

Serum Protein Profile in Women With Pregnancy Morbidity Associated With Antiphospholipid Syndrome

Angela M. Alvarez, Stefan Neubeck¹, Sergio Parra², Udo R. Markert¹, Angela P. Cadavid

Reproduction Group, Department of Microbiology and Parasitology, School of Medicine, Universidad de Antioquia, Medellín, Colombia, ¹Department of Obstetrics, University Hospital Jena, Jena, Germany, ²Department of Pharmacology and Toxicology, Universidad de Antioquia, Medellín, Colombia

ABSTRACT

Context: Antiphospholipid antibodies (aPL) are related with a high risk of pregnancy morbidity (PM) and also of vascular thrombosis. On the basis of recent studies, we expect that in women with PM associated with antiphospholipid syndrome (APS), further factors may be deregulated and involved in pathophysiology of the disease. Such factors may have the potential to become novel biomarkers for APS and its stages. **Settings and Design:** Descriptive study from a recurrent pregnancy loss program. **Aims:** To study the protein expression in sera from women with PM with or without aPL. **Materials and Methods:** Protein profiles were determined by surface enhanced laser desorption and ionization – time of flight mass spectrometry (SELDI-TOF MS) in the serum samples from women with PM, 10 of them with aPL and 12 without aPL. On the basis of the mass-to-charge ratio (*m/z*) of the protein, signals differentially expressed between the two groups were compared with data banks to approach candidate proteins. **Statistical Analysis Used:** To determine the differential expression of each protein, a no paired *t*-test was performed using CIPHERGEN Express Client 3.1 software. **Results:** SELDI-TOF analysis makes it possible to discriminate between several proteins in women with PM with and without aPL, although it does not allow protein identification. Nine proteins were found in significantly higher levels in aPL-positive women. **Conclusion:** The results underline that further factors beyond autoantibodies are involved in PM associated with APS and might lead to the development of new biomarkers.

KEYWORDS: Antiphospholipid antibodies, pregnancy loss, proteomics, SELDI-TOF MS

INTRODUCTION

Antiphospholipid syndrome (APS) is characterized by clinical manifestations of thrombosis (arterial, venous, or small vessel) in different vascular territories, pregnancy morbidities [early or late gestational losses, intrauterine growth restriction, and preterm labor or preeclampsia (PE)], and the persistent presence of antiphospholipid antibodies (aPL).^[1] aPL are a heterogeneous auto-antibody subset directed against negatively charged phospholipids, and they can also recognize phospholipid binding proteins such as annexin-V, proteins C and S, prothrombin, and β_2 glycoprotein I (β_2 GPI).^[2] Cell surfaces can potentially bind β_2 GPI from the bloodstream while negative phospholipids are exposed; however, trophoblasts can permanently expose these phospholipids, thus allowing β_2 GPI binding, and in addition, they can synthesize their own β_2 GPI and express it in

their plasma membrane, turning placenta into a target of anti- β_2 GPI antibodies.^[2-4]

aPL could induce its deleterious effects on the cells through antigen-antibody complex formation on cell surfaces. This complex induces activation of several signaling pathways, transcription processes, and expression of adhesion molecules and pro-coagulant proteins, leading to a thrombogenic and inflammatory state. These effects have been demonstrated using endothelial cells, monocytes, and platelets.^[5] In the

Address for correspondence: Dr. Angela Patricia Cadavid, PhD, Reproduction Group, Department of Microbiology and Parasitology, School of Medicine, Universidad de Antioquia, Carrera 53 No. 61-30, Laboratory 534, Medellín, Colombia. E-mail: angela.cadavid@udea.edu.co

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How to cite this article: 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;10:10-7.

Access this article online

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10.4103/0974-1208.204018

pregnancy context, current reports suggest that thrombotic mechanisms are not sufficient to explain the pregnancy losses associated with APS. Consequently, it is presently thought that pregnancy failure in women with obstetric APS involves alterations on normal trophoblast functions such as migration, invasion, differentiation, or hormone production that leads to an impaired placental development.^[2,3,6-8]

Currently, the laboratory criteria involved in APS diagnosis are the positive test for anticardiolipin antibodies (aCL), anti- β_2 GPI antibodies, or lupus anticoagulant (LA).^[1] However, another group of aPL, such as antiphosphatidic acid (aPA), antiphosphatidylinositol (aPI), and antiphosphatidylserine (aPS) antibodies, have been associated to APS and are known as non-criteria aPL.^[9] The most extensively investigated aPL in thrombosis and pregnancy morbidity (PM)-related APS are aPS antibodies, and some investigators have suggested that testing for these non-criteria aPL may help identify women with recurrent pregnancy loss who could benefit of the treatment for PM associated to APS.^[9]

The awareness for pregnancy losses associated with APS is rising because it has often remained undiagnosed and untreated with unfortunate consequences.^[10] The search for biomarkers for the diagnosis of APS is increasing, while clinical applications of proteomics have been focused on cardiovascular risk and thrombosis.^[11-14] Although there are very few qualified biomarkers for APS diagnosis that have already been identified through proteomics assessment,^[13] Ripoll *et al.*^[15] recently reported the upregulated expression of six proteins in human monocytes treated with polyclonal immunoglobulin G (IgG) from APS patients [both vascular thrombosis (VT) and PM groups]. In the human reproductive field, we recently used a surface enhanced laser desorption and ionization – time of flight (SELDI-TOF) approach to identify protein expression in seminal plasma from fertile and infertile men.^[16] Since it is still expected that advances in proteomic technology could help identify potential biomarkers of the disease and lead to improvement of their management and prognosis, in this study, we investigated serum protein targets associated with obstetric APS using the proteomic mass spectrometry technology, SELDI-TOF.

MATERIALS AND METHODS

The serum samples were obtained from 22 women who attended a recurrent pregnancy loss program in our group. The Ethics Review Committee of our University approved the collection of their serum, and written consent was obtained. All of them fulfilled clinical criteria for PM. In all the samples, aCL were tested using an in-house Enzyme-linked immunosorbent assay (ELISA),^[17,18] and were also tested using a commercial aCL ELISA kit (BioSystems, Barcelona, Spain). Anti- β_2 GPI antibodies were detected using the

Quanta Lite ELISA kit (INOVA Diagnostics, San Diego, USA). LA was measured using diluted Russell's Viper Venom Time test (Rochem Biocare, Bogotá, Colombia). All antibodies were tested twice, at least 12 weeks apart. In addition, other aPL were detected by in-house ELISA [aPA, aPI, aPS, and also anti-phosphatidylglycerol (aPG)]. Finally, 10 of them were positive to the presence of aPL (aPL-positive group), and 12 women presented PM in the absence of aPL (aPL negative group).

Stored serum samples were centrifuged at 14,000×g for 10 min at 4°C. The amount of total serum proteins was measured using a NanoDrop[®] spectrophotometer, and then the samples were diluted to 0.5 mg/ml in binding buffer (0.1 M Tris-HCl, 0.02% Tween 20%, pH 9.0). Anionic Q10 ProteinChip[®] (Ciphergen Biosystems, CA, USA) was used here. Protein chip analysis was performed according the manufacturer's specifications. Briefly, the spots were equilibrated three times with 5 μ l binding buffer for 5 min and then 5 μ l of each sample was placed on each spot. After incubation for 90 min in a humid chamber, each spot was washed twice with 5 μ l binding buffer and twice with 5 μ l distilled water. After air-drying, the spots were coated with 0.5 μ l of energy-absorbing matrix (EAM: 20 mg/ml sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid), to facilitate desorption and ionization of proteins. Chips were air-dried again during 5 min, and then another 0.5 μ l of EAM was applied.

The protein chip arrays thus prepared were placed in a ProteinChip SELDI System Series 4000 (Ciphergen) for reading. Arrays were exposed to 2200 nJ laser energy to detect proteins of low molecular weights (2000–20,000 Da), as well as to 3500 nJ for proteins in the high molecular weight range (20,000–200,000 Da) at a pressure of <150 μ Pa, according a protocol established in Placentabor – University Hospital, Jena-Germany. The sample reading was performed randomly to avoid bias handling of the samples or the protein chips. All these SELDI-TOF MS analyses were performed at the Institute for Human Genetics – University Hospital Jena.

The spectra were analyzed using the Ciphergen Express Client 3.1 software (Ciphergen) that generates a consistent peak for comparison between two groups, with automatic data analysis. Mean values were evaluated by the Student's *t*-test. Receiver operating characteristic curve analysis was also performed to assess the predictive value of the peaks observed and the results are expressed as area under the curve, and a cutoff value was selected for optimal sensitivity and specificity.

RESULTS

Demographic characteristics of the women from both groups are described in Table 1. There were no significant differences in the age or number of pregnancy losses between the groups. Sera samples had an average

Table 1: Summary of clinical and laboratory characteristics among the women included

Parameter	aPL-positive (n = 10)	aPL-negative (n = 12)
Age (mean ± SEM years)	33.30 ± 1.48	31.92 ± 1.44
Previous fetal losses no. (mean and range)		
<10 weeks of gestation	2.00 (1–4)	2.27 (1–2)
≥10 weeks of gestation	1.20 (1–2)	1.00
Preeclampsia at <34 weeks, no.	2	0
Arterial/venous thrombosis, no.	2	0
Positive aCL patients, no. (mean ± SEM GPL) [†]	4 (32.66 ± 12.86)	0
Positive anti-β ₂ GPI patients, no. (mean ± SEM SGU) [‡]	3 (59.86 ± 15.83)	0
Positive LA, no.	2	0
Positive aCL patients by in-house ELISA, no. (% of control) [§]	9 (56.5%)	0
Positive other aPL patients by in-house ELISA, no. (% of control) [§]	Anti-GPA: 3 (72%) Anti-GPI: 7 (67%) Anti-GPG: 5 (102%) Anti-GPS: 4 (73.5%)	0 0 0 0

Anti-GPA = anti phosphatidic acid antibodies, Anti-GPI = anti phosphatidyl inositol antibodies, Anti-GPG = anti phosphatidyl glycerol antibodies, Anti-GPS = anti phosphatidyl serine antibodies, LA = lupus anticoagulant, GPL = IgG phospholipid units, SGU = standard IgG units. [†]Positive at >10 GPL. [‡]Positive at >20 SGU. [§]Positive at >25% control.

protein concentration of 88.5 mg/ml. In aPL-positive group, two women presented VT in addition to their pregnancy losses and other women did not fit the international laboratory criteria of APS because they had other aPL such as aPA, aPI, aPG, and aPS antibodies. However, they were included because it is important to evaluate the relationship between the presence of other aPL and PM (i.e., non-criteria aPL).^[19] In addition, we recently found that sera from these women induced decreased trophoblast migration and trophoblast–endothelial cell interaction.^[19] Even though our research group is a reference center for women with recurrent pregnancy losses in Medellín, few women have been well characterized with international clinical and laboratory diagnostic criteria for APS; this is the reason for the low number of patients in the present study.

A representative spectrum in the range of 12,500–15,000 Da of a serum sample from an aPL-positive woman exposed to 2200 nJ laser energy to detect proteins in the high molecular weight range is shown in Figure 1. Two peaks in the low molecular weight range corresponding to small proteins (2000–20,000 Da) and seven peaks in the high-molecular weight range corresponding to large proteins (20,000–200,000 Da) were consistently present and at increased levels in the aPL-positive group [Figure 2], and their respective mass/charge (*m/z*) value was obtained. Differential expression of all the proteins was analyzed using a heat map. Figure 3a and b shows a hierarchically organized dendrogram based on protein similarities (*y*-axis) in each serum sample.

SELDI-TOF analysis provides a good discrimination of distinct proteins in a complex mixture such as human serum. Here, the protein profiles of women with or without aPL antibodies who suffered pregnancy losses

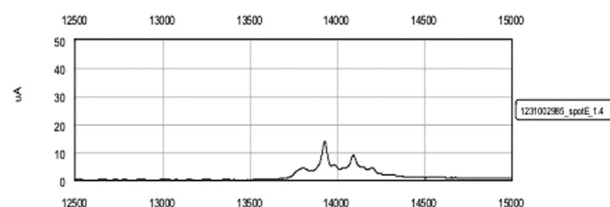


Figure 1: Representative spectrum with peaks in the range between 12,500 and 13,500 Da of one serum sample of an aPL-positive woman

were compared. The data reveal that several proteins are differentially expressed in these groups. These differences can be found only in distinct protein signals and not in the entire profile, as clearly demonstrated in the heat map. Moreover, SELDI-TOF analyses show that most of the *m/z* values of significantly different peaks are concentrated in clusters 1 and 3. This reflects the close relationship of these protein-based *m/z* signals and finally also of the proteins themselves.

With the aim of identifying these proteins, a search in several databases (Plasma Proteome Database, neXtProt, Phosphosite, PRIDE, and UniprotKB) and in the scientific literature was performed using the *m/z* value for these differentially expressed peaks, and allowed us to propose some proteins as summarized in Table 2.

DISCUSSION

Most of these proposed proteins have been described in a plasma proteome map generated by a multidimensional strong cation exchange – phase liquid chromatography – ion mobility spectrometry – mass spectrometry experiment combined with a database assignment approach.^[20]

Transthyretin (TTR)

Thyroid hormone-binding protein is a small protein highly abundant in plasma (200–400 µg/ml) and the cerebrospinal

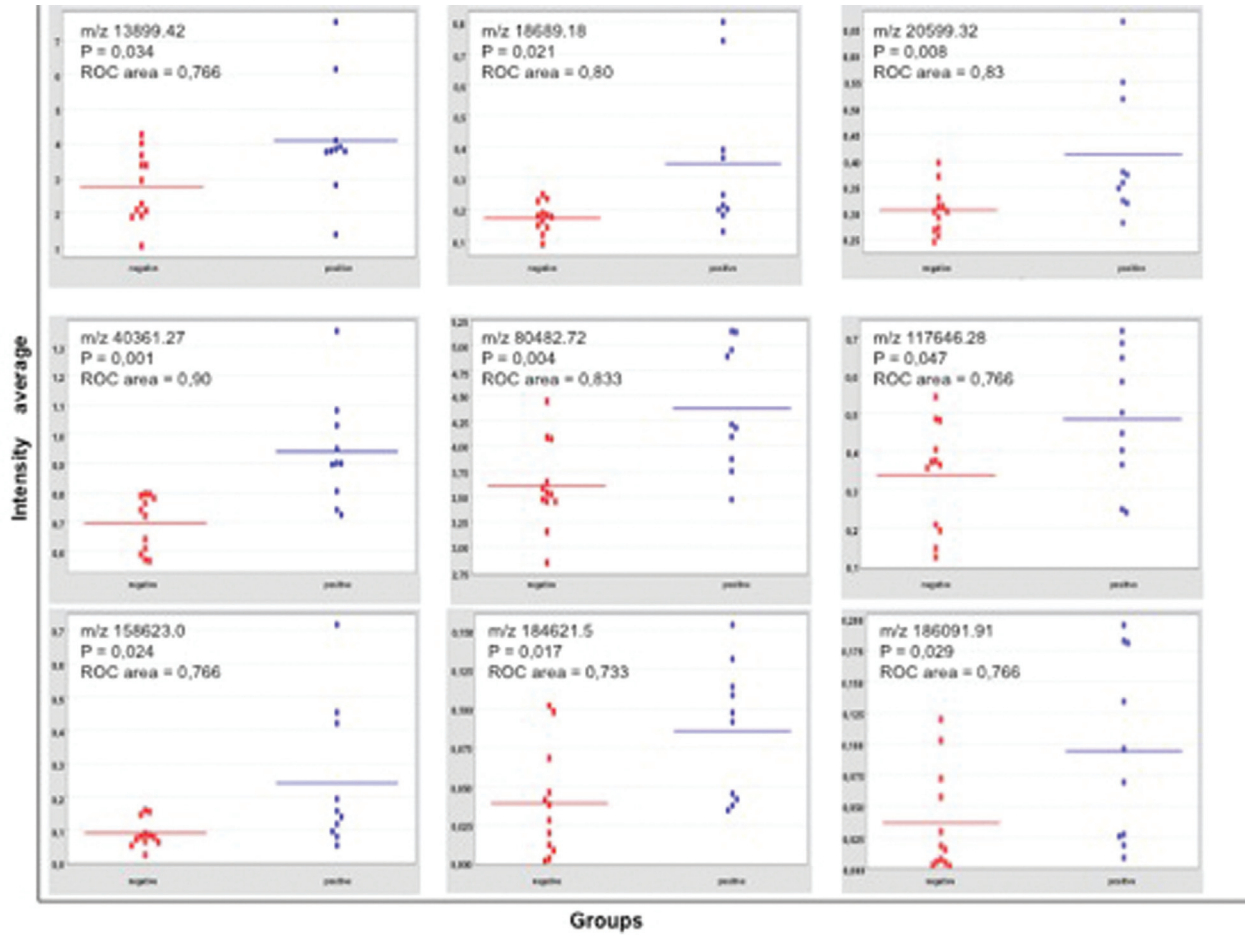


Figure 2: Dot plots of differentially expressed proteins in sera of both groups of women. All protein expression was increased in serum from the aPL-positive group

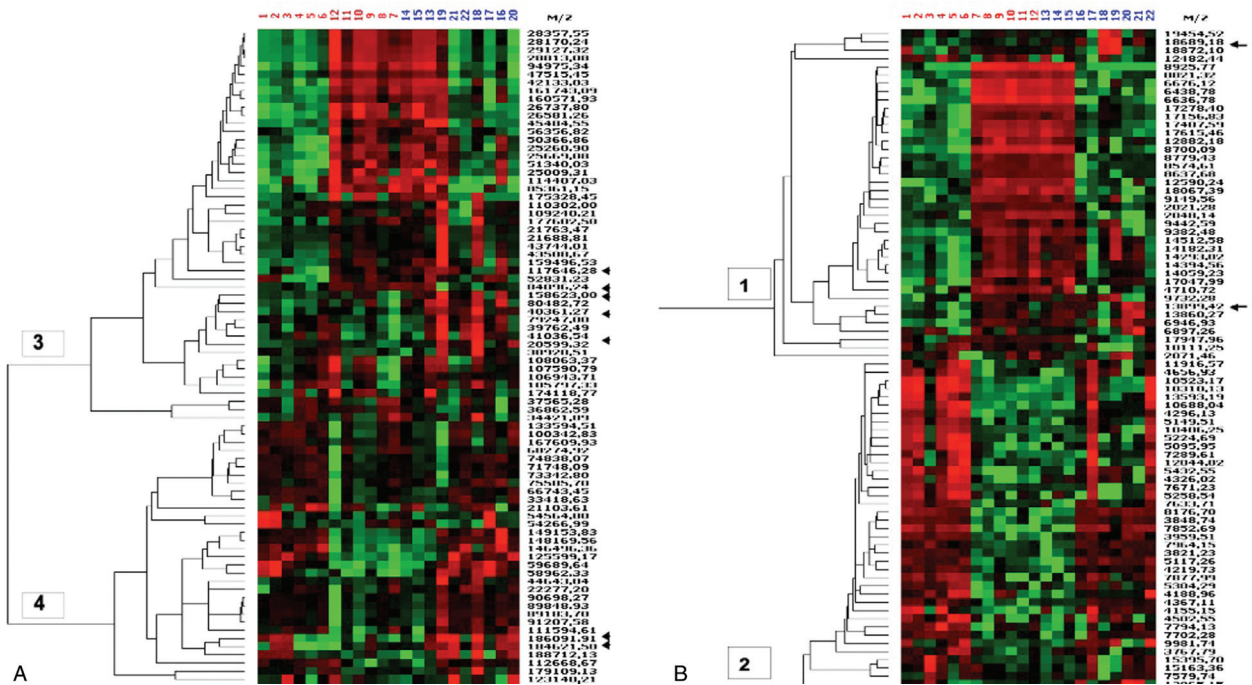


Figure 3: Heat map represents the relative expression of proteins in the serum samples of aPL-negative (numbers 1–12) and aPL-positive (numbers 13–22) women. Red represents overexpression, and green represents downregulated expression. Arrows identify the differentially expressed proteins. (a) Heat map for small proteins (2000–20,000 Da) and (b) heat map for large proteins (20,000–200,000 Da)

Table 2: Approach to protein identification

Cluster	Number UniProt	Name of related protein	Reported molecular weight (Da)	m/z found (Da)	% difference	Ref.
1	P02766	Transthyretin isoform	13,800	13,899.42	-0.72	[21,46]
	P01579	Interferon- γ (IFN- γ)	19,348	18,689.18	3.41	[20,47]
3	P22301	Interleukin-10 (IL-10)	20,517	20,599.32	-0.40	[48,49]
	P63096	Guanine nucleotide-binding protein G(i) alpha1 (GNAI1)	40,361	40,361.27	0.00	[20,50]
	P00736	Complement (C1r)	80,119	80,482.72	-0.45	[51,52]
	O75882	Unknown	158,537	11,7646.28		
4		Attractin	158,537	158,623.0	-0.05	[53,54]
		Unknown		184,621.5		
		Complement (C3)	187,148	186,091.91	0.56	[33,55]

fluid (CSF) (10–40 $\mu\text{g/ml}$). This 13.8 kDa protein is synthesized in the liver as well as in the choroid plexus, but it is also secreted by placental trophoblasts. It forms a homotetramer that transports thyroxine and retinol in both blood and CSF. TTR also plays an important role in the transport of thyroxine and retinol from maternal to fetal circulation that is critical for normal development of the human fetus. Moreover, about 100 point mutations in the TTR gene are known, and several of them are harmless but many play crucial roles in variants of hereditary TTR amyloidosis.^[21] Defects in TTR are a cause of hyperthyroxinemia dystransthyretinemic euthyroidal. It is a condition characterized by the elevation of total and free thyroxine in healthy, euthyroid people without detectable binding protein abnormalities.^[21] There are about nine TTR isoforms, due to numerous S-thiolation in a single cysteine residue at position ten.^[21] Chen and Zhang^[23] published a hypothesis concerning the relationship between PE and the deposit of TTR amyloid fibrils, and also Kalkunte *et al.*^[24] recently reported its dysregulation in women with PE using SELDI-TOF and a mouse model in addition.

Interferon-gamma (IFN- γ) and interleukin-10 (IL-10)

IFN- γ is produced by lymphocytes activated by specific antigens or mitogens. In addition to having antiviral activity, IFN- γ has important immunoregulatory functions. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells, and it can potentiate the antiviral and antitumor effects of type I interferons. It belongs to the type II (or gamma) interferon family.^[22] IL-10 is a cytokine with pleiotropic effects in immunoregulation and inflammation. It downregulates the expression of cytokines from type I T helper cells Th1 cytokines, major histocompatibility complex (MHC) class II antigens, and co-stimulatory molecules on macrophages. It acts in concert with IL-4 to induce activated B lymphocytes to grow, switch isotype, and ultimately differentiate into antibody-producing plasma cells.^[25]

Cytokines such as IFN- γ , IL-6, IL-17, and IL-21 are elevated in the plasma of mouse models of lupus, arthritis, and multiple sclerosis, and in subsets of patients with autoimmune

diseases.^[26] IFN- γ is intimately connected to autoimmunity and, specifically, has long been associated with lupus. Systemic autoimmunity owing to overactivity of follicular helper T cells (Tfh) and dysregulated germinal centers (GC) has been described in mice and humans. Although GC-driven autoimmunity probably results from the association of many dysregulated processes, cytokine imbalance plays a key role particularly centered around IFN- γ , IL-6, IL-17, and IL-21.^[26]

On the other hand, it is currently widely accepted that phagocytosis of apoptotic cells by bacterial lipopolysaccharide LPS-activated macrophages induces secretion of IL-10 and decreases the secretion of other inflammatory cytokines such as TNF- α , IL-1 β , and IL-12.^[27] It has been reported that accumulation of dead cells containing nuclear autoantigens in sites of immune selection may provide survival signals for autoreactive B-cells, and that the production of antibodies against nuclear structures determines the initiation of chronic autoimmunity in systemic lupus erythematosus.^[27] Moreover, high levels of IL-10 were found in cases of autoimmune lymphoproliferative syndrome (ALPS) and this could provide useful tools for ALPS diagnosis.^[28,29] Furthermore, plasma levels of IFN- γ and IL-10 among other cytokines were recently measured in patients with colorectal adenomas, and it was found that IFN- γ could contribute to cancer development.^[30]

Complement components

C1r is a serine protease that combines with C1q and C1s to form C1, the first component of the classical pathway of the complement system.^[22] Congenital defects in C1 and C4 are strongly associated with lupus, and this pattern along with laboratory evidence suggests that the activity of this classical pathway is highly important in safely eliminating and preventing immune complexes diseases.^[31] Similar immunological mechanisms might account for the vast majority of autoimmune diseases.

Likewise, C3 plays a central role in the activation of the complement system. Its processing by C3 convertase is the central reaction in both classical and alternative complement

pathways. C3a anaphylatoxin derived from proteolytic degradation of C3 is a mediator of the local inflammatory process. It induces the contraction of smooth muscle, increases vascular permeability, and causes histamine release from mast cells and basophilic leukocytes.^[22] Several studies have demonstrated that aPL can activate the complement system through the classical activation pathway, and also showed that C3 activation was required in aPL-induced fetal losses.^[32] Moreover, altered C3 expression was found in plasma samples from patients with polycystic ovary syndrome compared with healthy controls, using a Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF) approach.^[33]

Guanine nucleotide-binding protein G(i) alpha 1 (GNAI1)

Guanine nucleotide-binding proteins (G proteins) participate as modulators or transducers in various transmembrane signaling systems. GNAI1 is involved in biological processes as platelet activator, a response to peptide hormone stimulus, synaptic transmission, G protein coupled receptor protein signaling pathway, cell division, and blood coagulation.^[22] Recently, it was demonstrated that GNAI1 inhibits the class III β -tubulin isotype (β III-tubulin), a predictive biomarker in ovarian cancer and other solid tumor malignancies.^[34]

Attractin

Attractin is a protein involved in initial immune cell clustering during inflammatory response, and it may regulate the chemotactic activity of chemokines. Attractin may play a role in melanocortin signaling pathways that regulate energy homeostasis and pigmentation, and it may also be important in normal myelination in the central nervous system.^[35] It was first discovered as a circulating secreted molecule expressed by activated T lymphocytes; it was then examined as a potential marker of immune activity.^[36] Mass spectrometry and liquid chromatography analysis of plasma samples from patients with S-adenosylhomocysteine hydrolase (AHCY) deficiency revealed attractin as a potential biomarker, useful for routine diagnosis and management of AHCY deficiency.^[37] Mutations at the attractin locus were described as the cause of juvenile-onset neurodegeneration in animal mutants. The reassessment of earlier attractin mutants demonstrated that neurodegeneration, alterations in pigmentation regulation, and basal metabolic rates were common to all allelic variants.^[36]

The proposed proteins have been identified as biomarkers for other disorders, and some of them are proteins associated with the inflammatory response, which is not surprising given the APS characteristics.^[38,39] Even though the Giles group demonstrated changes in signaling pathways and proteins in human monocytes treated with IgG of different clinical types of APS patients,^[15,40] to our knowledge, this is one of the first reports about the protein profile in sera of women with

pregnancy losses associated with APS. It is also interesting that some of the proteins detected could be cytokines, given the elevated levels of inflammatory cytokines in autoimmune diseases. These cytokines play a role in promoting germinal centers that give rise to autoantibodies, so it is possible that monoclonal antibodies against these molecules will be effective in selected groups of patients, such as women with obstetric APS.^[26] In addition, our finding of two target proteins related with the complement system is interesting, since it has been widely demonstrated that complement activity mediates the aPL-induced pregnancy loss in APS patients.^[41-45]

Therefore, the best strategy to improve early diagnosis of obstetric APS could reside in the association between cytokine levels and the detection of other proteins. Maybe, the target candidates shown here raise the possibility of finding useful biomarkers in sera, since our findings highlight the need for additional investigation in this field. Novel research could be addressed to select some of these proteins and perform a complete identification of them by several proteomics methods, and then explore their expression in patients groups presented in this study. In addition, it will be interesting to explore protein post-translational modifications such enzymatic glycosylation. The search for glycan's patterns of proteins involved in immune response could be a novel pathway to understand this disease and support its diagnosis.

Acknowledgements

The authors thank Prof. F.v. Eggeling for his advice with the SELDI-TOF technique and Anne-Lise Haenni for her critical review.

Financial support and sponsorship

Administrative Department of Science, Technology and Innovation – Colciencias, Colombia (Grant #1115-493-26157). Universidad de Antioquia – UdeA. AMA is a fellow of Colciencias.

Conflicts of interest

There are no conflicts of interest.

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