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

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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 Expression^E 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 α chain, apolipoprotein C-III, and α-1-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

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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-$(\beta)_2$-glycoprotein I (anti-β_2GPI).[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

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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%).[4]

Currently, the presence of at least 1 clinical criterion (vascular thrombosis or gestational morbidity) associated with 1 laboratory criterion (LAC, aCL, or anti-β_2GPI) 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.[5] 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.[6]

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.[7] 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).[8]

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).[1] 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.[9] 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 Hemotherapy Center/HEMOCE, Walter Cantídio 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[5] 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.[5] 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.[10] 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 (ÄKTA 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^E 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-*oa*TOF; 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 Expression^E 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 Expression^E. 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.

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 (14.2%)	0 (0%)	

Abbreviations: %, percentage; ASA, acetylsalicylic acid; n, number of participants; *P*, level of significance; SD, standard deviation.

In group 1 (n = 14), 71.4%, 57.1%, and 21.4% were positive for aCL antibody, LAC, and anti-β_2GPI, 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-β_2GPI 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 α1 and α2 (IgA1 and IgA2, respectively), immunoglobulin heavy constant mu (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 2. Differentially Expressed Proteins Identified by Mass Spectrometry.[a]

Access	Description	Ratio: PAPS/ Control	Result
IGJ_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 PE1 SV2	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 1 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.
[a]List of upregulated and downregulated proteins in the PAPS group.

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[8] 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 (eg, 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 α 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 protein[18] 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 PAPS[22] 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

also induce insulin resistance and nuclear factor-κB activation in endothelial cells—a key regulator of inflammation in atherogenesis.[29] 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.[29]

Plasma α1GP1 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.[30] An association between acute-phase protein levels and cardiovascular disease has been reported, and evidence suggests that total α1GP1 concentration is positively associated with carotid plaque volume, in addition to being a predictor of atherosclerosis severity.[31-33] Changes in α1GP1 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.[33,34] Currently, little is known about the role of α1GP1 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 immunosorbent assay (ELISA) to identify the antigen–antibody complexes,[35] 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.[36] 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,[37] 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.[38,39] 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.[40] Particularly, Hb has been shown to reduce hydrogen peroxide–induced oxidative stress, indicating that an Hb-rich environment is protective against cell damage.[41] 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,[42] statins,[43] and estrogen[44] 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 α1GP1. These proteins are functionally involved in processes mainly associated with the induction of a procoagulant state and with autoimmune 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.

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