE reactivity among 14 cofactor-independent IgG patient serum samples (Figure 7). The data indicated that most of patient serum samples having high aPE reactivity on egg PE had even stronger aPE reactivity on 20:4 PE, with some samples were improved by 500% or more. Certain patient samples that were negative or marginally positive by egg PE showed strongly positive reactivity to 20:4 PE. Samples that were negative by 20:4 PE

were also negative by egg PE. Overall, the current formulation of 20:4 PE consistently led to a higher OD value than egg PE with  $P = 0.012$  ( $n = 14$ ).

### **The effect and optimization of synthetic phospholipid compositions on the ELISA performance of cofactor-dependent aPE**

We conducted screening studies using different synthetic PE compositions on the ELISA performance for cofactor-dependent aPE. Figures 8A to C demonstrated the relative aPE reactivity from representative cofactor-dependent aPE IgG, IgM and IgA, respectively. The data indicated, somewhat unexpectedly, that a single species of 18:1 PE (Figures 8A to C, formulation 4) was able to approach the reactivity level of egg PE. In addition, substitution or dilution of 18:1 PE with other PE species (for example, 16:0, 18:0, 20:4 PE or 22:6 PE), is more likely to result in a lower performance.

In a head-to-head comparison with egg PE, we used 18:1 PE to test for detecting cofactor-dependent aPE reactivity of 12 patient serum samples, which included 3 IgM aPE, 8 IgG aPE and 1 IgA aPE. The results (Figure 9) indicated that 18:1 PE delivered a similar sensitivity as egg PE for detecting aPE reactivity, with a  $P$  value of 0.99.

### **Testing ELISA performance with synthetic PE species by using purified total IgG**

In order to minimize uncertainties caused by potential confounding factors in the serum and variations in immunoglobulin contents, the total IgG was isolated from representative aPE patient serum samples and was used to validate the above findings in ELISA assays.

Microplates were coated using Egg PE, 20:4 PE or 18:1 PE for IgG aPE ELISA for normal human serum, a representative cofactor-dependent and cofactor-independent patient serum. As shown in Figure 10, the cofactor-independent sample had significant reactivity against egg PE and 20:4 PE, whereas the signal from 18:1 PE was near negligible. In contrast, for the cofactor-dependent sample, there was apparent reactivity against egg PE and 18:1 PE, but not 20:4 PE. To validate the difference in reactivity between 18:1 PE and 20:4 PE in cofactor-dependent aPE, total IgG from two other cofactor-dependent aPE samples were isolated, and their reactivity against egg PE, 20:4 PE, 18:1 PE and PS as a negative control was tested. The ELISA results (Figure 11) indicated that again, the cofactor-dependent aPE IgG sample strongly recognized egg PE and 18:1 PE, but not 20:4 PE or the negative control PS. These findings were consistent with 20:4 PE being a preferred antigen for cofactor-independent IgG aPE, and that 18:1 PE is a preferred antigen for cofactor-dependent IgG aPE. Additionally, the presence of ABP significantly diminished the reactivity of cofactor-independent aPE, suggesting that ABP may contain elements that potentially compete with cofactor-independent aPE. Similar observations have been documented in prior reports (23).

## **Discussion**

ELISA is the predominant method for detecting the presence of autoantibodies against PE. Existing reports in the current literature, which employed different natural PE extracts, indicated that the outcome of aPE ELISA may differ depending on the source of PE (6, 22, 23). These variations suggest that the composition and physicochemical properties of PE antigens could have a significant impact on the reactivity of aPE antibodies. The term PE



refers to a collection of species where, although they share the phosphorylethanolamine head group as a common structural feature, they have different fatty acyl chains (25–30). Relatively little is known about the actual impact in the variations of PE on the performance of aPE ELISA. Such knowledge, when available, could shed light on the nature of immunological reactivity of aPE and help develop standardized ELISA conditions using synthetic components.

The goal of the current study was to examine, using a series of synthetic positionally symmetrical PE species, how the outcome of aPE ELISA may be dependent on differences in PE. The phospholipids encompass a wide range of structural entities that vary in polarity, charge, hydrophobicity, size and shape (24, 32–34). The diversity of their biological functions is further enriched by controlled biosynthesis, compartmentalization and trafficking (32–34). Phospholipids that are identified by the same headgroup can have different species owing to properties of the acyl chains. These include differences in length, degree of unsaturation and stereoisomerism of the fatty tails, as well as positional asymmetry between sn-1 and sn-2 in the glycerol backbone (32–35). It is appreciated that in biological membranes, the fatty acyl chains are mostly asymmetrical, adding yet another layer of versatility to the membrane structure and functions (35). The PE species used in the current study differed in length and the degree of unsaturation, but were positionally symmetrical. While the latter was a choice made to limit the number of variables when creating a simplified model system, it inevitably introduced limitations where positionally symmetrical PE may not fully recapitulate the properties of naturally occurring biological membranes. Nevertheless, key findings from these experiments were that single and mixed synthetic PE species can indeed have a significant impact on the performance of aPE ELISA. Additionally, for the first time, the present data demonstrated that cofactor-dependent and -independent aPE may have distinct epitope preferences. These findings may open up new opportunities for adjusting aPE ELISA conditions with improved specificity and shed light on the nature of antibody-antigen interactions in aPE. The current findings warrant future studies using positionally asymmetrical PE species which, while introducing another degree of complexity in terms of lipid properties, may better represent natural PE in biological membrane systems.

Our data support the notion that different fatty acid length and saturation level can strongly influence the performance of aPE ELISA. For cofactor-independent IgM aPE, highly unsaturated PE has better performance than lower degrees of unsaturated PE and saturated PE; a longer fatty acid chain is preferred over a shorter one when comparing single PE species. The combination of one saturated and one unsaturated PE is comparable or better than either of the single species. Mixtures of multiple PE species at the right compositions may achieve an even higher performance, which allowed us to fine-tune the composition for optimization. These observations indicated that for cofactor-independent IgM aPE, the complexity of antigen combination may be important. It is possible that this complexity reflects specific solid/liquid phases that facilitate the interactions with IgM aPE.

Somewhat unexpectedly, the data revealed that cofactor-independent IgG aPE was preferentially reactive to 20:4 PE, whereas cofactor-dependent aPE was more selective for 18:1 PE. It indicated that different aPE antibodies may have distinct PE requirements. From



a lipid physics perspective, the relative hydrodynamic size of the head group versus the fatty acid tails plays a predominant role in dictating the shape of individual lipid molecules, which in turn, contributes to the structure of the lipid membrane (36). The phosphorylethanolamine head group in PE is relatively small. In the presence of bulky fatty acid tails, PE assumes an inverted cone shape, which favors hexagonal structures at physiological temperatures. Consistent with the shape-structure hypothesis, PE species with longer polyunsaturated fatty acids tend to form hexagonal tubes with smaller diameters (37, 38). This relationship has been demonstrated using electron microscopy with freeze-fractured lipid preparations (37). Without knowing the specific antigenic epitope(s) for aPE, these structural differences in the physical organization of PE membranes may offer clues in the preferential reactivity of aPE antibodies and cofactors to different PE species. In terms of making diagnostics for aPE, the ability to distinctly test for the presence of cofactor-dependent and -independent reactivity may help differentiate different forms of aPE and reduce ambiguity.

It is of interest to note that cofactor-independent IgM and IgG do not share a specific antigenic feature, where the former prefers longer fatty acids with a greater degree of unsaturation but the latter binds optimally to 20:4 PE. In contrast, among cofactor-dependent isotypes including IgM, IgG and IgA, there appears to be a common factor as the 18:1 PE. Relatively high levels of aPE reactivity could be consistently achieved when 18:1 PE was the sole or dominant component in the antigen composition. Reduction or substitution of 18:1 PE can be associated with significantly diminished aPE performance. A limitation for the current investigation is that only a relatively small number of serum samples were tested. Future studies involving larger cohorts are likely to provide further evidence on this observation.

The present data demonstrated that factors other than PE antigens could also have a significant impact on ELISA performance. The data showed that while the presence of ABP was necessary to produce a positive reactivity for cofactor-dependent aPE, its presence was consistently associated with a diminished ELISA reactivity for cofactor-independent aPE. Further evidence is warranted in order to determine, conclusively, whether this reduction was due to a competitive nature of ABP. A similar observation has been made in a prior report that the phospholipid contents in ABP were greater than fetal calf serum, and could have been a contributing factor that depletes aPE antibodies thus diminishes ELISA reactivity (23). As an alternative explanation, a PE-binding factor could sequester and competitively mask the antigen, thus prevents antibody-antigen interactions. Existing literature has identified the kininogens as PE-binding cofactors in at least a portion of cofactor-dependent aPE cases (39). It would be of interest to determine, in future studies, whether the kininogens or other unknown factors may indeed interact with PE species that have a specific physicochemical characteristic.

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## References

1. Wilson WA, Gharavi AE, Koike T, et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum.* 1999; 42:1309–1311. [PubMed: 10403256]
2. 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. [PubMed: 16420554]
3. Rodriguez-Garcia JL, Bertolaccini ML, Cuadrado MJ, et al. Clinical manifestations of antiphospholipid syndrome (APS) with and without antiphospholipid antibodies (the so-called 'seronegative APS'). *Ann Rheum Dis.* 2012; 71:242–244. [PubMed: 21953349]
4. Nayfe R, Uthman I, Aoun J, et al. Seronegative antiphospholipid syndrome. *Rheumatology (Oxford).* 2013; 52:1358–1367. [PubMed: 23502076]
5. Staub HL, Harris EN, Khamashta MA, et al. Antibody to phosphatidylethanolamine in a patient with lupus anticoagulant and thrombosis. *Ann Rheum Dis.* 1989; 48:166–169. [PubMed: 2494957]
6. McIntyre JA, Wagenknecht DR. Anti-phosphatidylethanolamine (aPE) antibodies: a survey. *J Autoimmun.* 2000; 15:185–193. [PubMed: 10968907]
7. Sher G, Fisch JD, Maassarani G, et al. Antibodies to phosphatidylethanolamine and phosphatidylserine are associated with increased natural killer cell activity in non-male factor infertility patients. *Hum Reprod.* 2000; 15:1932–1936. [PubMed: 10966989]
8. Yetman DL, Kutteh WH. Antiphospholipid antibody panels and recurrent pregnancy loss: prevalence of anticardiolipin antibodies compared with other antiphospholipid antibodies. *Fertil Steril.* 1996; 66(4):540–546. [PubMed: 8816614]
9. Matsubayashi H, Sugi T, Arai T, et al. Different antiphospholipid antibody specificities are found in association with early repeated pregnancy loss versus recurrent IVF-failure patients. *Am J Reprod Immunol.* 2001; 46:323–329. [PubMed: 11712760]
10. Sanmarco M, Bardin N, Camoin L, et al. Antigenic profile, prevalence, and clinical significance of antiphospholipid antibodies in women referred for in vitro fertilization. *Ann N Y Acad Sci.* 2007; 1108:457–465. [PubMed: 17894010]
11. Yamada H, Atsumi T, Kobashi G, et al. Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. *J Reprod Immunol.* 2009; 79:188–195. [PubMed: 19211151]
12. Gris JC, Quéré I, Sanmarco M, et al. Antiphospholipid and antiprotein syndromes in non-thrombotic, non-autoimmune women with unexplained recurrent primary early foetal loss. The Nîmes Obstetricians and Haematologists Study--NOHA. *Thromb Haemost.* 2000; 84:228–236. [PubMed: 10959694]
13. Balada E1, Ordi-Ros J, Paredes F, et al. Antiphosphatidylethanolamine antibodies contribute to the diagnosis of antiphospholipid syndrome in patients with systemic lupus erythematosus. *Scand J Rheumatol.* 2001; 30:235–241. [PubMed: 11578020]
14. Gonzales-Portillo F, McIntyre JA, Wagenknecht DR, et al. Spectrum of antiphospholipid antibodies (aPL) in patients with cerebrovascular disease. *J Stroke Cerebrovasc Dis.* 2001; 10:222–226. [PubMed: 17903828]
15. Sanmarco M, Gayet S, Alessi MC, et al. Antiphosphatidylethanolamine antibodies are associated with an increased odds ratio for thrombosis. A multicenter study with the participation of the European Forum on antiphospholipid antibodies. *Thromb Haemost.* 2007; 97:949–954. [PubMed: 17549297]
16. Karmochkine M, Cacoub P, Piette JC, et al. Antiphosphatidylethanolamine antibody as the sole antiphospholipid antibody in systemic lupus erythematosus with thrombosis. *Clin Exp Rheumatol.* 1992; 10:603–605. [PubMed: 1483313]
17. Berard M, Chantome R, Marcelli A, et al. Antiphosphatidylethanolamine antibodies as the only antiphospholipid antibodies. I. Association with thrombosis and vascular cutaneous diseases. *J Rheumatol.* 1996; 23:1369–1374. [PubMed: 8856615]

18. Sugi T, Katsunuma J, Izumi S, et al. Prevalence and heterogeneity of antiphosphatidylethanolamine antibodies in patients with recurrent early pregnancy losses. *Fertil Steril*. 1999; 71:1060–1065. [PubMed: 10360910]
19. Boffa MC, Berard M, Sugi T, et al. Antiphosphatidylethanolamine antibodies as the only antiphospholipid antibodies detected by ELISA. *J Rheumatol*. 1996; 23:1375–1379. [PubMed: 8856616]
20. Sugi T, Matsubayashi H, Inomo A, Dan L, et al. Antiphosphatidylethanolamine antibodies in recurrent early pregnancy loss and mid-to-late pregnancy loss. *J Obstet Gynaecol Res*. 2004; 30:326–332. [PubMed: 15238112]
21. Staub HL, Bertolaccini ML, Khamashta MA. Anti-phosphatidylethanolamine antibody, thromboembolic events and the antiphospholipid syndrome. *Autoimmun Rev*. 2012; 12:230–234. [PubMed: 22796282]
22. Bertolaccini ML, Amengual O, Atsumi T, et al. ‘Non-criteria’ aPL tests: report of a task force and preconference workshop at the 13<sup>th</sup> international congress on antiphospholipid antibodies, Galveston, TX, USA, April 2010. *Lupus*. 2011; 20:191–205. [PubMed: 21303836]
23. Sanmarco M. ELISA for antiphosphatidylethanolamine antibody detection: High impact of assay buffer on results. *J Immuno Methods*. 2010; 358:9–16.
24. Nelson, DL.; Cox, MM. *Lehninger principles of biochemistry*, fourth edition. W. H. Freeman; 2004.
25. Harper PE, Mannock DA, Lewis RN, et al. X-ray diffraction structures of some phosphatidylethanolamine lamellar and inverted hexagonal phases. *Biophys J*. 2001; 81:2693–2706. [PubMed: 11606282]
26. Toombes GE, Finnefrock AC, Tate MW, et al. Determination of L(alpha)-H(II) phase transition temperature for 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine. *Biophys J*. 2002; 82:2504–2510. [PubMed: 11964238]
27. Cullis PR, de Kruijff B. The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. A <sup>31</sup>P NMR study. *Biochim Biophys Acta*. 1978; 513:31–42. [PubMed: 31173]
28. Siakotos AN, Rouser G, Fleischer S. Phospholipid composition of human, bovine and frog myelin isolated on a large scale from brain and spinal cord. *Lipids*. 1969; 4:239–242. [PubMed: 4306678]
29. Hawke JC. The fatty acids of phosphatidylethanolamine and phosphatidylcholine from hen's egg. *Biochem J*. 1959; 71:588–592. [PubMed: 13638270]
30. Kleinig H, Zentgraf H, Comes P, et al. Nuclear membranes and plasma membranes from hen erythrocytes. II. Lipid composition. *J Biol Chem*. 1971; 246:2996–3000. [PubMed: 4324345]
31. Ke K, Strango ZI, Harper PE, et al. Influence of phosphatidylethanolamine concentration and composition on the detection of antiphosphatidylethanolamine antibodies by ELISA. *J Clin Lab Anal*. 2016 [epub ahead of print].
32. de Kroon AIPM, Rijken RJ, De smet CH. Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. *Prog Lipid Res*. 2013; 52:374–394. [PubMed: 23631861]
33. Hermansson M, Hokynar K, Somerharju P. Mechanisms of glycerophospholipid homeostasis in mammalian cells. *Prog Lipid Res*. 2011; 50:240–257. [PubMed: 21382416]
34. Dowhan W. Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu Rev Biochem*. 1997; 66:199–232. [PubMed: 9242906]
35. Schreiner M, Moreira RG, Hulan HW. Positional distribution of fatty acids in egg yolk lipids. *J Food Lipids*. 2006; 13:36–56.
36. Cullis PR, de Kruijff B. The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. A <sup>31</sup>P NMR study. *Biochim Biophys Acta*. 1978; 513:31–42. [PubMed: 31173]
37. Van Venetie R, Verkleij AJ. Analysis of the hexagonal II phase and its relations to lipidic particles and the lamellar phase. A freeze-fracture study. *Biochim Biophys Acta*. 1981; 645:262–269. [PubMed: 7272289]

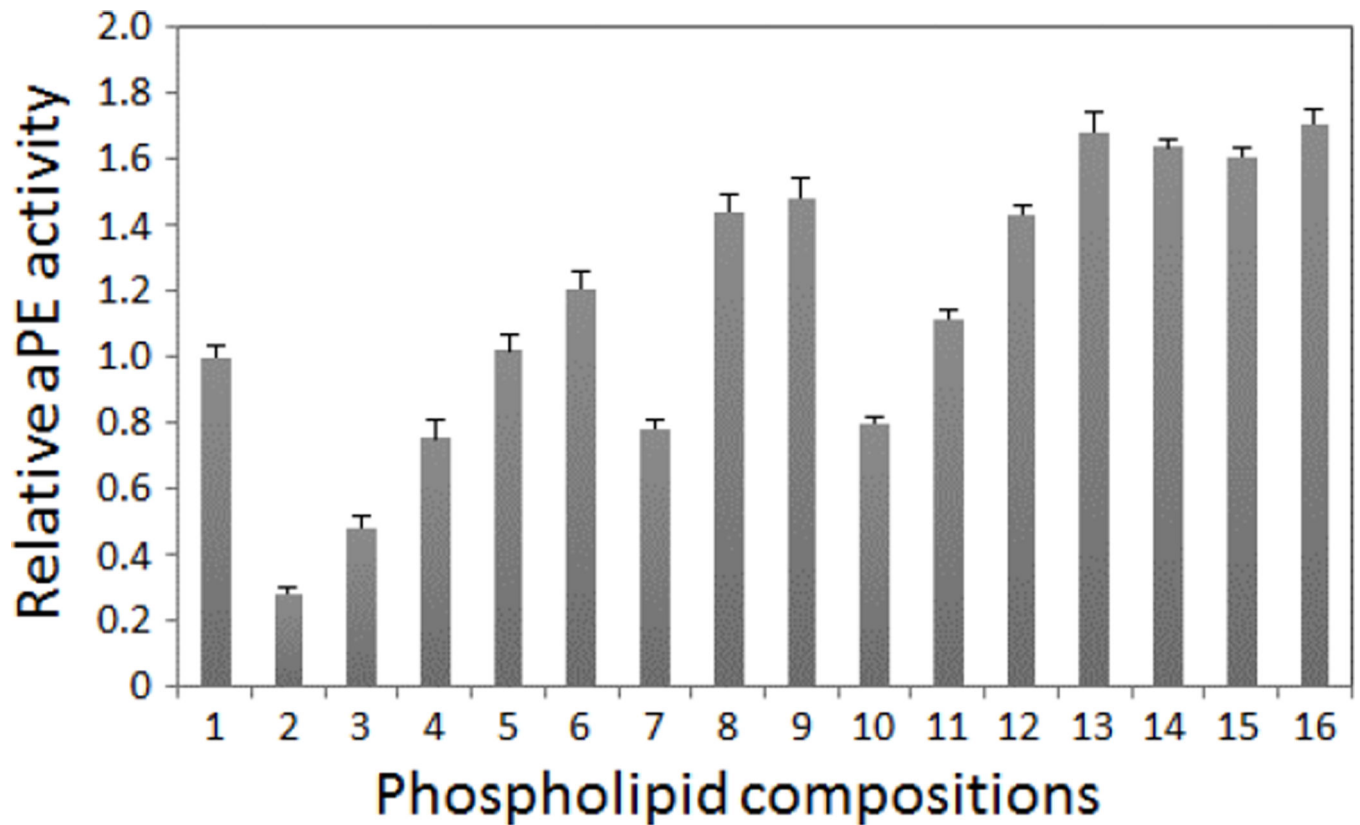
38. Dekker CJ, Geurts van Kessel WS, Klomp JP, et al. Synthesis and polymorphic phase behaviour of polyunsaturated phosphatidylcholines and phosphatidylethanolamines. *Chem Phys Lipids*. 1983; 33:93–106. [PubMed: 6627528]
39. Sugi T, McIntyre JA. Autoantibodies to phosphatidylethanolamine (PE) recognize a kininogen-PE complex. *Blood*. 1995; 86:3083–3089. [PubMed: 7579402]

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**Figure 1.**

The ELISA performance, relative to that of egg PE, of single and combinations of synthetic PE species using a representative IgM aPE patient plasma. The formulations are specified as follows.

- (1) Egg PE;
- (2) 16:0 PE;
- (3) 18:0 PE;
- (4) 18:1 PE;
- (5) 20:4 PE;
- (6) 22:6 PE;
- (7) 30% 16:0 PE and 70% 18:1PE;
- (8) 30% 16:0 PE and 70% 20:4 PE;
- (9) 30% 16:0 PE and 70% 22:6 PE;
- (10) 30% 18:0 PE and 70% 18:1PE;
- (11) 30% 18:0 PE and 70% 20:4 PE;
- (12) 30% 18:0 PE and 70% 22:6 PE;
- (13) 15% 16:0 PE, 15% 18:0 PE, 17.5% 18:1 PE and 52.5% 20:4 PE;
- (14) 15% 16:0 PE, 15% 18:0 PE, 23.3% 18:1 PE and 46.7% 20:4 PE;
- (15) 20% 16:0 PE, 10% 18:0 PE, 15.75% 18:1 PE, 47.25% 20:4 PE; and 7% 22:6 PE;
- (16) 20% 16:0 PE, 10% 18:0 PE, 21% 18:1 PE and 42% 20:4 PE and 7% 22:6 PE.

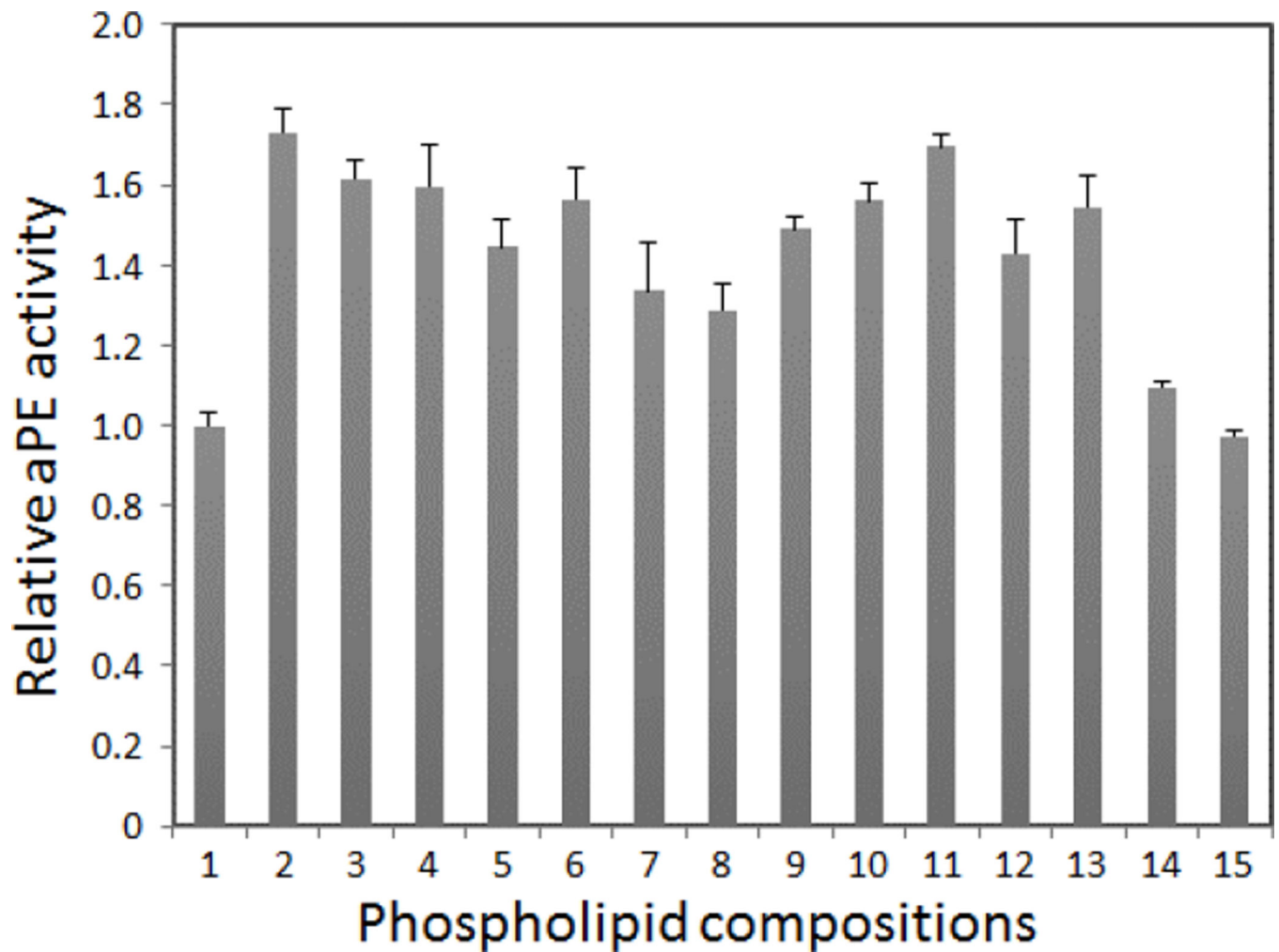
A total of 0.5 µg indicated PE was coated on each well of a 96 plate. OD<sub>405</sub> was measured, subtracted of normal control value, and normalized to the reactivity of egg PE. The results were obtained from triplicates.

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**Figure 2.**

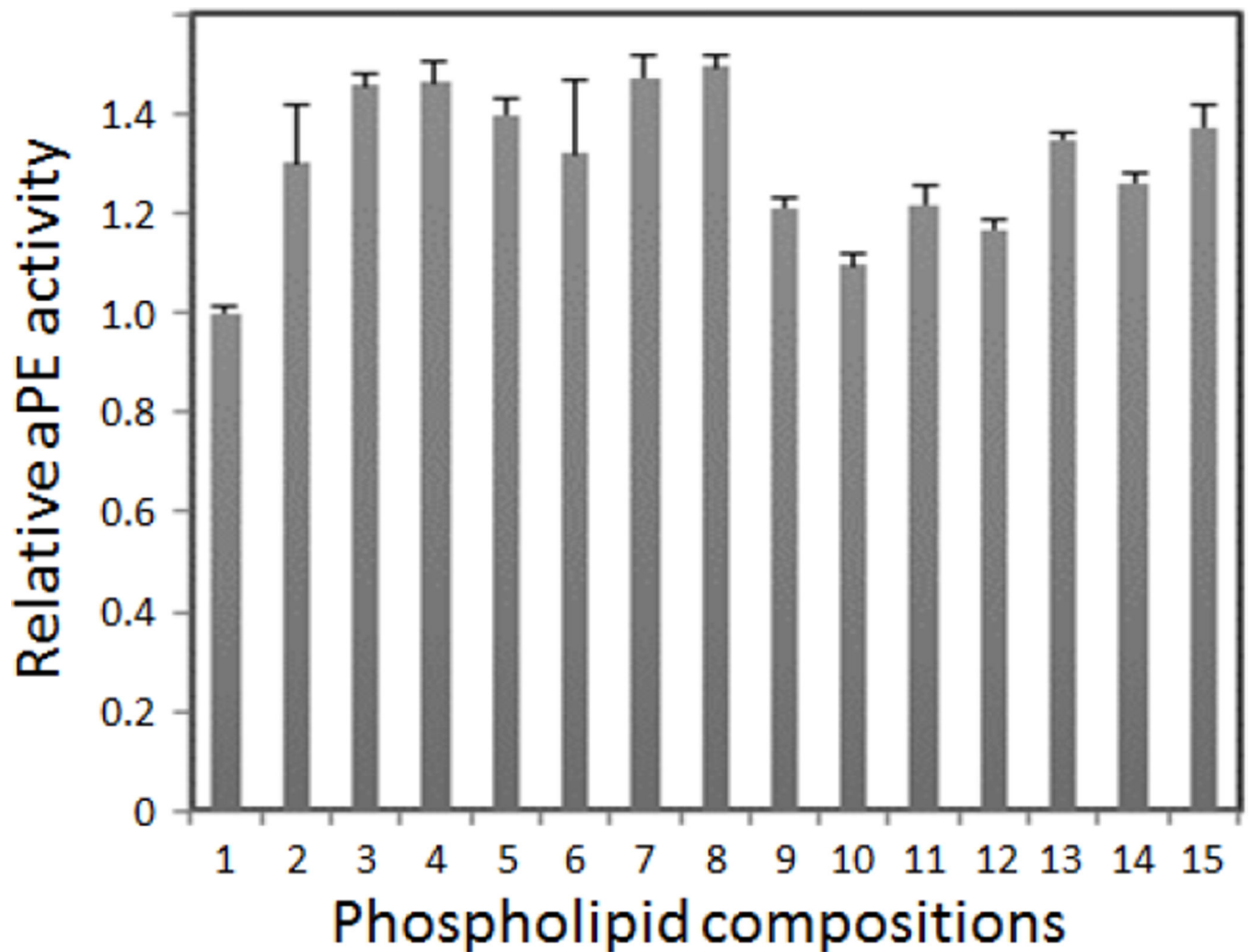
Testing for optimal phospholipid combinations for cofactor-independent IgM aPE ELISA using a representative IgM aPE patient plasma. The formulations are specified as follows.

- (1) egg PE;
- (2) 0% 18:0 PE, 16.6% 18:1 PE, 33.2% 20:4 PE, and 49.8% 22:6 PE;
- (3) 10% 18:0 PE, 15% 18:1 PE, 30% 20:4 PE, and 45% 22:6 PE;
- (4) 20% 18:0 PE, 13.3% 18:1 PE, 26.6% 20:4 PE, and 39.9% 22:6 PE;
- (5) 30% 18:0 PE, 11.6% 18:1 PE, 23.2% 20:4 PE, and 34.8% 22:6 PE;
- (6) 40% 18:0 PE, 10% 18:1 PE, 20% 20:4 PE, and 30% 22:6 PE;
- (7) 60% 18:0 PE, 6.6% 18:1 PE, 13.2% 20:4 PE, and 19.8% 22:6 PE;
- (8) 80% 18:0 PE, 3.3% 18:1 PE, 6.6% 20:4 PE, and 9.9% 22:6 PE;
- (9) 0% 18:0 PE, 16.6% 18:1 PE, 49.8% 20:4 PE, and 33.2% 22:6 PE;
- (10) 10% 18:0 PE, 15% 18:1 PE, 45% 20:4 PE, and 30% 22:6 PE;
- (11) 20% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE, and 26.6% 22:6 PE;
- (12) 30% 18:0 PE, 11.6% 18:1 PE, 34.8% 20:4 PE, and 23.2% 22:6 PE;
- (13) 40% 18:0 PE, 10% 18:1 PE, 30% 20:4 PE, and 20% 22:6 PE;
- (14) 60% 18:0 PE, 13.2% 18:1 PE, 6.6% 20:4 PE, and 19.8% 22:6 PE;



(15) 80% 18:0 PE, 3.3% 18:1 PE, 9.9% 20:4 PE, and 6.6% 22:6 PE.

A total of 0.5 µg of indicated PE was coated on each well of a 96 plate. OD<sub>405</sub> was measured, subtracted of normal control value, and normalized to the reactivity of egg PE. The results were obtained from triplicates.



**Figure 3.**

Characterizing the impact of unsaturated PE species on aPE ELISA performance for cofactor-independent IgM aPE using a representative IgM aPE patient plasma. The formulations are specified as follows.

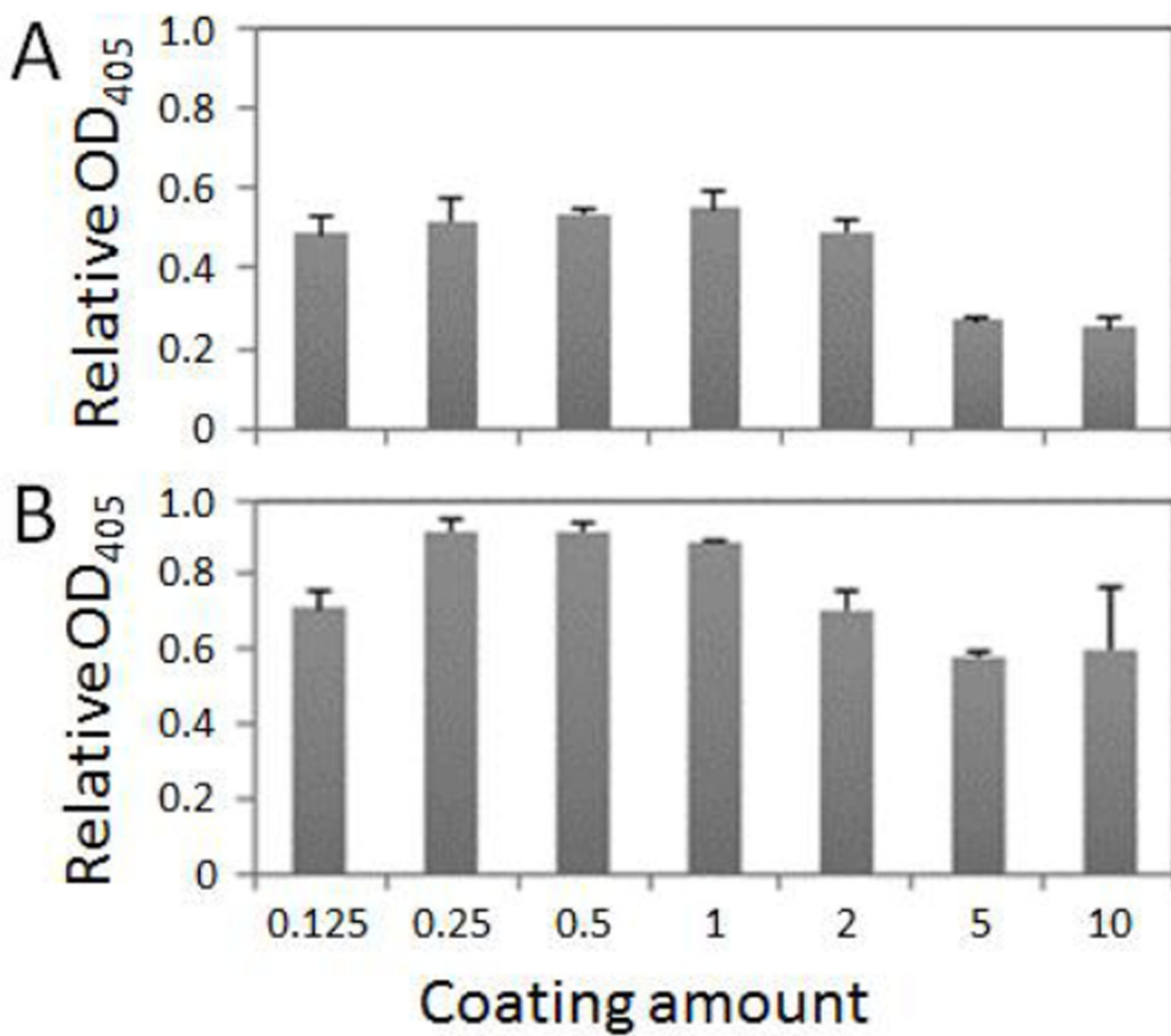
- (1) egg PE;
- (2) 20% 18:0 PE, 33.3% 18:1 PE, 33.3% 20:4 PE, and 33.3% 22:6 PE;
- (3) 20% 18:0 PE, 20% 18:1 PE, 60% 20:4 PE, and 0% 22:6 PE;
- (4) 20% 18:0 PE, 20% 18:1 PE, 0% 20:4 PE, and 60% 22:6 PE;
- (5) 20% 18:0 PE, 13.3% 18:1 PE, 66.5% 20:4 PE, and 0% 22:6 PE;
- (6) 20% 18:0 PE, 13.3% 18:1 PE, 0% 20:4 PE, and 66.5% 22:6 PE;
- (7) 20% 18:0 PE, 13.3% 18:1 PE, 26.6% 20:4 PE, and 39.9% 22:6 PE;
- (8) 20% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE, and 26.6% 22:6 PE;
- (9) 10% 18:0 PE, 30% 18:1 PE, 30% 20:4 PE, and 30% 22:6 PE;
- (10) 10% 18:0 PE, 22.5% 18:1 PE, 67.5% 20:4 PE, and 0% 22:6 PE;
- (11) 10% 18:0 PE, 22.5% 18:1 PE, 0% 20:4 PE, and 67.5% 22:6 PE;
- (12) 10% 18:0 PE, 15% 18:1 PE, 75% 20:4 PE, and 0% 22:6 PE;
- (13) 10% 18:0 PE, 15% 18:1 PE, 0% 20:4 PE, and 75% 22:6 PE;

(14) 10% 18:0 PE, 15% 18:1 PE, 30% 20:4 PE, and 45% 22:6 PE;

(15) 10% 18:0 PE, 15% 18:1 PE, 45% 20:4 PE, and 30% 22:6 PE.

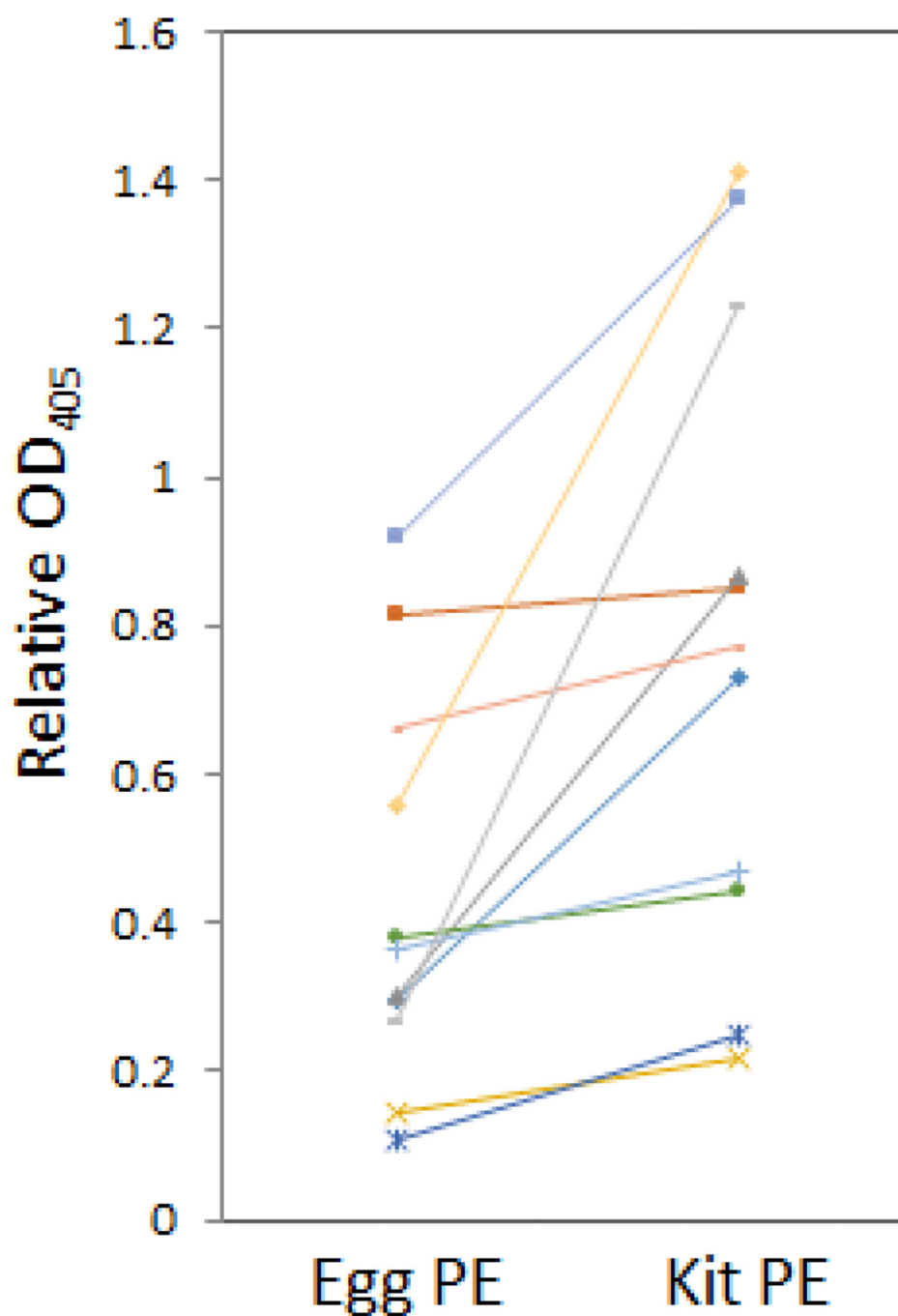
A total of 0.5 µg of indicated PE was coated on each well of a 96 plate. OD<sub>405</sub> was measured, subtracted of normal control value, and normalized to the reactivity of egg PE.

The results were obtained from triplicates.



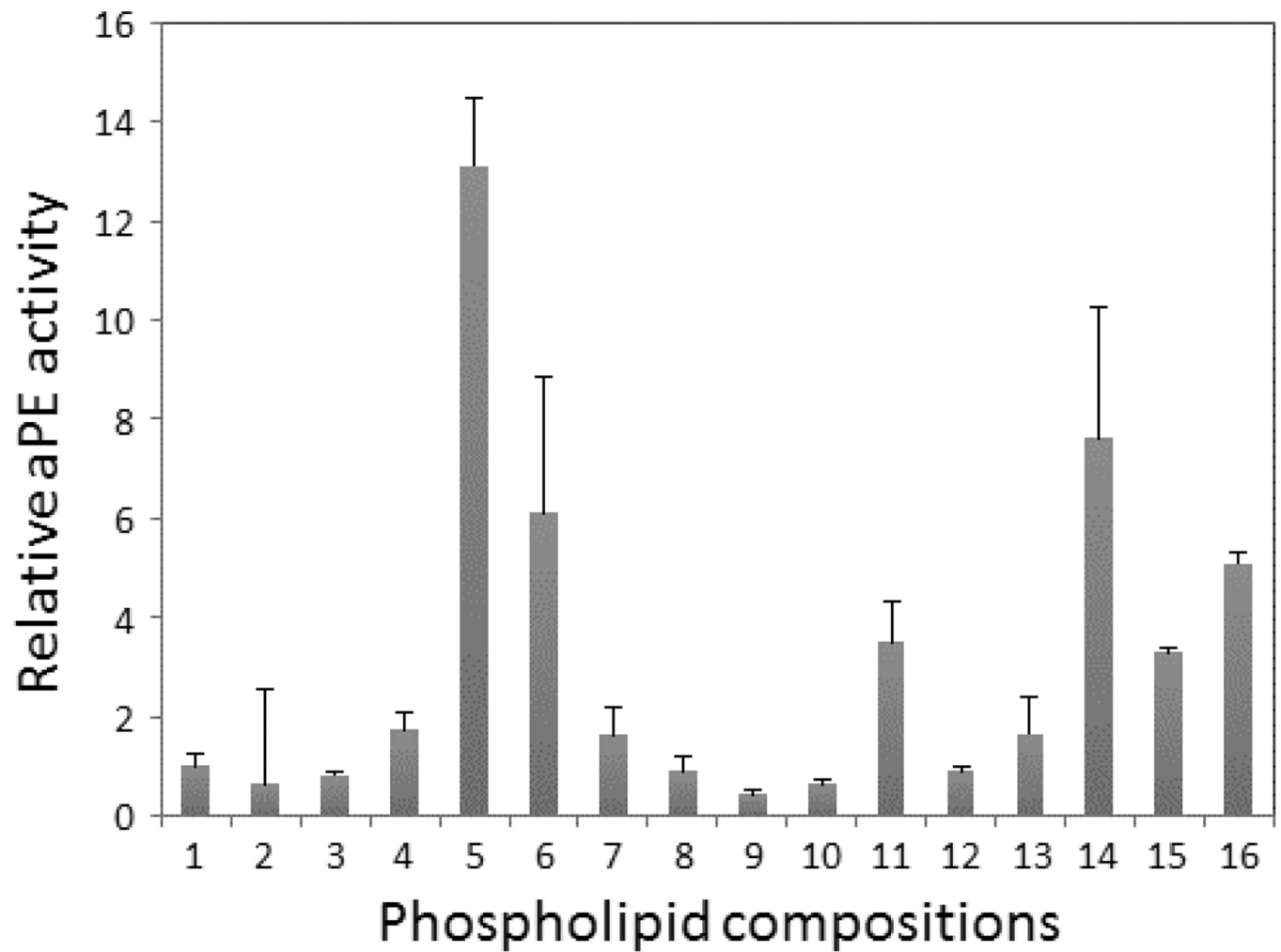
**Figure 4.**

Determination of the amount of PE coating and its impact on ELISA. The indicated amount of PE was coated on a 96 well plate and aPE ELISA was performed. The results were obtained from triplicates. A) Egg PE. B) 20% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE, and 26.6% 22:6 PE.



**Figure 5.**

A head-to-head comparison on the ELISA performance between egg PE and the current kit formulation using cofactor-independent IgM plasma samples. The ELISA plate was coated with 0.5  $\mu$ g of egg PE or the kit formulation (20% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE, and 26.6% 22:6 PE). OD<sub>405</sub> was measured after ELISA and the OD value from the normal human serum was subtracted. The net value was the mean of triplicates. A total 11 patient plasma samples were tested.  $P=0.006$ .



**Figure 6.**

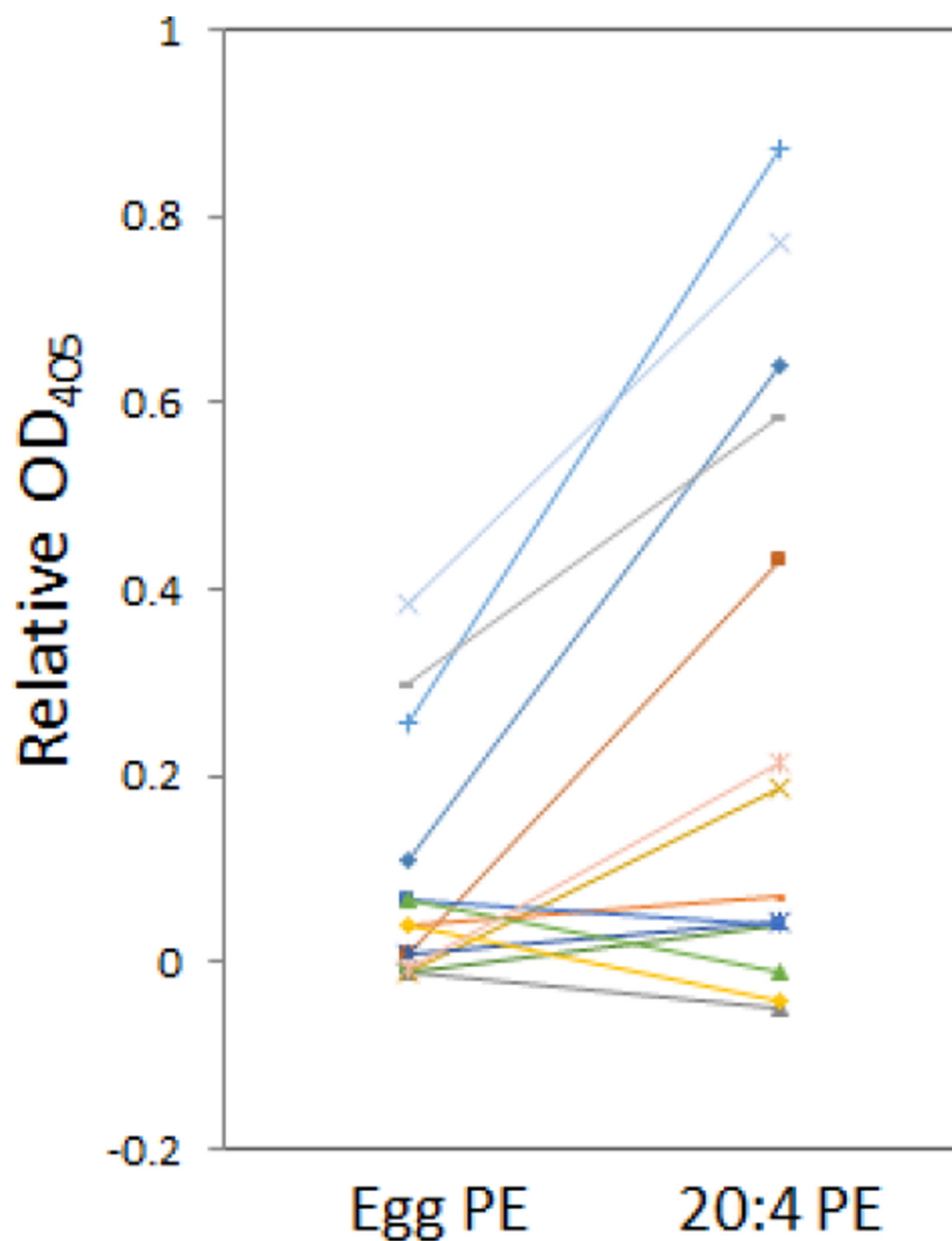
Screening of synthetic PE formulations for detecting cofactor-independent aPE IgG using a representative IgG aPE patient plasma. Formulations are specified as follows. The 20:4 PE was identified as a preferred antigen.

- (1) Egg PE;
- (2) 16:0 PE;
- (3) 18:0 PE;
- (4) 18:1 PE;
- (5) 20:4 PE;
- (6) 22:6 PE;
- (7) 20% 16:0 PE and 80% 18:1PE;
- (8) 20% 16:0 PE and 80% 20:4 PE;
- (9) 20% 16:0 PE and 80% 22:6 PE;
- (10) 20% 18:0 PE and 80% 18:1PE;
- (11) 20% 18:0 PE and 80% 20:4 PE;
- (12) 20% 18:0 PE and 80% 22:6 PE;
- (13) 10% 16:0 PE, 10% 18:0 PE, 13.3% 18:1 PE, 26.6% 20:4 PE and 39.9% 22:6 PE;

- (14) 10% 16:0 PE, 10% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE and 26.6% 22: PE;  
(15) 20% 16:0 PE, 10% 18:0 PE, 13.3% 18:1 PE, 26.6% 20:4 PE and 39.9% 22:6 PE;  
(16) 20% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE and 26.6% 22:6 PE.

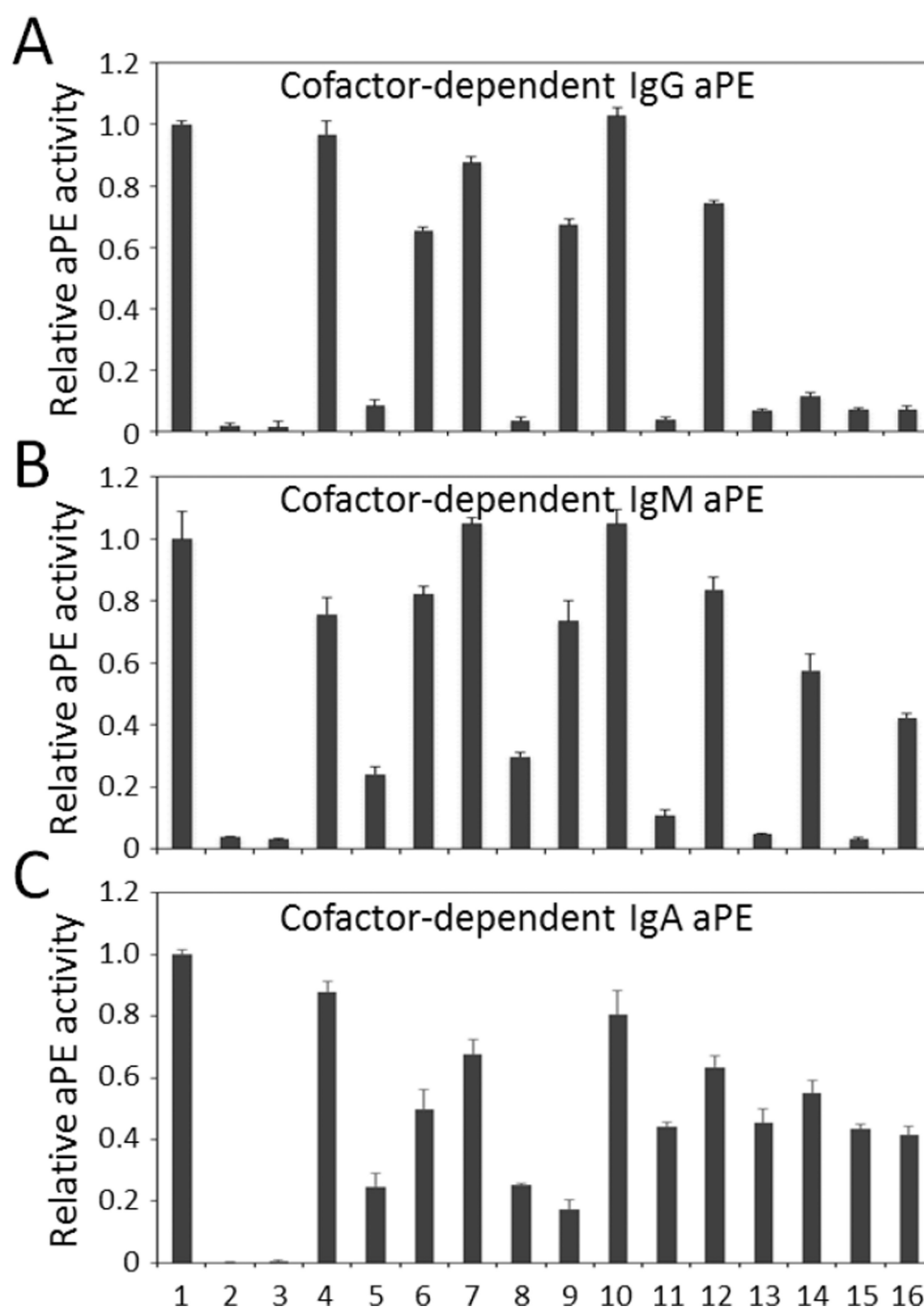
A total of 1 µg indicated PE was used to coat each well of a 96 plate. OD<sub>405</sub> was measured, subtracted of normal control value, and normalized to the reactivity of egg PE. The results were obtained from triplicates.





**Figure 7.**

Head-to-head comparison between egg PE and synthetic 20:4 PE on the ELISA for cofactor-independent IgG aPE. Each well of a 96 well plate was coated with 1  $\mu$ g of indicated PE. OD<sub>405</sub> was measured after ELISA and was subtracted of normal control value. The values are the means of triplicates. A total 14 patient serums were tested.  $P = 0.012$ .

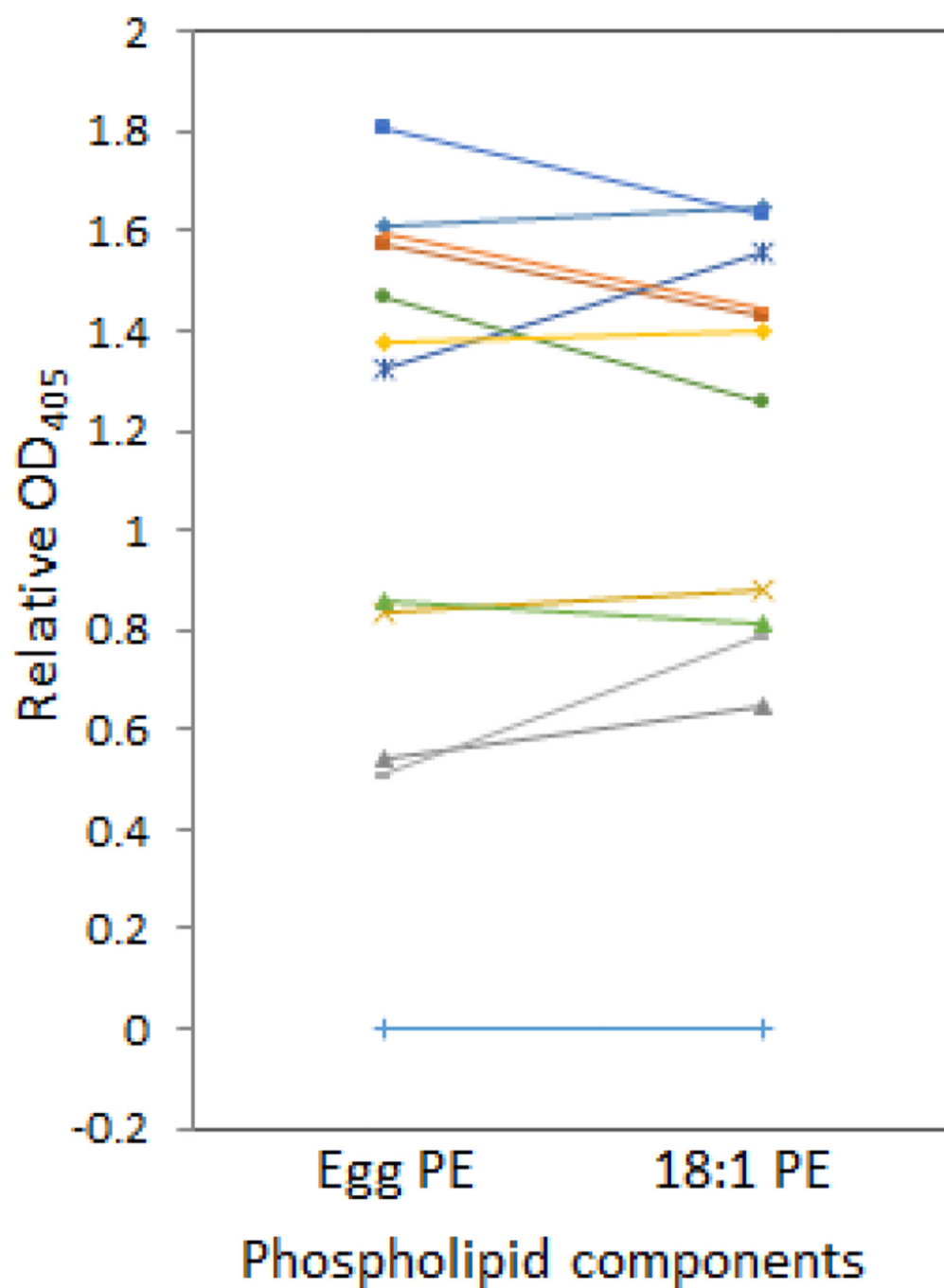


**Figure 8.** Screening of phospholipid combinations for ABP-dependent aPE using representative IgG, IgM and IgA aPE patient plasma samples. The 18:1 PE was identified as preferred antigen. Individual formulas are specified as follows.

- (1) Egg PE;
- (2) 16:0 PE;
- (3) 18:0 PE;
- (4) 18:1 PE;

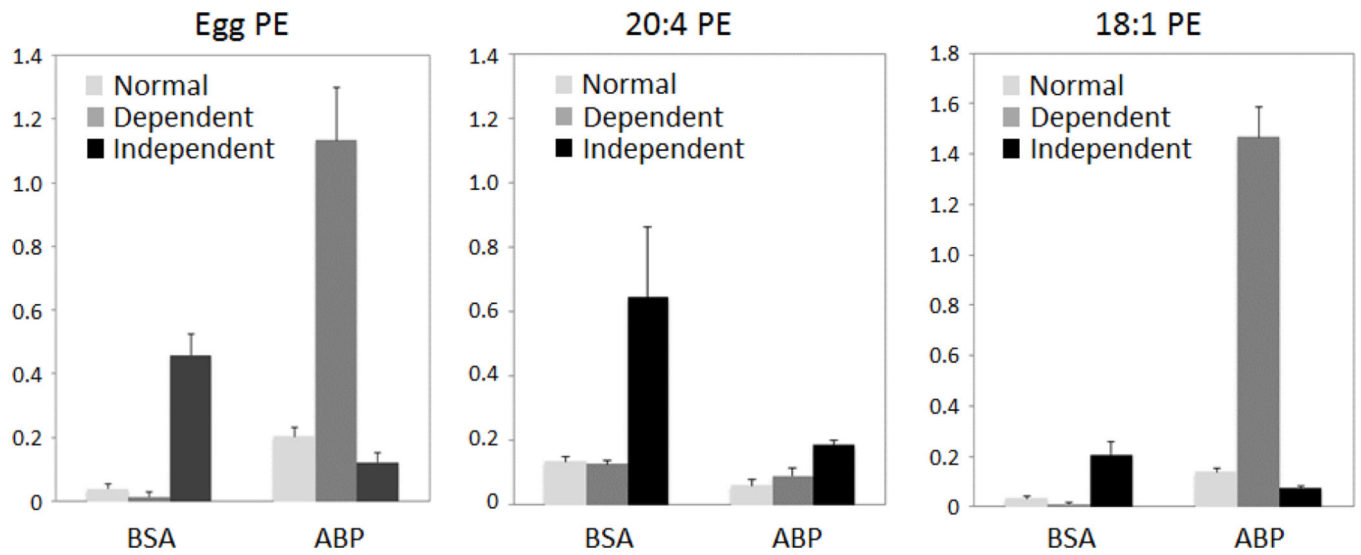
- (5) 20:4 PE;
- (6) 22:6 PE;
- (7) 20% 16:0 PE and 80% 18:1PE;
- (8) 20% 16:0 PE and 80% 20:4 PE;
- (9) 20% 16:0 PE and 80% 22:6 PE;
- (10) 20% 18:0 PE and 80% 18:1PE;
- (11) 20% 18:0 PE and 80% 20:4 PE;
- (12) 20% 18:0 PE and 80% 22:6 PE;
- (13) 10% 16:0 PE, 10% 18:0 PE, 13.3% 18:1 PE, 26.6% 20:4 PE and 39.9% 20:6 PE;
- (14) 10% 16:0 PE, 10% 18:0 PE, 13.3% 18:1 PE and 39.9% 20:4 PE and 26.6% 20:4 PE;
- (15) 20% 16:0% 18:0 PE, 13.3% 18:1 PE, 26.6% 20:4 PE and 39.9% 22:6 PE;
- (16) 20% 18:0 PE, 13.3% 18:1 PE, 39.9% 20:4 PE and 26.6% 22:6 PE.

A total of 1 µg indicated PE was used to coat each well of a 96 plate. OD<sub>405</sub> was measured, subtracted of normal control value, and normalized to the reactivity of egg PE. The results were obtained from triplicates.



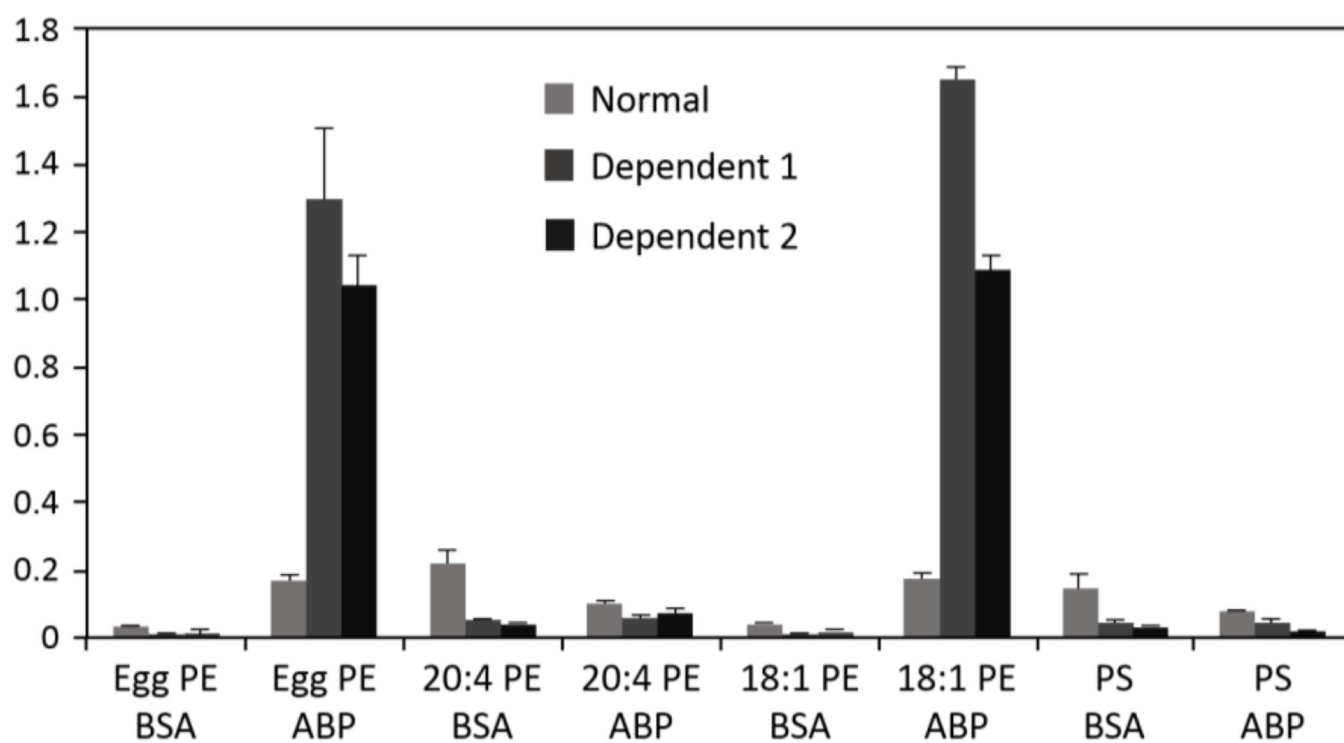
**Figure 9.**

A head-to-head comparison between egg PE and 18:1 PE on the ELISA for cofactor-dependent aPE samples. One  $\mu$ g indicated PE was used to coat each well of a 96 plate. OD<sub>405</sub> was measured, subtracted of normal control value, and normalized to the reactivity of egg PE. The values are means of triplicates. A total of 12 patient plasma samples were used, including 8 IgG aPE, 3 IgM aPE and 1 IgA aPE.  $P=0.9989$ .



**Figure 10.**

aPE reactivity to egg PE, 20:4 PE and 18:1 PE using affinity-purified total IgG from a cofactor-dependent and a cofactor-independent IgG aPE patient plasma. For each ELISA test, the total IgG was used at a concentration of 200 µg/ml. The data demonstrated that the cofactor-dependent and cofactor-independent IgG aPE prefer 18:1 PE and 20:4 PE, respectively. The values are means of triplicates.



**Figure 11.**

aPE activity to synthetic PE antigens using affinity purified total IgG from two different patients with cofactor-dependent IgG aPE. The data demonstrated a preference to 18:1 PE as an antigen. The values are means of triplicates.