Optimization of Unnicked β2-Glycoprotein I and High Avidity Anti-β2-Glycoprotein I Antibodies Isolation

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1. Introduction

Recent findings of antiphospholipid syndrome (APS) pathogenesis support the important role of β2-glycoprotein I (β2GPI), as one of the most studied antigens [1–3]. β2GPI is a ~50 kDa protein with a mean plasma concentration in the healthy population of ~180 mg/L. The protein consists of 326 amino acids folded into 5 domains [4, 5]. The first 4 domains contain approximately 60 amino acids, whereas the last, 5th domain consists of 82 amino acids containing specific segments of positively charged amino acids 281 CKNKEKKC 288 and a hydrophobic loop 313 LAFW 316, which along with 19 amino acids of the C-terminal extension form the binding site to negatively charged phospholipids [6, 7]. Plasmin can clip/nick β2GPI at amino acids L317T318 and consequently terminate its ability to bind phospholipids [8]. Furthermore, recently it was observed that β2GPI can exist in different conformations, that is, in a circular form that can change to an open (fishhook) conformation after exposure to anionic structures or negatively charged phospholipids, which can be stabilized by anti-β2GPI antibodies (anti-β2GPI) [9, 10].

The presence of anti-β2GPI in human sera or plasma is one of the defining laboratory criteria for classification of APS [3, 11]. High avidity IgG anti-β2GPI (HAV anti-β2GPI)
represents a subgroup of anti-β2GPI associated with thrombotic [12, 13] and obstetric [14] manifestations in APS patients. On the other hand, several studies in past decades implied that anti-β2GPI were associated with the development of atherosclerosis in autoimmune patients (as reviewed in [15]) and represent a non-traditional risk factor for atherosclerosis-based cardiovascular diseases in patients without overt autoimmunity (reviewed in [16]).

In the context of fully utilizing the preparatory fractions for the isolation of human un nicked β2GPI and HAV anti-β2GPI, as well as optimizing their yield, we evaluated and improved the protocols and procedures/methods involved [17, 18]. The primary aim was to gain unmodified, endogenous proteins in higher yields and purity.

2. Materials and Methods

2.1. Isolation of β2-Glycoprotein I. For isolation of human unnicked β2GPI, AB plasma pooled from apparently healthy donors was used. Isolation included a stepwise procedure combining perchloric acid (PA) precipitation, heparin affinity, and cationic exchange chromatography as determined by Cuncik et al. [17], with modifications performed in the precipitation step. In contrast to the previously used isolation procedure (standard protocol) [17], precipitation with PA was carried out in 3 aliquots of 80–90 mL of plasma starting volume (~250 mL) diluted with an equal volume of physiological solution of sodium chloride. Precipitation was carried out in an ice bath (0°C) using 60% PA, which was added dropwise to a final concentration of 0.285 M. The addition of PA in the optimized protocol lasted ~20 min per aliquot, as compared to the standard protocol which involved a precipitation step lasting ~60 min or more, due to the higher starting volume of plasma (~250 mL). Immediately after precipitation the suspension was centrifuged at 4°C and the pH of supernatants was adjusted to 8.0 with 1 M NaOH. Following precipitation, centrifugation and adjustment of pH, all aliquots were combined and dialyzed against 0.02 M Tris-HCl/0.03 M NaCl, pH 8.0 overnight. The following steps were without modifications as previously described [17]. Briefly, the dialyzed supernatants were concentrated using 350 mL Amicon Stirred Ultrafiltration cell unit (Millipore, Bedford, MA, USA) and Ultrafiltration Membranes from regenerated cellulose with a molecular weight cut-off (MWCO) lower than 10 kDa (Millipore, Carrigtwohill Co., Cork, Ireland). In the next step concentrated dialyzed supernatants were applied to a 10 mm × 20 cm Heparin Sepharose CL-6B column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), which was equilibrated with the same buffer. For elution of bound proteins 0.02 M Tris-HCl/0.35 M NaCl, pH 8.0 was used. Eluted fractions containing proteins were then pooled and dialyzed against 0.05 M acetate buffer/0.05 M NaCl, pH 4.8 overnight and finally applied to a 5 × 50 mm cation exchange column with polystyrene/divinylbenzene matrix and R-CH2-SO3− charged groups (Mono S 5/50 GL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). β2GPI was eluted over a linear gradient starting from 0.05 M NaCl at pH 4.8 to 0.65 M NaCl at pH 5.2 (Na+ in acetate buffer). After the last step (cationic exchange chromatography), three quantitatively different peaks were collected and dialyzed against phosphate buffered saline, pH 7.4 (PBS) with 0.02% NaN3 for 2 h (repeated twice). Protein concentrations were determined by NanoDrop 2000c Spectrophotometer (Thermo Fischer Scientific, Wilmington, Delaware, USA) at a wavelength of 280 nm using the excitation coefficient for β2GPI E1% cm = 10.0. The final preparations were aliquoted and stored at −80°C for further analysis and/or use.

2.2. Polyacrylamide Gel Electrophoresis. The purity of isolated β2GPI was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli protocol [19] in 4% stacking and 10% resolving gels on the Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, isolated protein samples were diluted in SDS sample buffer with 2-mercaptoethanol as reduction agent. Samples were then heated at 98°C for 5 min and afterwards applied to the gels. Electrophoresis was run at 125 V (stacking gel) and 250 V (resolving gel) at 4°C. Staining was carried out with Coomassie Brilliant Blue R250 (CBB) and destaining with a 10% acetic acid solution in 25% ethanol. Gels were scanned using G-Box (Syngene, Cambridge, United Kingdom).

2.3. Mass Spectrometry Analysis. Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS/MS) was used to determine an unknown protein band ~150–250 kDa coming from the 1st peaks of column chromatography and detected on 10% SDS-PAGE. Due to the higher purity demands for ESI-MS/MS analysis (to avoid keratin and any other contamination), samples were run on a premade 75% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) at 250 V in a laminar air flow chamber. All samples and buffers were filter sterilized. After staining with CBB and destaining with 30% methanol, the stained band was cut out and stored at −20°C in sterile autoclaved microtubes for further MS analysis. Further destaining was done with 25 mM ammonium bicarbonate/50% (V/V) acetonitrile and in-gel digestion was performed using mass spectrometry grade modified trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate overnight at 37°C. The resulting peptides were extracted with 50% acetonitrile/5% formic acid (V/V), concentrated and analyzed on an ion trap mass spectrometer 12000 series HPLC-Chip-LC/MSD Trap XCT Ultra (Agilent Technologies, Waldbronn, Germany). MS and MS/MS spectra were searched against the non-redundant National Center for Biotechnology Information (NCBI-nr) database using the Mascot software (Matrix Science Ltd., UK).

2.4. Size Exclusion Chromatography. Size exclusion chromatography was carried out using Sephacryl S-300 HR, as matrix on a 16 mm × 100 cm column (16/100 XK, Pharmacia Biotech, Uppsala, Sweden) in order to separate β2GPI from proteoglycan 4 (PRG4) in the 1st peaks with a fractionation
range for globular proteins from 10 to 1500 kDa [20]. The
1st peaks from several β2GPI isolations were collected and
concentrated with Amicon Ultra centrifugation filters from
regenerated cellulose with MWCO lower than 10 kDa (Millipore,
Carrrigtwohill Co., Cork, Ireland) at 2000 ×g. The final
volume of ~2.5 mL was applied to the column with PBS as the
mobile phase. The flow used was 10 mL/h and the volume of
collected fractions was ~2.3 mL. Fractions from corresponding
peaks were concentrated using Amicon Ultra centrifugation
filters from regenerated cellulose with MWCO 30 kDa
(Millipore Corporation, Billerica, MA, USA) and stored at
~20°C.

2.5. In-House Anti-β2-Glycoprotein I ELISA. In-house anti-
β2GPI ELISA was used to check the functionality of purified
 unnicked β2GPI [21, 22]. The test was conducted with seven
sera samples from different autoimmune patients, positive and
negative controls with respect to IgG, IgM, and IgA
subtypes. Results were presented in arbitrary IgG, IgM, and
IgA titer units (i.e., dividing the starting volume to smaller aliquots which
were precipitated separately) consequently shortened the
duration of precipitation with PA improved the
emphasis on duration of precipitation with PA improved the
isolation procedure. Lowering the precipitation volume
(i.e., dividing the starting volume to smaller aliquots which
were precipitated separately) consequently shortened the
duration of precipitation from an average of 63 ± 6 min to
20 ± 1 min per aliquot. Elution from cationic exchange chro-
matography resulted in three protein peaks (Figure 1). After
purity and functionality check with SDS-PAGE and anti-
β2GPI ELISA, the isolated mass of unnicked human β2GPI
rose significantly from 6.56 ± 1.38 mg (n = 12; range 3.63
to 8.85 mg) using the standard protocol of isolation [17] to

Table 1: β2-Glycoprotein I antigen isolation procedure: comparison of standard and optimized protocols.

|                     | Standard protocol | Optimized protocol | β2GPI isolation from the same starting material after both protocols |
|---------------------|-------------------|--------------------|---------------------------------------------------------------------|
| AB plasma donors (n)| 14                | 6                  | 2                                                                   |
| Number of isolations| 12                | 5                  | 1 (isolation I)                                                     |
| Starting volume, mean ± SD (mL) | 254 ± 24     | 246 ± 8            | 250                                                                 |
| Number of aliquots  | 1                 | 3                  | 1                                                                   |
| Precipitation duration, mean ± SD (min) | 63 ± 6        | 20 ± 1             | 61.25                                                              |
| Mass of isolated β2GPI, mean ± SD (mg) | 6.56 ± 1.38*    | 9.94 ± 1.57*       | 7.22                                                                |
| Efficiency improvement | 51.5%       | 41.4%              |                                                                     |

β2GPI, β2-glycoprotein I. *Significantly higher mass (P = 0.004) as compared to standard protocol (Student’s t-test, two-tail).

2.7. Statistical Analysis and Data Presentation. Data are presented in mean values ± standard deviation (SD). Where statistical analysis was applied, each set of data was normally distributed according to Shapiro-Wilk test. For statistical analysis the two-tail Student’s t-test with significance level P < 0.05 was used.

3. Results
Modification of the β2GPI isolation procedure with special emphasis on duration of precipitation with PA improved the efficiency of the procedure by 51.5% (Table 1, Scheme 1). The precipitation was carried out in three smaller aliquots (~80–90 mL) from similar starting volumes (~250 mL), regardless of the isolation procedure. Lowering the precipitation volume (i.e., dividing the starting volume to smaller aliquots which were precipitated separately) consequently shortened the duration of PA precipitation from an average of 63 ± 6 min to
20 ± 1 min per aliquot. Elution from cationic exchange chromatography resulted in three protein peaks (Figure 1). After purity and functionality check with SDS-PAGE and anti-
β2GPI ELISA, the isolated mass of unnicked human β2GPI rose significantly from 6.56 ± 1.38 mg (n = 12; range 3.63 to 8.85 mg) using the standard protocol of isolation [17] to

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9.94 ± 1.57 mg (n = 5; range 8.69 to 12.56 mg) (P = 0.004), following optimization of the standard protocol.

In one example, isolation data were compared after β2GPI isolation according to standard (isolation I) and optimized (isolation II) protocols from the same starting material, pooled from two AB plasma donors (Table 1, Figure 1). The starting volumes were 250 mL and 255 mL, with the precipitation time ~61 min according to standard and ~21 min per aliquot according to the optimized protocol, respectively. After elution from heparin affinity column the peak was higher in isolation II as compared to isolation I (Figure 1, Panel B). The next step yielded 3 peaks in isolations I and II (Figure 1, Panel C). Since in isolation I the total protein mass of the 3rd peak was very low and the amount of protein C inhibitor was negligible [17], this peak was not further separately examined. The purity check with 10% SDS-PAGE revealed 3 peaks with different quantity of β2GPI in isolation II, which were not different as compared to the band of unnicked β2GPI from the 2nd and 3rd peaks after the standard procedure (Figure 1, Panel D, bands >50 kDa). In the 1st peaks of both isolations a protein band with molecular weight over 250 kDa was present (described below). The functionality check with anti-β2GPI ELISA showed no differences between pure β2GPI isolated after the new optimized protocol (combined 2nd and 3rd peaks— is isolation II) as compared to the unnicked β2GPI isolated according to the standard protocol (2nd peak—isolation I) (Figure 1, Panel E). In this specific case, the mass of isolated unnicked β2GPI rose by more than 41% (from 7.22 mg to 10.21 mg) (Table 1).

In the optimized isolation procedure we observed an impurity of high molecular weight (150–250 kDa) in the 1st peak. The unknown protein impurity of high molecular weight (150–250 kDa) in the 1st peak was not further examined. All isolated samples were established as HAv anti-β2GPI (Table 2). The average efficiency from 48 tests (technical duplicates). Through the improvement of the isolation procedure, the calculated estimate for diagnostic use increased from ~6300 to ~9500 tests, gaining an additional ~3200 tests.

The average efficiency of the isolation procedure for polyclonal IgG anti-β2GPI from plasma on the protein G and β2GPI column was 8.9%, as determined by our in-house anti-β2GPI ELISA (Table 2). The average efficiency from different starting materials, such as immunoadsorption, sera, and plasma was 13.8% (ranging from 6% to 21.4%; data not shown). All isolated samples were established as HAv anti-β2GPI, due to their binding to antigen in PBS-Tw in a higher ionic strength environment (0.5 M NaCl) being ≥80% of the initial binding in PBS-Tw (0.15 M NaCl).

**Table 2**: Isolation of human polyclonal high avidity IgG antibodies against β2-glycoprotein I: a representation of isolation efficiency calculation.

| Starting material (plasma) | V₀ (mL) | 2GPI | m₀ (µg/mL) | mₐₙₜ (µg) |
|---------------------------|---------|------|-------------|------------|
| HAv anti-2GPI column      |         |      |             |            |
| 2GPI before isolation and before and after each step |         |      |             |            |
| 2GPI before isolation and before and after each step |         |      |             |            |

**Concentrations and calculated masses were determined by in-house anti-β2GPI ELISA**. β2GPI: β2-glycoprotein I; HAv anti-β2GPI: high avidity anti-β2GPI IgG antibodies; V₀: volume of plasma used for isolation; m₀: starting concentrations of HAv anti-β2GPI; mₐₙₜ: and mₐₙₛ: calculated masses of HAv anti-β2GPI before isolation and before and after each step; and η: estimated efficiency of isolation.
Panel: Isolation I-standard \[\text{Pooled AB plasma} \rightarrow 1x\] Isolation II-optimized \[\text{Pooled AB plasma} \rightarrow 3x\]

Panel A: Perchloric acid precipitation with the whole volume (V) (isolation I) and in 3 aliquots (isolation II) from the same starting material (pooled plasma from 2 AB donors, see Table 1).

Panel B: Elution chromatograms with NaCl gradient after heparin affinity chromatography.

Panel C: Elution chromatograms with NaCl gradient after cation exchange chromatography.

Panel D: Purity check of protein fractions collected after cationic exchange chromatography as detected by Coomassie Brilliant Blue stained 10% SDS-PAGE (\(\sim 5\) \(\mu\)g of proteins/lane). Indicated are \(\beta_2\) GPI in 2nd and 3rd peaks of isolations I and II, respectively, and PRG4 in 1st peak after optimized protocol (isolation II).

Panel E: Functionality check of isolated \(\beta_2\) GPI. Pure \(\beta_2\) GPI was used as antigen in anti-\(\beta_2\) GPI ELISA. Data is presented in arbitrary IgG, IgM, and IgA units—negative < 2, positive \(\geq\) 2, and high positive \(\geq\) 16. Legend: A.U.: absorbance units; \(\beta_2\) GPI: \(\beta_2\)-glycoprotein I; MM: molecular weight marker; mS/cm: millisiemens per centimeter; PRG4: proteoglycan 4; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

**Figure 1:** Flowchart and graphical data of \(\beta_2\)-glycoprotein I isolation procedure: comparison between isolation data after standard (isolation I) and optimized (isolation II) protocols. Panel A: the perchloric acid precipitation was carried out with the whole volume (V) (isolation I) and in 3 aliquots (isolation II) from the same starting material (pooled AB plasma, see Table 1). Panel B: elution chromatograms with NaCl gradient after heparin affinity chromatography. Panel C: elution chromatograms with NaCl gradient after cation exchange chromatography. Panel D: purity check of protein fractions collected after cationic exchange chromatography as detected by Coomassie Brilliant Blue stained 10% SDS-PAGE (\(\sim 5\) \(\mu\)g of proteins/lane). Indicated are \(\beta_2\) GPI in 2nd and 3rd peaks of isolations I and II, respectively, and PRG4 in 1st peak after optimized protocol (isolation II). Panel E: functionality check of isolated \(\beta_2\) GPI. Pure \(\beta_2\) GPI was used as antigen in anti-\(\beta_2\) GPI ELISA. Data is presented in arbitrary IgG, IgM, and IgA units—negative < 2, positive \(\geq\) 2, and high positive \(\geq\) 16. Legend: A.U.: absorbance units; \(\beta_2\) GPI: \(\beta_2\)-glycoprotein I; MM: molecular weight marker; mS/cm: millisiemens per centimeter; PRG4: proteoglycan 4; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
4. Discussion

β2GPI and anti-β2GPI are important proteins in the pathology of APS, especially since anti-β2GPI represent one of the diagnostic markers/laboratory criteria for the disease classification. The main pathological functions of anti-β2GPI in APS arise from complexes formed by anti-β2GPI binding to β2GPI in the fishhook-open conformation and impacting the vascular system [25].

In order to improve the existing procedure for isolation of unnicked β2GPI, we found that the time of protein exposure to low pH was important. According to recent findings by Ağar et al. [9], we assume that the isolated and solubilized unnicked form of β2GPI is present in circular conformation, whereas in the anti-β2GPI ELISA β2GPI is expected to be unfolded, due to the physical and chemical characteristics of the microtiter plates. This would allow for hydrophobic and ionic binding (with respect to negatively charged carboxyl groups) to the fishhook conformation of β2GPI, making detection of anti-β2GPI possible and reproducible [21, 22]. Brighton et al. reported that purification of β2GPI using PA precipitation yields from 49.7–90.8% of unnicked β2GPI, whereas 9.2–50.3% of purified β2GPI was reported to be in the cleft form, depending on the elution peaks collected in the last step. Observed cleavage occurred at Lys\(^{317}\)-Thr\(^{318}\) and to a lesser extent at Ala\(^{314}\)Phe\(^{315}\) as determined by N-terminal sequencing. Furthermore, in ELISA binding assays they observed no detectable binding to solid-phase anionic groups to the fishhook conformation of β2GPI, making detection of anti-β2GPI possible and reproducible [21, 22].

For diagnostic purposes of APS, the presence and quantity of anti-β2GPI are crucial. A subpopulation of anti-β2GPI specifically HA\(^{v}\) anti-β2GPI, was successfully isolated in the current report. The procedure involved multiple steps [24] including affinity binding onto a column with immobilized unnicked β2GPI. The average and the highest isolation efficiency from human sera, plasma, or immunoadsorption were determined by in-house anti-β2GPI ELISA as 13.8% and 21.4%, respectively. HA\(^{v}\) anti-β2GPI present a clinically relevant subpopulation of anti-β2GPI, which can correlate with thrombosis in patients with APS [12, 13]. This correlation, as well as association of HA\(^{v}\) anti-β2GPI with obstetric complications, was recently reported by Cucnik et al. [14, 30]. These data were confirmed by the European multicenter study analysing HA\(^{v}\) anti-β2GPI that enrolled 226 out of 479 patients with primary APS and APS associated with other autoimmune diseases as well as patients with other non-APS autoimmune diseases [14]. Recently, it was also observed that HA\(^{v}\) anti-β2GPI influenced human coronary artery endothelial cells to release chemotactic and inflammatory cytokines which consequently resulted in a higher migration of peripheral blood mononuclear cells to preconditioned supernatants [24].

5. Conclusions

Modification of the in-house isolation and purification procedures for unnicked β2GPI and polyclonal IgG anti-β2GPI of high avidity led to increased purity of both as well as a substantial elevation in the number of diagnostic tests performed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.
Acknowledgments

This study was approved by the National Ethical Committee (no. 163/02/09) and is in accordance with the Helsinki Declaration of 1975, as revised in 1983, and was supported by the National Research Programme no. P3-0314 from the Ministry of Higher Education, Science and Technology, Slovenia.

References

[1] A. A. Mehdi, I. Uthman, and M. Khamashta, "Antiphospholipid syndrome: pathogenesis and a window of treatment opportunities in the future," European Journal of Clinical Investigation, vol. 40, no. 5, pp. 451–464, 2010.

[2] B. de Laat, K. Mertens, and P. G. de Groot, "Mechanisms of disease: antiphospholipid antibodies—from clinical association to pathologic mechanism," Nature Clinical Practice Rheumatology, vol. 4, no. 4, pp. 192–199, 2008.

[3] E. J. Favalaro and R. C. W. Wong, "Laboratory testing for the antiphospholipid syndrome: making sense of antiphospholipid antibody assays," Clinical Chemistry and Laboratory Medicine, vol. 49, no. 3, pp. 447–461, 2011.

[4] J. Lozier, N. Takahashi, and F. W. Putnam, "Complete amino acid sequence of human plasma β2-glycoprotein I," Proceedings of the National Academy of Sciences of the United States of America, vol. 81, no. 12, pp. 3640–3644, 1984.

[5] F. Lin, R. Murphy, B. White et al., "Circulating levels of β2-glycoprotein I in thrombotic disorders and in inflammation," Lupus, vol. 15, no. 2, pp. 87–93, 2006.

[6] J. E. Hunt, R. J. Simpson, and S. A. Krillis, "Identification of a region of β2-glycoprotein I critical for lipid binding and anti-cardiolipin antibody cofactor activity," Proceedings of the National Academy of Sciences of the United States of America, vol. 90, no. 6, pp. 2141–2145, 1993.

[7] R. Schwarzenbacher, K. Zeth, K. Diederichs et al., "Crystal structure of human β2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome," EMBO Journal, vol. 18, no. 22, pp. 6228–6239, 1999.

[8] D. A. Horbach, E. Van Oort, T. Lisman, J. C. M. Meijers, R. H. W. M. Derksen, and P. G. de Groot, "β2-glycoprotein I is proteolytically cleaved in vivo upon activation of fibronolysis," Thrombosis and Haemostasis, vol. 81, no. 1, pp. 87–95, 1999.

[9] Ç. Ağar, G. M. A. Van Os, M. Mörgelin et al., "β2-Glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome," Blood, vol. 116, no. 8, pp. 1336–1343, 2010.

[10] B. De Laat, R. H. W. M. Derksen, M. Van Lummel, M. T. T. Pennings, and P. G. De Groot, "Pathogenic anti-β2-glycoprotein I antibodies recognize domain I of β2-glycoprotein I only after a conformational change," Blood, vol. 107, no. 5, pp. 1916–1924, 2006.

[11] S. Miyakis, M. D. Lockshin, T. Atsumi et al., "International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS)," Journal of Thrombosis and Haemostasis, vol. 4, no. 2, pp. 295–306, 2006.

[12] S. Čučnik, B. Božič, T. Kveder, M. Tomšič, and B. Rozman, "Avidity of anti-β2-glycoprotein I and thrombosis or pregnancy loss in patients with antiphospholipid syndrome," Annals of the New York Academy of Sciences, vol. 1051, pp. 141–147, 2005.

[13] B. de Laat, R. H. W. M. Derksen, and P. G. de Groot, "High-avidity anti-β2 glycoprotein I antibodies highly correlate with thrombosis in contrast to low-avidity anti-β2 glycoprotein I antibodies," Journal of Thrombosis and Haemostasis, vol. 4, no. 7, pp. 1619–1621, 2006.

[14] S. Cucnik, T. Kveder, A. Artenjak et al., "Avidity of anti-beta2-glycoprotein I antibodies in patients with antiphospholipid syndrome," Lupus, vol. 21, no. 7, pp. 764–765, 2012.

[15] Y. Shoenhfeld, R. Gerli, A. Doria et al., "Accelerated atherosclerosis in autoimmune rheumatic diseases," Circulation, vol. 112, no. 21, pp. 3337–3347, 2005.

[16] A. Artenjak, K. Lakota, M. Frank et al., "Antiphospholipid antibodies as non-traditional risk factors in atherosclerosis based cardiovascular diseases without overt autoimmunity. A critical updated review," Autoimmunity Reviews, vol. 11, no. 12, pp. 873–882, 2012.

[17] S. Cucnik, I. Krizaj, B. Rozman et al., "Concomitant isolation of protein C inhibitor and unnicked beta2-glycoprotein I," Clinical Chemistry and Laboratory Medicine, vol. 42, no. 2, pp. 171–174, 2004.

[18] U. Žager, Š. Irman, M. Lunder et al., "Immunohistochemical properties and pathological relevance of anti-β2-glycoprotein I antibodies of different avidity," International Immunology, vol. 23, no. 8, pp. 511–518, 2011.

[19] J. Omersel, U. Žager, T. Kveder, and B. Božič, "Alteration of antibody specificity during isolation and storage," Journal of Immun assay and Immunochromatography, vol. 31, no. 1, pp. 45–59, 2010.

[20] J. Omersel, I. Avberske-Lužnik, P. A. Grabnar, T. Kveder, B. Rozman, and B. Božič, "Autoimmune reactivity of IgM acquired after oxidation," Redox Report, vol. 16, no. 6, pp. 248–256, 2011.

[21] S. Cucnik, A. Ambrozic, B. Bozic, M. Skitek, and T. Kveder, "Anti-β2-glycoprotein I ELISA: methodology, determination of cut-off values in 434 healthy Caucasians and evaluation of monoclonal antibodies as possible international standards," Clinical Chemistry and Laboratory Medicine, vol. 38, no. 8, pp. 777–783, 2000.

[22] T. Avcin, A. Ambrozic, B. Bozic et al., "Estimation of anticardiolipin antibodies, anti-beta2-glycoprotein I antibodies and lupus anticoagulant in a prospective longitudinal study of children with juvenile idiopathic arthritis," Clinical and Experimental Rheumatology, vol. 20, no. 1, pp. 101–108, 2002.

[23] S. Čučnik, T. Kveder, I. Krizaj, B. Rozman, and B. Božič, "High avidity anti-β2-glycoprotein I antibodies in patients with antiphospholipid syndrome," Annals of the Rheumatic Diseases, vol. 63, no. 11, pp. 1478–1482, 2004.

[24] A. Artenjak, M. Kozelj, K. Lakota et al., "High avidity anti-beta2-glycoprotein I antibodies activate human coronary artery endothelial cells and trigger peripheral blood mononuclear cell migration," European Journal of Immunology, vol. 42, no. 11, pp. 385–396, 2013.

[25] P. L. Meroni, M. O. Borghi, E. Raschi, and F. Tedesco, "Pathogenesis of antiphospholipid syndrome: understanding the antibodies," Nature Reviews Rheumatology, vol. 7, no. 6, pp. 330–339, 2011.

[26] T. A. Brighton, Y. P. Dai, P. J. Hogg, and C. N. Chesterman, "Microheterogeneity of beta-2 glycoprotein I: implications for binding to anionic phospholipids," Biochemical Journal, vol. 340, no. 1, pp. 59–67, 1999.

[27] B. L. Steele, M. C. Alvarez-Veronesi, and T. A. Schmidt, "Molecular weight characterization of PRG4 proteins using multi-angle laser light scattering (MALLS)," Osteoarthritis Cartilage, vol. 21, no. 3, pp. 498–504, 2013.

[28] T. A. Schmidt, N. S. Gestelm, Q. T. Nguyen, B. L. Schumacher, and R. L. Sah, "Boundary lubrication of articular cartilage: role
of synovial fluid constituents,” *Arthritis and Rheumatism*, vol. 56, no. 3, pp. 882–891, 2007.

[29] T. E. Ludwig, J. R. McAllister, V. Lun et al., “Diminished cartilage-lubricating ability of human osteoarthritic synovial fluid deficient in proteoglycan 4: restoration through proteoglycan 4 supplementation,” *Arthritis & Rheumatism*, vol. 64, no. 12, pp. 3963–3971, 2012.

[30] S. Čučnik, T. Kveder, G. Z. Ulcova et al., “The avidity of anti-β2-glycoprotein i antibodies in patients with or without antiphospholipid syndrome: a collaborative study in the frame of the European forum on antiphospholipid antibodies,” *Lupus*, vol. 20, no. 11, pp. 1166–1171, 2011.