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Standardized antigen preparation to achieve comparability of anti-β2-glycoprotein I assays

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ABSTRACT

Introduction: The Sydney classification for diagnosis of the antiphospholipid syndrome (APS) first introduced the determination of anti-β2-glycoprotein I (anti-β2-GPI)-antibodies in serum as laboratory criteria. In this context, widely differing results of anti-β2-GPI assays are a concerning issue. Considerable efforts have been made to optimize ELISAs, however little attention was hitherto spent to the antigen preparation. We evaluated the influence of different β2-GPI preparations on the ability to separate ill and healthy patients and on the comparability of anti-β2-GPI-assays.

Materials and Methods: Microplates were coated with various β2-GPI preparations and anti-β2-GPI IgG- and IgM-ELISAs were performed for 21 APS patients and 21 controls using the monoclonal calibrators HCAL and EY2C9. Subsequently, by use of a surface plasmon resonance (SPR) biosensor, affinity constants for the HCAL- and EY2C9-interaction with each β2-GPI preparation were determined and antigen binding of sera of APS patients and controls was studied.

Results: All ß2-GPI preparations showed good discrimination ability ill vs. healthy but poor inter-assay comparability in the ELISAs. Affinity constants for HCAL and EY2C9 were independent of the β2-GPI variant (K_A 0.105 – 0.200 and 0.449 – 1.04×10^{10} M^{-1}; K_D 50.0 – 95.5 and 9.61 – 22.3×10^{-11} M, respectively). In the biosensor, reactivity to the different β2-GPIs was negligible for the controls and varied considerably for patients.

Conclusion: Inter-assay comparability of anti-β2-GPI ELISAs is highly dependent upon the β2-GPI preparation. Only agreement on one common β2-GPI preparation will improve the requested inter-assay comparability.

Introduction

The antiphospholipid syndrome (APS) is characterized by thromboses and pregnancy complications. Antiphospholipid antibodies (aPL) form the serological correlate of APS with the phospholipid (PL) Cardiolipin (CL) and the PL-binding serum protein β2-glycoprotein I (β2-GPI) being the main antigenic factors.

β2-GPI consists of five distinct complement control protein modules (domains I to V) with a positively charged amino acid sequence in domain V as phospholipid binding site [1]. The epitopes recognized by anti-β2GPI are discussed to be either native or cryptic and expressed after interaction with anionic phospholipids. Domain I is the major target for most anti-β2-GPI. For a subset of anti-β2-GPI, however, domain IV appears to be critical for the exposure of cryptic epitopes.

According to the currently effective Sydney-classification [2], a definite diagnosis of APS requires the presence of at least one of the laboratory criteria: lupus anticoagulant (LA), anti-CL-IgG or -IgM and, deviant from previous classifications, the anti-β2-GPI IgG or IgM. Anti-β2-GPI having now been included in the criteria requires well-known limitations in their determination to be addressed. Almost all studies [3–5] note the poor standardization of anti-β2-GPI ELISAs. Especially for low-positive and IgM-positive samples, variation of cut-off values, widely differing calibration curves (obtained with positive patients sample pool as well as with monoclonal antibodies) and significantly

Abbreviations: aCL, anti-cardiolipin; aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; AUC, area under the curve; β2-GPI, β2-glycoprotein I; bPha, bovine β2-GPI from Phadia; CL, cardiolipin; EY2C9, monoclonal IgM-standard; FC, flow cell; HCAL, monoclonal IgG-standard; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HRP, horseradish peroxidase; HSA, human serum albumin; huIgG1κ, human IgG1κ fraction; K_A, association constant; K_D, dissociation constant; LA, lupus anticoagulant; mab-Acr, monoclonal anti-β2-GPI antibody from Acris; MW, molecular weight; nCal, native human β2-GPI from Calbiochem; n. d., not detectable; nPha, native human β2-GPI from Phadia; nSci, native human β2-GPI from Scipac; PL, phospholipid; rDia, recombinant human β2-GPI from Diarect; ROC–curve, receiver operating characteristic curve; rPha, recombinant human β2-GPI from Phadia; RU, resonance unit; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; TMB, 3,3′,5,5′-tetramethylbenzidine.

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differing results were observed, leading to poor inter-method agreement, which greatly affects patient classification ill vs. healthy. To the best of our knowledge, only one previously published study showed good agreement between different anti-β2-GPI assays [6]. However, the methodological approach of the study was strongly criticized [7].

To overcome the above mentioned problems considerable efforts were made to standardize aPL-ELISAs. The first standardized measurement of anti-β2-GPI based on reference calibrators was reported by Lewis et al. [8]. These calibrators were aliquots of an APS-patient plasma arbitrarily assigned a certain "unit" number [9]. Since then, influences of the microplate surface, coating buffer, composition of blocking solution, dilution buffer and enzyme-conjugate on the anti-β2-GPI assay have been studied [10–12]. Minimal requirements for aPL-ELISAs including the use of monclonal antibodies as calibration standards have been defined [13,14]. Yet, there is consensus, that standardization and inter-laboratory reproducibility of anti-β2-GPI ELISAs should be improved [15]. The approaches to achieve this aim, however, remain a matter of controversy [16–23].

Aforementioned standardization attempts were not focused on the molecular structure of the antigen, even though it is well known that β2-GPIs from different sources vary in their ability to bind patient autoantibodies. So far, systematic studies on the impact of different antigen preparations on quantitative test results do not exist. As well, the benefits of calibration with monoclonal standards have not yet been properly evaluated. The aim of our investigation was therefore to assess the influence of various β2-GPI preparations on the differentiation ability ill vs. healthy and the comparability of anti-β2-GPI ELISAs calibrated with the proposed monoclonal standards.

A major drawback in the exploration of anti-β2-GPI antibodies is the lack of a second established independent analytical technique additional to the ELISA, such as immunofluorescence. To close this gap, we used a surface plasmon resonance (SPR)-biosensor chip with covalently immobilized β2-GPI which was recently presented by our group [24,25] to further elucidate the observed phenomenons. This biosensor device enables the measurement of kinetic on- and off-rates and the calculation of functional affinity constants for the interaction of antigen and specific antibody.

Materials and methods

Patients

21 consecutive APS-patients (median 49 years, 17 women, 4 men) fulfilling the Sydney criteria [2] were recruited from the University Hospital of the Technische Universität München and the University Hospital of Würzburg. 14 patients displayed primary and 7 secondary APS. 17 suffered from thromboembolic events, 2 from pregnancy complications and 2 from a combination of both. Laboratory findings were as follows: anti-cardiolipin (aCL)-IgG were detected in 7, aCL-IgM in 3, both aCL-isotypes in 9, anti-β2-GPI IgG in 8, anti-β2-GPI IgM in 2 and both anti-β2-GPI-isotypes in 8 patients; LA was found positive in 19 patients. Single, double and triple positivity of the investigated patients in the laboratory tests for APS are depicted in Table 1. Serological parameters were determined by the Alegria system from Orgentec (Mainz, Germany). LA was detected with the BCS XP from Siemens Healthcare Diagnostics (Eschborn, Germany). An activated partial thromboplastin time and a dilute Russell`s viper venome time (Pathromtin SL and LA1 Screening Reagent/LA2 Confirmation Reagent, respectively, both from Siemens Healthcare Diagnostics) were run as screnning tests for LA. In case of a prolonged coagulation time in at least one of these tests, diagnosis of LA was established in accordance with international recommendations [26]. An age- and sex-matched control group of 21 healthy subjects was recruited from the laboratory staff. Serum aliquots were stored at -80 °C. The study was approved by the institutional ethics committee. All participants gave written informed consent. No financial compensation was provided.

Chemicals

Details of the six examined β2-GPI protein preparations are depicted in Table 2. The monoclonal anti-β2-GPI antibody (mab-Acr) was from Acris Antibodies (Herford, Germany) and a human IgG1κ fraction (huIgG1κ) from Sigma (St. Louis, MO, USA). The monoclonal standards HCAL for IgG [27] and EY2C9 for IgM [28] were from Inova Diagnostics Inc. (San Diego, CA, USA). Both recognize β2-GPI/CL-complexes and β2-GPI solely. Triton X-100 and Tween-20 were from Fluka (Buchs, Switzerland). All other reagents were from Sigma unless otherwise indicated.

Gel electrophoresis and western blot

10% sodium dodecylsulfate polyacrylamide gel electrophoreses (SDS-PAGE) and Western Blots were run in the Mini-Protean 3 Cell system (Bio-Rad, München, Germany). Slots were loaded with 200 ng of β2-GPI or 5 µL of Rainbow Marker (Amersham Pharmacia Biotech, Freiburg, Germany). The gels were either dyed over night with Coomassie solution (3.0 mM Coomassie Brilliant Blue in 9:9:2 methanol, H_2O and acetic acid) or subjected to Western Blotting. For the latter, proteins were transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad) and incubated with a goat anti-human β2-GPI primary antibody (US Biological, Swampscott, MA, USA) and a rabbit anti-goat-IgG horseradish peroxidase (HRP)–conjugated secondary antibody (Acris Antibodies). Blots were developed with the Super Signal West Dura ECL Kit (Pierce, Rockford, IL, USA).

ELISA

Anti-β2-GPI-ELISAs were performed on Nunc MaxiSorp microplates (Nunc, Langenselbold, Germany) according to a modified protocol of Nojima et al. [29]. Samples were run in duplicate with HCAL (2.0 - 197 ng mL^{-1}) and EY2C9 (1.6 - 156 ng mL^{-1}) as calibrators as recommended by the European Forum on antiphospholipid antibodies [13,14]. Signal detection was realized by a HRP–conjugated secondary antibody and 3,3´,5,5´-tetramethylbenzidine (TMB). Plates were read at 450 and 630 nm and the signal was calculated as difference of both measurements (see supporting information).

Biosensor measurements

Biosensor measurements were performed on a BIAcore X from GE Healthcare (Freiburg, Germany) at 25 °C, as described previously [24]. SPR biosensors allow to monitor biomolecular interactions in real-time by a change in mass concentration on the chip surface (see supporting information).

Antigen immobilization was realized with the BIAcore amine coupling kit (GE Healthcare) using HBS-buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 500 mM NaCl, pH 7.5). Carboxyl groups of the surface were activated by a 35 µL-injection of 100 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 25 mM *N*-hydroxysuccinimide at 5 µL min^{-1}. After reducing the flow to 2 µL min^{-1}, 75 µg of β2-GPI in 100 µL HBS were injected over the specific flow cell (FC) 2. Human transferrin was injected in place of β2-GPI on the reference cell FC1. The net sensorgrams were calculated as difference FC2 – FC1 to correct for refractive bulk effects, unspecific binding phenomena and baseline drift. Biosensor signals were expressed in arbitrary resonance units (RU).

Measurements were conducted at 10 µL min^{-1} with 20 mM HEPES, pH 7.5, supplemented with 300 mM NaCl, 0.2% Tween-20 and 0.1% human serum albumin (HSA) as running and dilution buffer. 45 µL of

Table 1
Single, double and triple positivity of the investigated patients in the laboratory tests for APS.

Positive laboratory tests for APS	Number of patients
only LA	1
only aCL	0
only anti-β2-GPI	0
aCL and LA	2
anti-β2-GPI and LA	0
aCL and anti-β2-GPI	2
aCL and anti-β2-GPI and LA	16

a 10 µg mL^{-1} mab-Acr- or huIgG1κ-solution or of 1:100-diluted serum samples were injected during the association phase. The 300 s long dissociation period was started with a switch to running buffer. Regeneration was performed by a double injection of 5 µL 50 mM glycine NaOH buffer, pH 12.0, containing 0.5% Triton X-100. In the same manner, six HCAL- and EY2C9-concentrations (0.313 – 10 and 0.0313 – 1 nM, respectively) were measured in duplicate to determine affinity constants (according to Regnault *et al.* [30]). Kinetic data were analysed using the BIAevaluation software (GE Healthcare) applying the 1:1 Langmuir binding model. For each β2-GPI preparation, the six APS-sera with the highest binding rates were selected to demonstrate the biosensor binding curves to be caused by antibodies. 3 µl of each serum were mixed with 97 µl HBS and 90 µl of a 1:1:1 mixture of anti-Fc agarose beads against human IgG, IgA and IgM and incubated for 60 min at 25 °C under agitation. The mixture was then centrifuged and the supernatant filled up to a total volume of 270 µl. Finally, 30 µl of 1.65 M NaCl, 2% Tween and 1% HSA in HBS werde added, thereby creating a 1:100-dilution of the initial serum sample in running buffer. By this procedure, >90% of the immunoglobulins of each of the three isotypes were depleted (data not shown). For control purposes, the respective sera were incubated with 90 µl HBS-buffer instead of anti-Fc agarose beads. Every ten measurement cycles, 10 µg mL^{-1} mab-Acr were injected for internal surface stability control.

Table 2
Characteristics of the six investigated β2-GPIs (manufacturers information).

Abbrev.	Manufacturer	Origin	Purification method
nSci	Scipac Ltd. (Sittingbourne, UK)	human serum	1) precipitation with ethanol 2) precipitation with perchloric acid 3) ion exchange chromatography 4) protein A affinity chromatography 5) gel filtration chromatography
nCal	Calbiochem (Merck Biosciences, Darmstadt, Germany)	human plasma	1) precipitation with perchloric acid 2) heparin sepharose chromatography 3) ion exchange chromatography (SP sepharose)
nPha	Phadia GmbH (Freiburg, Germany)	human plasma	1) ligand affinity chromatography 2) gel permeation chromatography
rDia	Diarect AG (Freiburg, Germany)	Sf9 cells	1) immobilized metal ion affinity chromatography 2) ion exchange chromatography
rPha	Phadia GmbH	Sf9 cells	1) ion exchange chromatography 2) hydrophobic interaction chromatography 3) gel permeation chromatography
bPha	Phadia GmbH	bovine serum	two step ligand affinity chromatography

Statistics

The Analyse-it™ (Analyse-it Software, Leeds, UK) add-in program for Windows Excel was used for statistical analysis. Receiver operating characteristic (ROC)–curves were plotted and areas under the curves (AUC) as a measure of the differentiation ability ill vs. healthy were determined; $p_{0.5}$ was calculated to check whether the individual AUCs are significantly different from 0.5 [31] and p_c to test whether the AUCs of two assays differ significantly from each other [32] (significance limit $p_{0.5}/p_c<0.05$). For ROC analysis, all 21 patients were considered to be ill even if negative in the anti-β2-GPI routine assay.

Results

Gel electrophoresis and western blot

The native β2-GPI preparations showed a molecular weight (MW) of ~50 kDa (Fig. 1), whereas the recombinant proteins exhibited a lower and the bovine a higher MW. rPha appeared as double band. For bPha and nPha, an additional band at 70 kDa was visible. In the β2-GPI specific Western Blot (Fig. 1), positive signals corresponding to the bands of the gel around 50 kDa were detected. For bPha, however, no specific band could be observed.

ELISA

In all ELISAs, AUCs significantly exceeded 0.5 ($p_{0.5}<0.0001$ for each isotype with all preparations, Table 3). For the IgG-ELISA, the highest AUCs were found with nSci, and rPha. AUCs decreased with nPha, rDia and bPha and were lowest with nCal (Table 3). These differences were significant when comparing nCal vs. nSci, rPha and nPha (Table 4). IgM-ELISAs except for nCal generally yielded lower AUCs than the respective IgG-ELISA. When comparing the order of decreasing AUCs in the IgM-assay (nPha > nCal > rPha > nSci > rDia > bPha) to that of the IgG-assay particularly nPha and nCal performed better and nSci worse in relation to the other preparations (Table 3). AUC-differences, however, were only significant for nPha compared to bPha (Table 4).

A titer ranking of the patient results of each ELISA system was established and groups with low, medium and high antibody titers of 7 patients in each group were established. When comparing the sample classification across the different β2-GPIs (Table 5) only about one third of the sera were assigned to the same titer class. Most sera switched between the adjacent classes low/medium and medium/high. For a small number of sera even more discrepant results were found in terms of switching between low and high classes or allocation to all three classes.

Biosensor measurements

With huIgG1κ (negative control) no binding event to any of the β2-GPI preparations could be observed, whereas mab-Acr (positive control) exhibited a biospecific binding to all β2-GPIs (211 to 792 RU), except for bPha.

Iterative fitting of the binding curves of the HCAL- and EY2C9-dilutions resulted in the affinity constants shown in Table 6. For HCAL, association (K_A) and dissociation constants (K_D) could be determined for all except one β2-GPI preparation. nSci, nCal, nPha rPha and bPha showed comparable K_A and K_D values ($0.105 - 0.200\times10^{10}$ M^{-1} and $50.0 - 95.5\times10^{-11}$ M, respectively), whereas for rDia affinity constants could not determined as only minute antibody binding occured. The affinity constants for the interaction of EY2C9 with nPha, nSci and rPha were all in the same order of magnitude (K_A 0.449 –

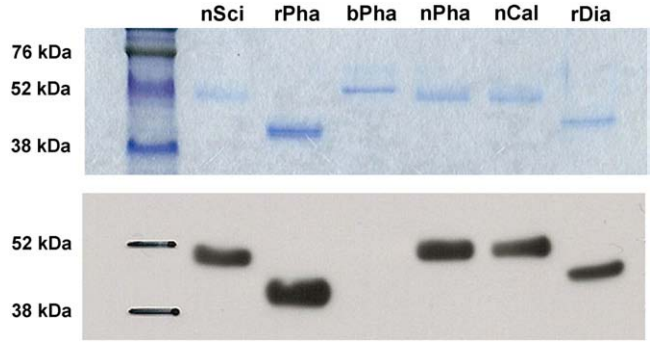

Fig. 1. 10% SDS-PAGE (upper part) and respective Western Blot (lower part) of the six β2-GPI preparations. First lane contains molecular weight marker.

$1.04 \times 10^{10}\ M^{-1}$, K_D 9.61 – 22.3×10^{-11} M). For bPha, nCal and rDia, however, determination of K_A and K_D was not viable due to the lack of significant EY2C9 binding.

The healthy controls exhibited negligible interaction with all biosensor surface-immobilized β2-GPI preparations (Fig. 2). Binding of patient sera was similarly high for nSci and nPha with at maximum 150 RU, except for one APS serum, which even showed a binding of ~200 RU to nSci. Lower maximum binding of patient sera occured with nCal and rPha (~75 RU) with a better discrimination between the study groups on the rPha coated sensor surface. With bPha, only one patient serum raised clearly above the controls (maximum binding 140 RU) whereas all others were found below the threshold level of the controls. Both study groups exhibited virtually no binding to rDia. After serum depletion of IgG, IgM and IgA, specific binding of the APS sera to the immobilized antigen was no longer detectable (Fig. 3) irrespective of the β2-GPI preparation used.

In the SPR biosensor, the discrimination ability was best for nSci and nPha. AUC values decreased from rPha to bPha and were lowest with nCal and rDia (Fig. 4). Except for the two latter preparations, all AUCs were significantly higher than 0.5 (Table 3). When comparing nSci vs. nPha/rPha/bPha, nCal vs. rDia/rPha/bPha and rPha vs. bPha differences in AUCs were insignificant, contrary to the remaining comparisons, which all exhibited significant p-values (Table 7).

Discussion

In this study the influence of six β2-GPI preparations on anti-β2-GPI assay results was investigated. Surprisingly, the MW of the different β2-GPI preparations exhibited major differences in SDS-PAGE which also could be confirmed in the β2-GPI specific Western Blot. Bands of nSci, nCal and nPha match the MW of the native protein (50 kDa) [33]. rDia and rPha were expressed in insect cells and their lower MWs reflect the principal existing, albeit rudimental, glycosylation ability of these cells. The higher MW of bPha can be explained by a differing glycosylation pattern of the bovine protein [34], its missing Western Blot signal by the specificity of the primary antibody for the human protein. Due to the lack of a corresponding Western Blot band, the 70 kDa-bands of bPha and nPha resemble protein contaminations. The double band of rPha might constitute partial protein degradation or subtle glycosylation differences. Yet, the impurities did not affect the discrimination ability of the ELISA and biosensor system.

For evaluation of the discrimination abilities of the investigated test systems, we applied a ROC analysis. We are well aware, that application of ROC analysis to APS is complicated as a clear diagnostic gold standard does not exist. We considered a patient fulfilling the Sydney classification [2] to have APS and therefore this classification as reference. The approach seemed to be justified as these criteria were designed to better define the condition of APS, especially in clinical trials, as they are accepted worldwide, and as therapeutic interventions in clinical routine are based on them. The ROC analysis is the best method to gain information on the overall performance of diagnostic assays. Even if its application to APS is not perfect, it still constitutes the most suitable statistical procedure we have in the setting of this work. Further, for adequate interpretation of our results, the study design has to be kept in mind. 21 consecutive patients diagnosed to have APS were enrolled. No preselection was performed and only patients fulfilling the Sydney criteria were included. This approach resulted in a well characterized study group comprising only patients definitely suffering from APS and not just exhibiting a temporary elevation of – not necessarily APS-associated - anti-β2-GPI antibodies. However, the approach also resulted in a patient group consisting mainly of high risk patients as illustrated by the fact that 16 out of 21 patients were positive in all three APS laboratory tests. Hence, we explicitely point out, that our results as well as the inferences described below are only valid for the examined high risk patients and can not easily be extrapolated to other patient populations.

In all performed ELISAs, the calculated AUCs were significantly higher than 0.5 thereby demonstrating the ability of all β2-GPI preparations to discriminate between ill and healthy states. AUC-differences were only significant when comparing best- with worst-differentiating β2-GPI variants. Hence, the discrimination ability ill vs. healthy of anti-β2-GPI ELISAs is not essentially dependent on the antigen preparation. In relation to the other preparations, bPha surprisingly only performed worse when comparing it to the best discriminating preparation (nPha) in the IgM-assay. The bovine β2-

Table 3
AUC and $p_{0.5}$-values of the six β2-GPIs in the ELISA and biosensor. Significant values bold.

β2-GPI preparation	IgG-ELISA (HCAL-calibration)		IgM-ELISA (EY2C9-calibration)		SPR-biosensor	
	AUC	$p_{0.5}$	AUC	$p_{0.5}$	AUC	$p_{0.5}$
nSci	0.974	<**0.0001**	0.846	<**0.0001**	0.906	<**0.0001**
nCal	0.837	<**0.0001**	0.869	<**0.0001**	0.535	0.3546
nPha	0.957	<**0.0001**	0.882	<**0.0001**	0.985	<**0.0001**
rDia	0.952	<**0.0001**	0.832	<**0.0001**	0.501	0.4952
rPha	0.961	<**0.0001**	0.848	<**0.0001**	0.802	<**0.0001**
bPha	0.931	<**0.0001**	0.785	<**0.0001**	0.744	**0.0009**

Table 4
p_c-values for comparison of the AUC of anti-β2-GPI IgG- (upper right half) and IgM-ELISAs (lower left half) when using different β2-GPIs. Significant values bold.

	nSci	nCal	nPha	rDia	rPha	bPha	
nSci		**0.0198**	0.2234	0.4776	0.1880	0.1315	**IgG**
nCal	0.5718		**0.0307**	0.0650	**0.0264**	0.0823	
nPha	0.2691	0.6539		0.8945	0.7436	0.1437	
rDia	0.7578	0.3472	0.1327		0.7738	0.6247	
rPha	0.9543	0.5414	0.1639	0.5571		0.2553	
bPha	0.3139	0.1128	**0.0411**	0.2827	0.1944		
	IgM						

Table 5
Variations across the six different β2-GPI preparations when classifying the patient samples anti-β2-GPI ELISA results into low, medium and high titers.

Titer ranking in the ELISA with different β2-GPI preparations	IgG-ELISA (HCAL-calibration)	IgM-ELISA (EY2C9-calibration)
no switch	7	9
switch low/medium or medium/high	11	9
switch low/high	0	2
switch low/medium/high	3	1

Table 6
K_A and K_D of HCAL and EY2C9 for the interaction with different β2-GPI preparations (n. d., not detectable).

β2-GPI preparation	HCAL		EY2C9	
	K_A [$\times 10^{10} M^{-1}$]	K_D [$\times 10^{-11} M$]	K_A[$\times 10^{10} M^{-1}$]	K_D [$\times 10^{-11} M$]
nSci	0.200	50.0	1.04	9.61
nCal	0.112	89.6	n. d.	n. d.
nPha	0.105	95.5	0.449	22.3
rDia	n. d.	n. d.	n. d.	n. d.
rPha	0.148	67.4	0.708	14.1
bPha	0.138	72.5	n. d.	n. d.

GPI had been included in the investigation as the prototype of a poorly discriminating preparation. Yet, its discrimination ability turned out to be comparably good as four out of the five human preparations. One might speculate about the proportion of APS-sera with species-specific anti-β2-GPI in our patient group being lower than in other studies which detected insufficient discrimination ability of the bovine protein [35]. Further, it is known, that some patients exhibit anti-β2-GPI that only react in ELISAs coated with bovine β2-GPI but not with human β2-GPI [36]. In case such sera are included in our patient group, they might counterbalance patients only recognizing the human protein. In total, the discrimination ability of the human and the bovine protein, as examined in the presented work, would not be distinguishable. The lower AUCs obtained with the IgM- compared to the IgG-ELISAs in five of the six β2-GPI variants originate from the lower prevalence of anti-β2-GPI IgM in our patient group. Yet, even with HCAL- and EY2C9-calibration, inter-assay comparability was unsatisfactory for both IgG- and IgM-titers. Thus, despite satisfactory discrimination ability, the applied β2-GPI preparation considerably influences the determined anti-β2-GPI titer on the individual patient level. This is due to the fact that the respective protocols for the determination of anti-β2-GPI in the IgG-ELISAs, IgM-ELISAs and SPR-assays were not altered except for the applied β2-GPI preparation. β2-GPI is the only factor varied.

We further elucidated this observation by use of a SPR-biosensor which enables the covalent attachment of β2-GPI to the surface *via* amide bonds formed with one or more of the 30 lysine residues of human β2-GPI [33]. Amine coupling is a standard bioconjugation chemistry successfully applied to the exploration of antigen-antibody interactions in numerous settings. Its suitability for analysis of β2-GPI anti-β2-GPI interactions has been shown by Metzger *et al.* [24]. When used in the biosensor, real-time monitoring of the amine coupling immobilization process assures immobilization of reproducible amounts of protein by setting up a certain association level in the sensorgramm. Hence, the biosensor yields a well defined surface, highly suitable for multiple biomolecular interaction analyses. In this work, the successful immobilization of the different β2-GPI variants as well as good specificity and stability of the chip, as described previously [24], were confirmed by injection of specific control antibodies. The limited reactivity of mab-Acr to bPha is caused by its specificity for the human protein. In this context, we report on K_A and

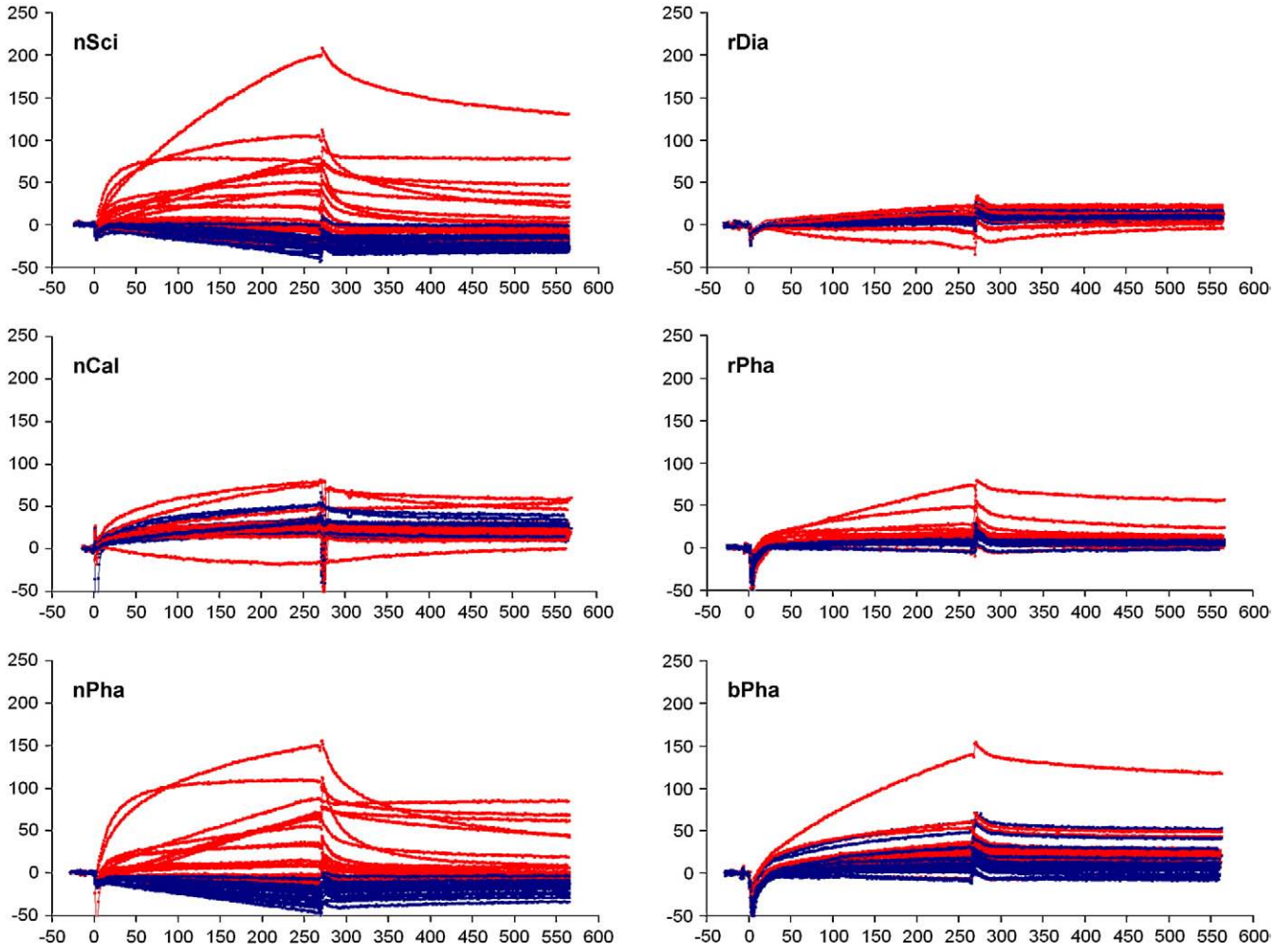

Fig. 2. Sensorgrams of 1:100-diluted sera of APS-patients (red) and healthy controls (blue) on different β2-GPI preparations (x-axis: time [s]; y-axis: binding [ΔRU]).

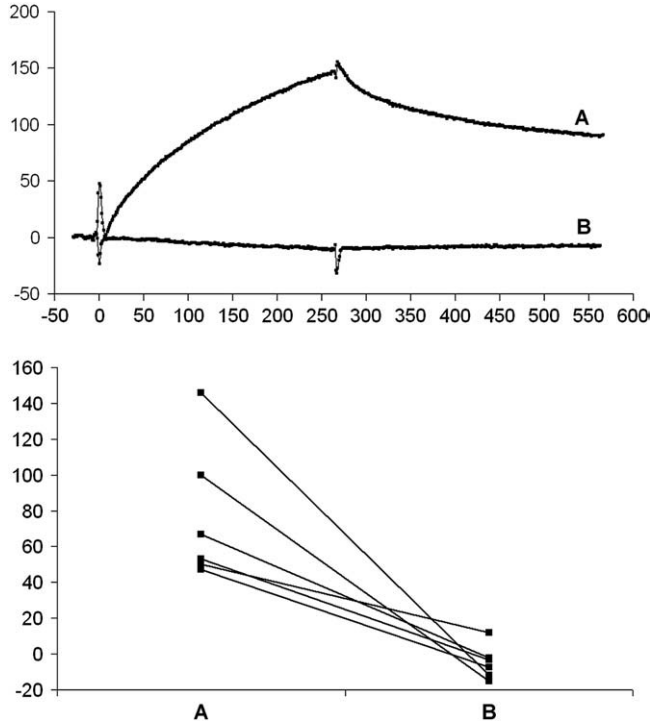

Fig. 3. Immunoglobulin depletion experiments exemplified with nPha (A, undepleted; B, immunogobulin depleted). Upper half: Sensorgrams of the APS-serum with highest binding to nPha (x-axis: time [s]; y-axis: binding [ΔRU]). Lower half: Maximum binding rates of the six selected APS-sera with highest binding to nPha (y-axis: binding [ΔRU]).

K_D data for the HCAL and EY2C9 interaction with β2-GPI. The magnitude of the calculated values is consistent with that of other monoclonal antibodies interacting with β2-GPI [30]. If K_A and K_D were determinable for the respective β2-GPI preparation, values were comparable with those of other preparations for HCAL as well as for EY2C9. Due to the lack of significant antibody binding, valid kinetic analyses were, however, impossible to perform for one β2-GPI with HCAL and three β2-GPIs with EY2C9. This observation clearly demonstrates, that the strength of the monoclonal antibody/β2-GPI interaction varies considerably with the β2-GPI preparation used.

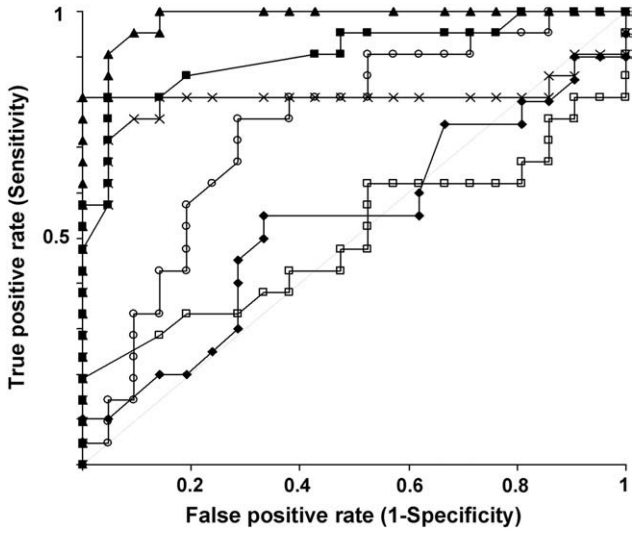

Fig. 4. ROC-curves for discrimination APS-patients vs. healthy controls on different β2-GPI preparations in the biosensor (■, nSci; ♦, nCal; ▲, nPha; □, rDia; ×, rPha; ○, bPha).

Table 7
p_c-values for comparison of the AUCs when using different β2-GPIs in the biosensor system. Significant values bold.

	nSci	nCal	nPha	rDia	rPha	bPha
nSci		**0.0003**	0.0690	**0.0004**	0.2307	0.0819
nCal			<**0.0001**	0.8231	0.0569	0.1294
nPha				<**0.0001**	**0.0261**	**0.0021**
rDia					**0.0016**	**0.0087**
rPha						0.4569
bPha						

Obviously, when different β2-GPI preparations are immobilized, HCAL and EY2C9 target epitopes in domain IV of human β2-GPI [37] are presented to varying extents, resulting in variations of the calibration standards affinities and finally in the poor inter-assay comparability of anti-β2-GPI ELISAs. Thus, the monoclonal calibrators only help to achieve comparable autoantibody titers if the preparation of β2-GPI is carefully characterized.

The study shows large variations of APS-sera binding rates in the biosensor dependent on the immobilized β2-GPI. Significant differences of the various β2-GPIs discrimination abilities in the SPR system exist. Both findings additionally point to a clearly differing immunogenicity of the individual β2-GPI variants. A major drawback of the SPR-biosensor is the sole detection of mass changes at the sensor surface. Hence, APS patients positive binding signals in principle might also be caused by serum components other than antibodies. As a check for selectivity depletion of the endogenous immunoglobulins from the sera resulted in the nearly complete loss of β2-GPI binding activity. Thus, it is evident that the biosenor signals originate from the binding of anti-β2-GPI autoantibodies. As reactivity of serum anti-β2-GPI is considerably affected by the antigen preparation, the heterogeneous behaviour of HCAL and EY2C9 discussed above seems plausible because their F_{ab}-parts originate from lymphocytes of APS patients [27,28]. These observations are in accordance with other studies, showing that purification methods can impair immunoreactivity of β2-GPI to varying extents [38–40] due to protein polymorphisms, variations in glycosylation [39,41,42], proteolytic cleavage [43] and oxidative modifications [44]. Hence, heterogeneous reactivity of serum anti-β2-GPI-autoantibodies to different β2-GPI-variants is a further major cause for the poor inter-assay comparability of the ELISAs.

Our data support the finding that the biosensor is a more sensitive system to untangle variable antigenicity of different β2-GPI variants. This might be true for several reasons: β2-GPI is covalently attached to the chip by a clearly defined bioconjugation strategy which improves reproducibility and comparability of measurement surfaces as compared to the ELISA-coating procedures just relying on unspecific adsorption processes; no washing steps are necessary during interaction analysis, thereby preventing influence of surface active agents; and finally, label-free monitoring in real-time yields deeper insights into the interaction processes than just a single, amplified measurement signal at the end of several incubation and washing steps. We use the SPR-biosensor as a second independent method to support the ELISA data in this study. Despite these advantages we point out that the ß2-GPI chip was not designed to replace the ELISA in the clinical laboratory.

A recent study of Cavazzana *et al.* [45] investigating different β2-GPI variants in the anti-β2-GPI ELISA also observed different levels of protein purity, but found no significant differences in the obtained antibody titers. There are some methodological differences compared to our study. In particular, we calibrated our ELISAs with HCAL and EY2C9 and applied numerous different ß2-GPI preparations. Yet, the primary reason for the discrepant results might be the different approaches in ELISA data analysis. Cavazzana *et al.* distinguished the patients into “negative” and “positive” (according to the respective cut-off) reflecting the discrimination ability. We also showed

comparably good discrimination power of all β2-GPI preparations by ROC-analysis in our study. However, we demonstrated poor inter-assay comparability when classifying the patients into three titer groups according to their anti-β2-GPI titers. This quantitative dimension is missed by just discriminating negative/positive. In a study by Sanmarco *et al.* [46] only APS-patients negative in the in-house standard anti-β2-GPI ELISA were reexamined with another human and an additional bovine antigen preparation. HCAL/EY2C9 calibration was not performed. The repetition of the ELISA did not yield additional information. Also this study design is suboptimal for quantitatively comparing the anti-β2-GPI test results gained with different β2-GPI preparations as it is possible with our data. This is due to the fact that Sanmarco *et al.* only retested a minority (~10%) of the sera and results were compared just semiquantitatively. Our findings, however, agree with a further study of Sanmarco *et al.* [47] which showed variable reactivity of APS-sera towards different β2-GPI-preparations. We confirm these results by an independent method (SPR-biosensor), extend them to IgM isotypes and additionally disclose a similar heterogenous reactivity of the monoclonal calibrators.

In summary, we demonstrated that, in a high risk patient population, despite HCAL and EY2C9 calibration, ELISA-measured anti-β2-GPI titers vary significantly dependent on the antigen preparation. Our biosensor experiments revealed different reactivity of the monoclonal standards and of serum anti-β2-GPI autoantibodies to the six β2-GPIs to be major reasons for the poor inter-assay comparability. It is tempting to assume that the described problems are at least comparable, if not worse with low-positive titers. However, further studies are needed to clarify this issue. Due to their incomplete glycosylation, concerns on the use of rDia and rPha in anti-β2-GPI ELISAs arise. As well, bPha is obviously less suitable to detect antibodies against human β2-GPI: the anti human β2-GPI antibody used for Western Blotting did not recognize bPha, thereby showing convincingly that not all antibodies interacting with human β2-GPI will be identified with a bovine preparation. nCal performed significantly worse than nSci and nPha in the IgG-ELISA and the SPR-system. So, altogether, from the six examined β2-GPI variants, nSci and nPha appear to be the most suitable preparations for the anti-β2-GPI ELISA with a slight advantage for nSci in the IgG- and for nPha in the IgM-assay. However, only the nSci antigen is commercially available. It seems essential that researchers and clinical laboratorians agree on one and just one common β2-GPI preparation irrespective of its actual origin, purification procedure and EY2C9- and HCAL-reactivity. Only a commonly used antigen with defined traceability will improve the requested inter-assay comparability of anti-β2-GPI ELISAs.

Conflict of interest statement

The authors declare that they do not maintain any financial or personal relationships with other people or organisations that could inappropriately influence the presented work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.thromres.2010.05.022.

References

[1] Schwarzenbacher R, Zeth K, Diederichs K, Gries A, Kostner GM, Laggner P, et al. Crystal structure of human beta2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. EMBO J 1999;18:6228–39.
[2] Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295–306.
[3] Reber G, Schousboe I, Tincani A, Sanmarco M, Kveder T, de Moerloose P, et al. Inter-laboratory variability of anti-beta2-glycoprotein I measurement. A collaborative study in the frame of the European Forum on Antiphospholipid Antibodies Standardization Group. Thromb Haemost 2002;88:66–73.
[4] Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa MC. Variability of anti-beta2 glycoprotein I antibodies measurement by commercial assays. Thromb Haemost 2005;94:665–72.
[5] Andreoli L, Malacarne F, Ceribelli A, Kutschera I, Tincani A. Anti-cardiolipin and anti-beta2-glycoprotein I antibodies: performance of new commercial ELISA kits. Ann N Y Acad Sci 2007;1109:531–7.
[6] Audrain MA, Colonna F, Morio F, Hamidou MA, Muller JY. Comparison of different kits in the detection of autoantibodies to cardiolipin and beta2glycoprotein 1. Rheumatology (Oxford) 2004;43:181–5.
[7] Ambrozic A, Kveder T, Bozic B, Rozman B. Comparison of different kits in the detection of autoantibodies to cardiolipin and [beta]2-glycoprotein 1: comment on the article by Audrain et al. Rheumatology (Oxford) 2004;43:1062–3.
[8] Lewis S, Keil LB, Binder WL, DeBari VA. Standardized measurement of major immunoglobulin class (IgG, IgA, and IgM) antibodies to beta2glycoprotein I in patients with antiphospholipid syndrome. J Clin Lab Anal 1998;12:293–7.
[9] Erickson Jr EN, Najmey SS, Keil LB, El-Kadi HS, DeBari VA. Reference calibrators for IgG antibodies to beta2-glycoprotein I: preparation, properties and availability to investigators. Clin Chem 1996;42:1116–7.
[10] Cucnik S, Ambrozic A, Bozic B, Skitek M, Kveder T. Anti-beta2-glycoprotein I ELISA: methodology, determination of cut-off values in 434 healthy Caucasians and evaluation of monoclonal antibodies as possible international standards. Clin Chem Lab Med 2000;38:777–83.
[11] Tsutsumi A, Ichikawa K, Matsuura E, Sawada KI, Koike T. Heterogeneous behavior of anti-beta2-glycoprotein I antibodies on various commercially available enzyme immunoassay plates coated with beta2-glycoprotein I. J Rheumatol 2000;27:391–6.
[12] Cavazzana A, Ruffatti A, Tonello M, Bortolati M, De Moerloose P, Reber G. An analysis of experimental conditions influencing the anti-beta2-glycoprotein I ELISA assay results. Ann N Y Acad Sci 2007;1109:484–92.
[13] Tincani A, Allegri F, Balestrieri G, Reber G, Sanmarco M, Meroni P, et al. Minimal requirements for antiphospholipid antibodies ELISAs proposed by the European Forum on antiphospholipid antibodies. Thromb Res 2004;114:553–8.
[14] Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa MC. Proposals for the measurement of anti-beta2-glycoprotein I antibodies. Standardization group of the European Forum on Antiphospholipid Antibodies. J Thromb Haemost 2004;2:1860–2.
[15] Tincani A, Filippini M, Scarsi M, Galli M, Meroni P. European attempts for the standardisation of the antiphospholipid antibodies. Lupus 2009;18:913–9.
[16] Pierangeli SS, Harris EN. A quarter of a century in anticardiolipin antibody testing and attempted standardization has led us to here, which is? Semin Thromb Hemost 2008;34:313–28.
[17] Reber G, Boehlen F, de Moerloose P. Technical aspects in laboratory testing for antiphospholipid antibodies: is standardization an impossible dream? Semin Thromb Hemost 2008;34:340–6.
[18] de Groot PG, Derksen RH, de Laat B. Twenty-two years of failure to set up undisputed assays to detect patients with the antiphospholipid syndrome. Semin Thromb Hemost 2008;34:347–55.
[19] Andreoli L, Rizzini S, Allegri F, Meroni P, Tincani A. Are the current attempts at standardization of antiphospholipid antibodies still useful? Emerging technologies signal a shift in direction. Semin Thromb Hemost 2008;34:356–60.
[20] Favaloro EJ, Wong RC. Laboratory testing and identification of antiphospholipid antibodies and the antiphospholipid syndrome: a potpourri of problems, a compilation of possible solutions. Semin Thromb Hemost 2008;34:389–410.
[21] Galli M, Reber G, de Moerloose P, de Groot PG. Invitation to a debate on the serological criteria that define the antiphospholipid syndrome. J Thromb Haemost 2008;6:399–401.
[22] Pengo V. A contribution to the debate on the laboratory criteria that define the antiphospholipid syndrome. J Thromb Haemost 2008;6:1048–9.
[23] Wahl D, Thiebaugeorges O, Regnault V, Dalloul A, Lecompte T. Pursuing the debate on the serologic criteria that define the antiphospholipid syndrome. J Thromb Haemost 2008;6:1433–5.
[24] Metzger J, von Landenberg P, Kehrel M, Buhl A, Lackner KJ, Luppa PB. Biosensor analysis of beta2-glycoprotein I-reactive autoantibodies: evidence for isotype-specific binding and differentiation of pathogenic from infection-induced antibodies. Clin Chem 2007;53:1137–43.
[25] Thaler M, Buhl A, Welter H, Schreiegg A, Kehrel M, Alber B, et al. Biosensor analyses of serum autoantibodies: application to antiphospholipid syndrome and systemic lupus erythematosus. Anal Bioanal Chem 2009;393:1417–29.
[26] Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: an update. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. Thromb Haemost 1995;74:1185–90.
[27] Ichikawa K, Tsutsumi A, Atsumi T, Matsuura E, Kobayashi S, Hughes GR, et al. A chimeric antibody with the human gamma1 constant region as a putative

standard for assays to detect IgG beta2-glycoprotein I-dependent anticardiolipin and anti-beta2-glycoprotein I antibodies. Arthritis Rheum 1999;42:2461–70.
[28] Ichikawa K, Khamashta MA, Koike T, Matsuura E, Hughes GR. beta 2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the antiphospholipid syndrome. Arthritis Rheum 1994;37:1453–61.
[29] Nojima J, Kuratsune H, Suehisa E, Futsukaichi Y, Yamanishi H, Machii T, et al. Association between the prevalence of antibodies to beta(2)-glycoprotein I, prothrombin, protein C, protein S, and Annexin V in patients with systemic lupus erythematosus and thrombotic and thrombocytopenic complications. Clin Chem 2001;47:1008–15.
[30] Regnault V, Arvieux J, Vallar L, Lecompte T. Both kinetic data and epitope mapping provide clues for understanding the anti-coagulant effect of five murine monoclonal antibodies to human beta2-glycoprotein I. Immunology 1999;97: 400–7.
[31] Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982;143:29–36.
[32] DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 1988;44:837–45.
[33] Lozier J, Takahashi N, Putnam FW. Complete amino acid sequence of human plasma beta 2-glycoprotein I. Proc Natl Acad Sci U S A 1984;81:3640–4.
[34] Bendixen E, Halkier T, Magnusson S, Sottrup-Jensen L, Kristensen T. Complete primary structure of bovine beta 2-glycoprotein I: localization of the disulfide bridges. Biochemistry 1992;31:3611–7.
[35] Arvieux J, Darnige L, Hachulla E, Roussel B, Bensa JC, Colomb MG. Species specificity of anti-beta 2 glycoprotein I autoantibodies and its relevance to anticardiolipin antibody quantification. Thromb Haemost 1996;75:725–30.
[36] Arvieux J, Regnault V, Hachulla E, Darnige L, Roussel B, Bensa JC. Heterogeneity and immunochemical properties of anti-beta2-glycoprotein I autoantibodies. Thromb Haemost 1998;80:393–8.
[37] Igarashi M, Matsuura E, Igarashi Y, Nagae H, Ichikawa K, Triplett DA, et al. Human beta2-glycoprotein I as an anticardiolipin cofactor determined using mutants expressed by a baculovirus system. Blood 1996;87:3262–70.
[38] Regnault V, Arvieux J, Vallar L, Lecompte T. Immunopurification of human beta2-glycorprotein I with a monoclonal antibody selected for its binding kinetics using a surface plasmon resonance biosensor. J Immunol Meth 1998;211:191–7.
[39] Brighton TA, Dai YP, Hogg PJ, Chesterman CN. Microheterogeneity of beta-2 glycoprotein I: implications for binding to anionic phospholipids. Biochem J 1999;340(Pt 1):59–67.
[40] Passam F, Krilis S. Laboratory tests for the antiphospholipid syndrome: current concepts. Pathology 2004;36:129–38.
[41] Schousboe I. Characterization of subfractions of beta 2-glycoprotein I: evidence for sialic acid microheterogeneity. Int J Biochem 1983;15:35–44.
[42] Gries A, Nimpf J, Wurm H, Kostner GM, Kenner T. Characterization of isoelectric subspecies of asialo-beta 2-glycoprotein I. Biochem J 1989;260:531–4.
[43] Hunt JE, Simpson RJ, Krilis SA. Identification of a region of beta 2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity. Proc Natl Acad Sci U S A 1993;90:2141–5.
[44] Arvieux J, Regnault V, Hachulla E, Darnige L, Berthou F, Youinou P. Oxidation of beta2-glycoprotein I (beta2GPI) by the hydroxyl radical alters phospholipid binding and modulates recognition by anti-beta2GPI autoantibodies. Thromb Haemost 2001;86:1070–6.
[45] Cavazzana A, Pengo V, Tonello M, Noventa F, Grossi C, Borghi MO, et al. Anti-beta (2)-glycoprotein I ELISA assay: The influence of different antigen preparations. Thromb Haemost 2009;101:789–91.
[46] Sanmarco M, Roll P, Gayet S, Oksman F, Johanet C, Escande A, et al. Combined search for anti-beta2-glycoprotein I and anticardiolipin antibodies in antiphospholipid syndrome: contribution to diagnosis. J Lab Clin Med 2004;144:141–7.
[47] Sanmarco M, Bardin N, Blank M, Pascal V, Christine Alessi M, Dignat-George F, et al. Heterogeneity of anti-beta2-glycoprotein I antibodies. A factor of variability in test results. Thromb Haemost 2005;93:80–7.