Proteins Involved in the Induction of Procoagulant Activity and Autoimmune Response in Patients With Primary Antiphospholipid Syndrome

Débora Medeiros Araújo, MSc1, Carlos Ewerton Maia Rodrigues, PhD2, Nidyedja Goyanna Gomes Gonçalves, PhD3, Carlos Nobre Rabelo-Júnior, PhD4, Marina Duarte Pinto Lobo, PhD5, Renato de Azevedo Moreira, PhD5, and Ana Cristina de Oliveira Monteiro-Moreira, PhD5

Abstract
The aim of this study was to determine the plasma protein profile of patients with primary antiphospholipid syndrome (PAPS) compared to healthy controls and identify proteins that might be used in the evaluation, diagnosis, and prognosis of this condition. The sample consisted of 14 patients with PAPS and 17 sex- and age-matched controls. Plasma samples were submitted to proteomic analysis (albumin and immunoglobulin G depletion, concentration, digestion, and label-free data-independent mass spectrometry). The software ExpressionE was used to quantify intergroup differences in protein expression. The analysis yielded 65 plasma proteins of which 11 were differentially expressed (9 upregulated and 2 downregulated) in relation to controls. Four of these are known to play a role in pathophysiological mechanisms of thrombosis: fibrinogen α chain, fibrinogen β chain, apolipoprotein C-III, and α1-glycoprotein-1. Our analysis revealed autoimmune response and the presence of proteins believed to be functionally involved in the induction of procoagulant activity in patients with PAPS. Further studies are necessary to confirm our findings and may eventually lead to the development of significantly more accurate diagnostic tools.

Keywords
mass spectrometry, protein profile, antiphospholipid syndrome

Date received: 14 August 2019; revised: 02 January 2020; accepted: 15 January 2020.

Introduction
Antiphospholipid syndrome (APS) is an autoimmune disease characterized by repeated thrombotic events and/or gestational morbidity associated with positivity for and often persistently high levels of antiphospholipid antibodies (aPL), including anticardiolipin antibodies (aCL), lupus anticoagulant (LAC), and/or anti-(β2)2-glycoprotein I (anti-β2GPI).1 Primary APS (PAPS) is defined as the presence of aPL in patients with idiopathic thrombosis but no evidence of other autoimmune diseases or other triggering factors such as infection, malignancy, hemodialysis, and drug-induced aPL.2 The prevalence of aPL (1%-5% in apparently healthy individuals) increases with age, especially in association with chronic diseases.3 The mean age at onset of clinical manifestations is 31 years, and the most
common presentations are deep venous thrombosis (DVT; 31.7%), thrombocytopenia (21.9%), stroke (13.1%), superficial thrombophlebitis (9.1%), pulmonary embolism (9.0%), fetal loss (8.3%), transient ischemic attack (7.0%), and hemolytic anemia (6.6%).

Currently, the presence of at least 1 clinical criterion (vascular thrombosis or gestational morbidity) associated with 1 laboratory criterion (LAC, aCL, or anti-β2GPI) is required for the diagnosis of APS. Positive laboratory findings should be confirmed after 12 or more weeks. All 3 parameters should be evaluated to determine the patient’s aPL profile. Different mechanisms have been proposed to explain the role of aPL in the development of thrombosis in patients with APS, such as participation in cell-mediated events (platelets, monocytes, and endothelial cells), activation of the coagulation and complement systems, and inhibition of fibrinolysis.

Advances in genomics and proteomics have helped identify pathogenic mechanisms in a wide range of diseases and made it possible to develop biomarkers signaling changes in protein and peptide levels or posttranslational processes. These new techniques have yielded promising results in various areas of medical research. For example, a study using plasma proteomic analysis to evaluate patients with systemic lupus erythematosus (SLE) identified potential protein biomarkers for APS (apolipoprotein A-1, prothrombin, albumin, transthyretin, and haptoglobin).

In a study on patients with APS, purified monocytes were submitted to proteomic analysis in order to identify proteins associated with the induction of procoagulant activity (annexin A1, annexin A2, Nedd8, RhoA, PDI, and Hsp60). The results of that study are supported by a study on serum protein expression in aPL and non-aPL carriers with gestational morbidity showing a pattern of 9 proteins in the aPL carrier group which might be used as biomarkers in this patient population. However, to our knowledge, no study has used proteomic analysis to compare the plasma protein profile of patients with PAPS and healthy controls. The purpose of the present study was therefore to establish the plasma protein profile of patients with PAPS for use in the evaluation, diagnosis, and prognosis of the condition.

**Patients and Methods**

**Patients**

This study was based on clinical data from an anticoagulation outpatient service in Northeastern Brazil (Ceará Hematology and Hemothrapy Center/HEMOCE, Walter Cantidio University Hospital/HUWC) covering the period from August 2016 to January 2017. Group 1 consisted of 14 patients with PAPS receiving warfarin, classified according to the revised criteria of APS after excluding other causes of thrombosis. Group 2 consisted of 17 sex- and age-matched healthy controls from the community. All participants were submitted to a clinical interview, physical examination, and blood draw. The variables included current age (years), sex, disease duration, thrombotic and obstetric manifestations, aPL profile, medication history (eg, warfarin, aspirin, statins, and estrogens) as well as laboratory test results obtained from medical records or during interviews. The exclusion criteria for group 1 were (1) APS secondary to other connective tissue disorders, (2) other causes of thrombophilia, (3) age under 18 years, and (4) insufficient information to meet the diagnostic criteria for APS. The exclusion criteria for group 2 were (1) clinical suspicion of infection, (2) chronic disease, (3) malignancy, (4) dialysis therapy, and (5) use of any medication at the time of the evaluation.

All participants gave their informed consent. The study protocol complied with the guidelines set forth in Resolution 466/2012 of the National Health Council and was approved by the Research Ethics Committee of the University of Fortaleza and HUWC (file #1.540.556).

**Methods**

**Blood Sampling**

Blood collected by peripheral venous puncture was placed in tubes containing EDTA and centrifuged for 15 minutes. The supernatant (plasma) was stored in polypropylene microtubes at −80°C.

**Protein Quantification**

The plasma samples were individually quantified by absorbance at 280 nm using a NanoVue Plus spectrophotometer (GE Healthcare, Sunnyvale, California), followed by the preparation of a pool for each of the 2 study groups (patients with PAPS and controls). The pools were prepared with equivalent amounts of protein mass from the individual samples contained in each group, totaling 1 pool for each group, with a final concentration of 100 μg protein and a final volume of 1 mL.

**Immunodepletion**

Immunodepletion was performed as described by Lobo et al. For each sample, albumins and immunoglobulin G (IgGs) were removed from the plasma to enrich for less abundant proteins using an albumin and IgG depletion column (HiTrap; GE Healthcare) on a fast protein liquid chromatography system (AKTA Purifier 10; GE Healthcare), following the manufacturer’s instructions. The nonretained material (flow-through fractions) was dialyzed against ultrapure water and concentrated using centrifugal concentrators set to a molecular mass cutoff of 3 kDa (VivaSpin6; GE Healthcare).

**Protein Trypsin Digestion**

Briefly, samples containing 100 μg protein were denatured with 0.2% RapiGest SF (Waters Co, Milford, Massachusetts), reduced with 10 mM dithiothreitol, alkylated with 10 mM iodoacetamide, and enzymatically digested with trypsin (Promega, Madison, Wisconsin). The samples were then centrifuged, and the supernatant was transferred to vials (Waters
Co., Manchester, United Kingdom), and a mixture of enzyme alcohol dehydrogenase (ADH) peptides, 3% acetonitrile, and 0.1% formic acid was added to achieve a final ADH concentration of 100 fmol/μL.10

**Label-Free Data-Independent Mass Spectrometry Analysis**

Quantitative and qualitative nano-UPLC tandem nano-ESI-MS experiments were performed as described by Lobo et al.10 Tryptic peptides were separated using a nanoACQUITY UPLC system (Waters) equipped with an HSS T3 C18 reverse-phase column (1.8 μm, 75 μm × 20 mm). The data-independent analysis (Mass Spectrometry [MS]E)) of tryptic peptides was conducted with a Synapt HDMS mass spectrometer (nanoESI-Qq-oaTOF; Waters, Manchester).

**Data Management, Protein Identification, and Quantification**

All samples were tested in triplicate, and protein identification and quantitative analyses were carried out with algorithms dedicated to searching species-specific databases. The softwares MassLynx version 4.1 and ProteinLynx version 2.4 were used to collect and process the spectra, and the package Protein Lynx Global Server (PLGs) version 2.4 (which contains the software ExpressionE version 2.4) was used to search the appropriate database. The databases UniProtKB/Swiss-Prot 57.1 and UniProtKB/TrEMBL 40.1 were also used, with search parameters set to taxonomy, Homo sapiens (human). The identified proteins were organized by PLGs as a list corresponding to a single protein for both conditions (PAPS vs healthy controls).

**Statistical Analysis**

Clinical and demographic characteristics were expressed as mean values ± standard deviation (continuous variables) or as frequencies and percentages (categorical variables). The collected data were analyzed with the software IBM SPSS Statistics, version 17.0. The groups were compared to Student t test (continuous variables) or Fisher exact test (categorical variables). The logarithmic ratio between the groups was plotted on a scatter plot to visualize intergroup differences. Mean quantitative values were calculated for all samples, and differences between replicates were expressed in P values (P < .05) using the software ExpressionE. Control–PAPS ratios below 0.66 were interpreted as PAPS downregulation, while ratios above 1.5 were interpreted as upregulation. Ratios between 0.66 and 1.5 were considered to be in the normal range.10

**Results**

The sample consisted of 31 individuals (n = 31; Table 1). Group 1 (PAPS, n = 14) consisted of 12 females and 2 males (6:1) aged 42.2 ± 8.9 years. Group 2 (controls n = 17) consisted of 13 females and 4 males aged 36.7 ± 7.3 years. The groups were matched for age (P = .07) and sex (P = .6). All participants in group 1 were receiving warfarin, with international normalized ratio within the therapeutic range of 2 to 3. The controls received no medication.

In group 1 (n = 14), 71.4%, 57.1%, and 21.4% were positive for aCL antibody, LAC, and anti-β2GPI, respectively. When assessed individually, the aPL profiles were associated with aCL positivity only (29%, n = 4), LAC positivity only (29%, n = 4), combined aCL and LAC positivity (21%, n = 3), combined aCL and anti-β2GPI positivity (14%, n = 2), or triple positivity (7%, n = 1). The main clinical manifestations were DVT (64.2%), thrombocytopenia (35.7%), acute myocardial infarction (35.7%), fetal loss (28.5%), central venous thrombosis (14.2%), ischemic stroke (7.14%), and arterial thrombosis (7.14%).

The proteomic analysis yielded 65 plasma proteins of which 11 were differentially expressed (9 upregulated and 2 downregulated) in relation to controls (Table 2). In group 1, the most important upregulated proteins were fibrinogen α chain, fibrinogen γ chain, apolipoprotein C-III (apo-CIII), alpha-1-acid glycoprotein 1 (α1GP1), immunoglobulin heavy constant μ (IgM), lambda immunoglobulin, and J chain immunoglobulin. In contrast, apo-AII and hemoglobin (Hb) subunit delta were downregulated.

**Discussion**

To our knowledge, this is the first study to determine the plasma protein profile of patients with PAPS in relation to healthy controls. The observed profile indicated that our patients with PAPS were functionally at increased risk of thrombotic events and autoimmune manifestations, suggesting that plasma protein profiling may be useful in the assessment and prognosis of this patient population. Our clinical and demographic data allowed to draw a representative profile of APS, while the proteomic analysis revealed a plasma protein pattern that could help clarify the immunopathological mechanism of the disease.

Sex- and age-matched healthy controls with no evidence of autoimmunity or prothrombotic disorders were included in the study to increase the reliability of the proteomic analysis,

---

**Table 1. Profile of Study Participants.**

| Variables | Group 1, n = 14 | Group 2, n = 17 | P Value |
|-----------|----------------|---------------|---------|
| Age, years ± SD | 42.2 ± 8.9 | 36.7 ± 7.3 | .07 |
| Sex, n (%) | | | |
| Female | 12 (85.7%) | 13 (76.6%) | .6 |
| Male | 2 (14.3%) | 4 (23.5%) | |
| Warfarin, n (%) | 14 (100%) | 0 (0%) | |
| ASA, n (%) | 6 (42.8%) | 0 (0%) | |
| Statins, n (%) | 3 (21.4%) | 0 (0%) | |
| Estrogens, n (%) | 2 (14.3%) | 0 (0%) | |

Abbreviations: %, percentage; ASA, acetylsalicylic acid; n, number of participants; P, level of significance; SD, standard deviation.
considering the possibility of APS secondary to SLE associated with other thrombogenic factors inherent to SLE. For example, in a serum protein analysis of patients with SLE, Kazemipour et al.10 identified 15 differentially expressed proteins (7 upregulated and 8 downregulated in relation to healthy controls) which may be used to further explore the mechanisms involved in SLE.

The female–male ratio (6:1) and mean age (42.2 \pm 8.9 years) in our sample match the literature.4 Deep venous thrombosis was the most prevalent clinical manifestation (62.4%), and all patients with PAPS were using warfarin. The observed aPL profile (prevalence of aCL, IgG, and 7% triple positivity) was also compatible with the literature, according to which aCL is the most common antibody found in patients with APS.4,6,11-13

The plasma protein identified with proteomic techniques may be useful in early diagnosis, investigation of pathogenic mechanisms, and monitoring of treatment response and disease progression.13 However, so far, few proteomic analyses of patients with APS have been conducted, and only 1 study has evaluated the protein expression in monocytes of patients with APS. The available evidence suggests that novel proteins may be involved in the pathogenic mechanisms associated with thrombosis in this disease.1

Using serum or plasma for proteomic analysis is advantageous due to the ease of sampling and the high protein content. The disadvantages include low concentrations of plasma protein for the organ or tissue of origin, difficulties in determining whether the protein is involved in the disease process at the time of sampling, and the impossibility of establishing the origin of the protein.14

Moreover, the presence of certain proteins (e.g., albumin and IgG) at high plasma concentrations can make it difficult to identify rare proteins. To circumvent this, methods capable of reducing differences in protein concentration may be used.15 Prior protein depletion allows to enhance the analytical sample power by drastically reducing the concentration of the most abundant components while increasing the concentration of the most diluted ones.16

In this study, patients with PAPS and healthy controls differed with regard to the expression of 11 proteins, some of which play a potential role in the development of APS-related thrombosis.17 All patients with PAPS were using warfarin. To our knowledge, no other study has correlated warfarin use with plasma protein expression investigated by proteomic analysis.

Fibrinogen α chain and fibrinogen β chain were upregulated in our patients with PAPS. In another study, increased plasma fibrinogen concentrations were associated with higher rates of fibrin polymerization—a possible additional risk factor for thrombosis in autoimmune diseases.17 Moreover, fibrinogen is an acute-phase protein18 and a potent predictor of cardiovascular disease that promotes platelet aggregation and stimulates muscle cell proliferation and thrombus formation.17,19-21

Other studies have found high levels of fibrinogen in plasma to be strongly associated with increased risk of vascular death in patients with PAPS22 by inducing endothelial dysfunction and favoring the emergence of atherosclerosis and thromboembolic complications.23 The pathophysiological mechanisms of aPL in thrombotic events in patients with APS are not fully understood, but high plasma fibrinogen levels do appear to predispose patients with PAPS to vascular thrombosis.

Another relevant finding in our study was the upregulation of apo-CIII in patients with PAPS. Despite its small structural value, apo-CIII has deleterious effects on lipoprotein metabolism and on the cell functions involved in atherosclerosis.24 Apo-CIII is found on the surface of 40% to 60% of very low-density lipoproteins (VLDL) and 10% to 20% of low-density lipoproteins (LDL). It interferes with VLDL binding to liver receptors, inhibiting VLDL plasma clearance.25 The association between LDL/apo-CIII and risk of cardiovascular disease is not dependent on LDL concentration, suggesting elevated atherogenicity in apo-CIII-containing lipoproteins.25

In addition to impairing lipoprotein metabolism, apo-CIII has recently been shown to have direct effects on vascular and inflammatory functions.26,27 It stimulates the induction of adhesion molecules to vascular endothelial cells, with subsequent recruitment of circulating monocytes, promoting an inflammatory process that feeds atherosclerosis through the activation of Toll-like receptor 2 in monocytes,28 but it can

### Table 2. Differentially Expressed Proteins Identified by Mass Spectrometry

| Access         | Description                                                                 | Ratio: PAPS/Control | Result    |
|----------------|------------------------------------------------------------------------------|---------------------|-----------|
| IGI_HUMAN      | Immunoglobulin J chain OS Homo sapiens GN JCHAIN PE 1 SV 4                  | 2.00 Up             |           |
| IGLC3_HUMAN    | Immunoglobulin lambda constant 3 OS Homo sapiens GN IGLC3 PE 1 SV 1        | 1.79 Up             |           |
| IGHA1_HUMAN    | Immunoglobulin heavy constant alpha 1 OS Homo sapiens GN IGHA1 PE 1 SV 2   | 1.75 Up             |           |
| IGA2_HUMAN     | Immunoglobulin alpha 2 heavy chain OS Homo sapiens PE 1 SV 1               | 1.69 Up             |           |
| APOC3_HUMAN    | Apolipoprotein C III OS Homo sapiens GN APOC3 PE 1 SV 1                    | 1.64 Up             |           |
| FIBG_HUMAN     | Fibrinogen gamma chain OS Homo sapiens GN FGG PE 1 SV 3                     | 1.64 Up             |           |
| FIBA_HUMAN     | Fibrinogen alpha chain OS Homo sapiens GN FGA PE 1 SV 2                    | 1.59 Up             |           |
| A1AG1_HUMAN    | Alpha 2 acid glycoprotein 1 OS Homo sapiens GN ORM1 PE 1 SV 1              | 1.59 Up             |           |
| IGHM_HUMAN     | Immunoglobulin heavy constant mu OS Homo sapiens GN IGHM PE 1 SV 4         | 1.54 Up             |           |
| APOA2_HUMAN    | Apolipoprotein A II OS Homo sapiens GN APOA2 PE 1 SV 1                     | 0.56 Down           |           |
| HBD_HUMAN      | Hemoglobin subunit delta OS Homo sapiens GN HBD PE 1 SV 2                  | 0.29 Down           |           |

Abbreviation: PAPS, primary antiphospholipid syndrome.
*List of upregulated and downregulated proteins in the PAPS group.
also induce insulin resistance and nuclear factor-κB activation in endothelial cells—a key regulator of inflammation in atherogenesis. These observations suggest apo-CIII is a strong predictor of cardiovascular risk and make its role in atherogenesis more evident. Nevertheless, further studies are required to establish the practical aspects of the laboratory measurement of apo-CIII and new therapeutic targets in view of the fact that the reduction in this protein can reduce the risk of inflamed atherosclerotic plaques and acute thrombotic complications in patients with APS.

Plasma z1GP1 levels were higher in patients with PAPS than in controls. Alpha-1-acid glycoprotein 1 is an acute-phase reagent, disease marker, and immunity modulator capable of mediating drug binding and transport, capillary barrier function maintenance, and sphingolipid metabolism, among others. An association between acute-phase protein levels and cardiovascular disease has been reported, and evidence suggests that total z1GP1 concentration is positively associated with carotid plaque volume, in addition to being a predictor of atherosclerosis severity. Changes in z1GP1 glycoforms have been associated with different pathological conditions, including cardiovascular diseases (acute myocardial infarction and stroke), suggesting this protein may be used as a biomarker for atherosclerosis. Currently, little is known about the role of z1GP1 and its association with cardiovascular outcomes in this patient population.

Levels of IgA, IgM, IgL, and J chain levels were higher in patients with PAPS than in controls. The samples were not submitted to enzyme-linked immunosorbsorbent assay (ELISA) to identify the antigen–antibody complexes, making it impossible to evaluate the immunoglobulin profile, but the observation of high plasma immunoglobulin concentrations in PAPS carriers suggests an association with antigens commonly detected in this condition, such as phospholipids.

Concentrations of apo-AII were low in our patients. The second-most abundant protein component of high-density lipoproteins (HDL), apo-AII, is synthesized almost exclusively in the liver. The contribution of apo-AII to the antiatherogenic effect of HDL is still a matter of dispute. Thus, while some experimental studies have shown that apo-AII reduces susceptibility to atherosclerosis, others have found the overexpression of apo-AII to have a proatherogenic and proinflammatory action due to its ability to stimulate lipid hydroperoxide formation and monocyte transmigration. Further investigations are necessary to fill the gaps in our knowledge of the role of apo-AII in HDL metabolism and to confirm its putative antiatherogenic properties and association with cardiovascular risk in patients with PAPS.

Hemoglobin subunit delta concentrations were also low in our patients with PAPS. According to some authors, the overexpression of Hb in plasma is associated with antioxidant activity. Particularly, Hb has been shown to reduce hydrogen peroxide–induced oxidative stress, indicating that an Hb-rich environment is protective against cell damage. Although the role of Hb in tissue homeostasis during inflammation, tissue injury, and hemolytic and vascular diseases is not yet fully understood, our results support the notion that low plasma Hb concentrations in patients with PAPS favor oxidative stress induced by aPL in the process of clot formation and development of atherosclerosis.

Our study has some limitations: (1) warfarin, statins, and estrogen can influence plasma protein composition and the proteomics of patients with PAPS; (2) no other technique (ELISA, Western blotting, immunohistochemistry, or real-time polymerase chain reaction) was employed to corroborate the protein profile found by MS; (3) the causal relationship between the protein expression pattern and the time frame of these proteins in relation to the clinical event (especially with regard to clinical manifestations and antiphospholipid antibody status) could not be assessed due to the study’s cross-sectional design; and (4) protein levels could not be compared between APS-related and non-APS-related, between APS with and without venous thromboembolism (VTE) or between non-APS-related autoimmune disorders and VTE.

Four proteins apparently related to thrombotic risk prediction and autoimmune response in individuals with PAPS were identified: fibrinogen α-chain, fibrinogen γ-chain, apoC-III, and z1GP1. These proteins are functionally involved in processes mainly associated with the induction of a procoagulant state and with autoimmunity response, but further studies are necessary to confirm our findings. The use of these proteins could help identify patients at increased risk of thromboembolic complications and eventually lead to the development of significantly more accurate diagnostic tools.

Acknowledgments
The authors would like to thank all the patients and controls who participated in the study.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD
Ana Cristina de Oliveira Monteiro-Moreira https://orcid.org/0000-0002-3606-6808

References
1. López-Pedrera C, Cuadrado MJ, Herández V, et al. Proteomic analysis in monocytes of antiphospholipid syndrome patients: deregulation of proteins related to the development of thrombosis. Arthritis Rheum. 2008;58(9):2835-2844.
2. Piette JC, Wechsler B, Frances C, Papo T, Godeau P. Exclusion criteria for primary antiphospholipid syndrome. J Rheumatol. 1993;20(10):1802-1804.
3. Petri M. Epidemiology of the antiphospholipid antibody syndrome. J Autoimmun. 2000;15(2):145-151.
4. Cervera R, Piette JC, Font J, et al. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum.* 2002;46(4):1019-1027.

5. 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(2):295-306.

6. Sciascia S, Amigo MC, Roccatello D, Khamashta M. Diagnosing antiphospholipid syndrome: “extra-criteria” manifestations and technical advances. *Nat Rev Rheumatol.* 2017;13(9):548-560.

7. Cuadrado MJ, Aguirre MA, Barbarroja N, Khamashta MA, Lopez-Pedrera Ch. Proteomics in antiphospholipid syndrome: a review. *Lupus.* 2010;19(4):385-388.

8. Kazemipour N, Qazizadeh H, Sepehrimanesh M, Salimi S. Biomarkers identified from serum proteomic analysis for the differential diagnosis of systemic lupus erythematosus. *Lupus.* 2015;24(6):582-587.

9. Alvarez AM, Neubeck S, Parra S, Markert UR, Cadavid AP. Serum protein profile in women with pregnancy morbidity associated with antiphospholipid syndrome. *J Hum Reprod Sci.* 2017;10:10-17.

10. Lobo MD, Moreno FB, Souza GH, et al. Label-free proteome analysis of plasma from patients with breast cancer: stage-specific protein expression. *Front Oncol.* 2017;7:14. doi:10.3389/fonc.2017.00014.

11. Houghton D, Mol S. Antiphospholipid antibodies. *Vasc Med.* 2015;22:545-550.

12. Brock CO, Brohl AS, Obiˇcan SG. Incidence, pathophysiology, and clinical manifestations of antiphospholipid syndrome. *Birth Defects Res C Embryo Today.* 2015;105(3):201-208.

13. Chan KC, Lucas DA, Hise D, et al. Analysis of the human serum proteome. *Clin Proteomics.* 2004;1(2):101-225.

14. Di Girolamo F, Del Chierico F, Caenaro G, Lante I, Muraca M, Putignani L. Human serum proteome analysis: new source of markers in metabolic disorders. *Biomark Med.* 2012;6(6):759-773.

15. Righetti PG, Boschetti E, Lomas L, Citterio A. Protein equalizer technology: the quest for a “democratic proteome”. *Proteomics.* 2006;6(3):3980-3992.

16. Keidel E, Ribitsch D, Lottspeich F. Equalizer technology—equal rights for disparate beads. *Proteomics.* 2010;10(11):2089-2098.

17. de Pablo P, Ramirez A, Cortina E, et al. Increased fibrin polymerization rate in patients with primary antiphospholipid syndrome and systemic lupus erythematosus. *Clin Appl Thromb Hemost.* 2003;9(3):221-225.

18. Amrani D. Regulation of fibrinogen biosynthesis: glucocorticoid and interleukin-6 control. *Blood Coagul Fibrinolysis.* 1990;1(4-5):443-446.

19. Ernst E, Resch KL. Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature. *Ann Intern Med.* 1993;118(12):956-963.

20. Wang J, Tan GJ, Han LN, et al. Novel biomarkers for cardiovascular risk prediction. *J Geriatr Cardiol.* 2017;14(2):135-150.

21. Papageorgiou N, Tousoulis D, Miliou A, et al. Combined effects of fibrinogen genetic variability on atherosclerosis in patients with or without stable angina pectoris: focus on the coagulation cascade and endothelial function. *Int J Cardiol.* 2013;168(5):4602-4607.

22. Ames PR, Merashi M, Chis Ster I, et al. Survival in primary antiphospholipid syndrome. A single-centre cohort study. *Thromb Haemost.* 2016;115(6):1200-1208.

23. Stalc M, Poredos P, Peternel P, Tomsic M, Sebestjen M, Kveder T. Endothelial function is impaired in patients with primary antiphospholipid syndrome. *Thromb Res.* 2006;118(4):455-461.

24. Yao Z. Human apolipoprotein C-III—a new intrahepatic protein factor promoting assembly and secretion of very low density lipoproteins. *Cardiovasc Hematol Disord Drug Targets.* 2012;12(2):133-140.

25. Mendivil CO, Rimm EB, Furtado J, Chiuve SE, Sacks FM. Low-density lipoproteins containing apolipoprotein C-III and the risk of coronary heart disease. *Circulation.* 2011;124(19):2065-2072.

26. Chan DC, Chen MM, Ooi EM, Watts GF. An ABC of apolipoprotein C-III: a clinically useful new cardiovascular risk factor? *Int J Clin Pract.* 2008;62(5):799-809.

27. Lacey B, Herrington WG, Preiss D, Lewington S, Armitage J. The role of emerging risk factors in cardiovascular outcomes. *Curr Atheroscler Rep.* 2017;19(6):28.

28. Kawakami A, Osaka M, Aikawa M, et al. Toll-like receptor 2 mediates apolipoprotein C-III-induced monocyte activation. *Circ Res.* 2008;103(12):1402-1409.

29. Kawakami A, Aikawa M, Alcaide P, Luscsinskas FW, Libby P, Sacks FM. Apolipoprotein CIII induces expression of vascular cell adhesion molecule-1 in vascular endothelial cells and increases adhesion of monocytic cells. *Circulation.* 2006;114(7):681-687.

30. Luo Z, Lei H, Sun Y, Liu X, Su DF. Orosomucoid, an acute response protein with multiple modulating activities. *J Physiol Biochem.* 2015;71(2):329-340.

31. Råstam L, Lindberg G, Folsom AR, Burke GL, Nilsson-Ehle P, Lundblad A. Association between serum sialic acid concentration and carotid atherosclerosis measured by B-mode ultrasound. The ARIC investigators. Atherosclerosis risk in communities study. *Int J Epidemiol.* 1996;25(5):953-958.

32. Gronnholt ML, Sillesen H, Wiebe BM, Laursen H, Nordestgaard BG. Increased acute phase reactants are associated with levels of lipoproteins and increased carotid plaque volume. *Ear J Vasc Endovasc Surg.* 2001;21(3):227-234.

33. Berntsson J, Östling G, Persson M, Smith JG, Hedblad B, Engström G. Orosomucoid, carotid plaque, and incidence of stroke. *Stroke.* 2016;47(7):1858-1863.

34. Puerta A, Martin-Alvarez PJ, Ongay S, Diez-Masa JC, de Frutos M. Immunoaffinity, capillary electrophoresis, and statistics for the differential protein expression in plasma from patients with breast cancer: stage-specific protein expression. *Proteomics.* 2007;759-773.

35. Willis R, Papalardo E, Nigel Harris E. Solid phase immunoassay and clinical and immunologic manifestations and patterns of disease. *Int J Geriatr Cardiol.* 2017;14(2):135-150.
37. Tailleux A, Bouly M, Luc G, et al. Decreased susceptibility to diet-induced atherosclerosis in human apolipoprotein A-II transgenic mice. *Arterioscler Thromb Vasc Biol*. 2000;20(11):2453-2458.

38. Escolà-Gil JC, Julve J, Marzal-Casacuberta A, Ordóñez-Llanos J, González-Sastre F, Blanco-Vaca F. ApoA-II expression in CETP transgenic mice increases VLDL production and impairs VLDL clearance. *J Lipid Res*. 2001;42(2):241-248.

39. Warden CH, Hedrick CC, Qiao JH, Castellani LW, Lusis AJ. Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II. *Science*. 1993;261(5120):469-472.

40. Liu W, Baker SS, Baker RD, et al. Up-regulation of hemoglobin expression by oxidative stress in hepatocytes and its implication in nonalcoholic steatohepatitis. *PLOS One*. 2011;6:1-8.

41. Widmer CC, Pereira CP, Gehrig P, et al. Hemoglobin can attenuate hydrogen peroxide-induced oxidative stress by acting as an antioxidative peroxidase. *Antioxid Redox Signal*. 2010;12(2):185-198.

42. Bai J, Sadrolodabae L, Ching CB, Chowbay B, Ning Chen W. Comparative proteomic analysis of HepG2 cells incubated by S(-) and R(+) enantiomers of anti-coagulating drug warfarin. *Proteomics*. 2010;10(7):1463-1473.

43. López-Pedrera C, Ruiz-Limón P, Aguirre MA, et al. Global effects of fluvastatin on the prothrombotic status of patients with antiphospholipid syndrome. *Ann Rheum Dis*. 2011;70(4):675-682.

44. Kaliterna DM, Radić M, Ljutić D. Does estrogen stimulate the pathogenic sort of anticardiolipin antibodies? *Isr Med Assoc J*. 2014;16:197-198.