

Using Proteomic Analysis for Studying the Skin Fibroblast Protein Profile in Systemic Sclerosis

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1. Introduction

Increased efforts have been made during the last few decades to develop new technologies capable of identifying and quantifying the expression proteome in different cellular systems in physiological and physiopathological conditions for determining illness biomarkers, pharmaceutical targets and/or posttranslational modifications (PTM) by means of proteomic techniques. 2D gel electrophoresis, with immobilized pH gradients, associated with mass spectrometry, is one of the fundamentals steps in studying proteomics. The 2D technique can be used in studying the quantitative expression of protein profiles according to iso-electric point (Ip), molecular weight (Mr), protein solubility and the relative abundance of the above. This methodology provides a protein profile reflecting changes in protein expression levels, isoforms and PTM.

Proteins can be classified into those known by their structure and function, those recognized by determined domains and about which there is some knowledge, and those whose function is still not known. Proteomics is defined as the large-scale study of proteins expressed for a specific tissue from a genome, (global proteomics) or differentially expressed proteins (differential proteomics). Determining differentially expressed proteins, or proteins suffering a change in physiological circumstances, is the clue to understanding such pathology's cellular mechanisms. Although an expressed gene in specific tissues (as an answer to biologic alterations) could be analyzed by a mRNA expression study (transcriptomics), these results do not always coincide with the expected expression profiles since the number and activity of proteins associated with the same regulation in different stages could be modified. Genomic data integration is required, as well as transcritomics,

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proteomics, variome, peptidomics, and metabolome to understand physiological phenomenon in a comprehensive manner.

Systemic sclerosis (SSc) is a chronic illness of the connective tissue having unknown etiology; it has a variable course and severity and is characterized by intercellular matrix alterations and secondary fibrosis of enormous amounts of connective tissue. This results in hardening and thickening of the skin, alterations in the microvasculature and the large vessels, secondary to changes in the endothelial cells together with Raynaud's phenomenon, self-immunity alterations, and musculoskeletal and visceral degenerative fibrotic changes (1).

Despite recent advances having been made in understanding some molecular pathways involved in SSc, its etiopathogenesis remains unknown. Treatment for these patients has had very limited effectiveness, and the natural course of the illness inevitably leads to a fatal outcome. A better understanding of the illness' physiopathology is required to be able to orientate suitable therapeutic measures, carry out effective monitoring of its response, and determine severity criteria indicating a bad prognosis for the illness. This is where genomics, micro-array analysis and proteomics appear as valuable therapeutic and diagnosis tools.

Several groups have reported gene expression profiles for SSc-patient's tissues and cells (2-6).

Zhou found that fibroblasts in SSc patients showed different RNAm expression profiles for fibrilarine autoantigens, B centromere protein, P27 centromeric autoantigen, RNA polymerase I, DNA topoisomerase I, and PMScl (2). Luzina found high chemokine and cytokine levels in bronchoalveolar lavage (LAB) in SSc patients (3). Whitfield examined skin biopsies in four SSc patients, identifying 2,776 genes which expressed themselves in different ways to that of healthy controls (4). Tan (using a fibroblast culture) identified 62 genes which expressed themselves in different ways in SSc (5). Zhou reported fibroblast micro-array analysis results for fibroblasts from 18 sets of discordant twins in SSc (6,7). Protein analysis using two-dimensional electrophoresis electrophoresis on polyacrylamide gel (2D PAG) will contribute to and extend the knowledge produced by analyzing gene expression, especially for proteins undergoing crucial PTM in their function.

Proteomic analysis involves many methodologies orientated towards identifying and characterizing altered proteins as a result of illness. Thousands of proteins are evaluated in just one trial in such studies, leading to detecting expression profiles as a consequence of abnormality in cell function or interaction. Traditional methods used in proteome analysis have included 2D PAG where proteins are first separated according to their electric charge and then by their mass in the second direction before being stained, thus allowing mixtures of 1,000 to 3,000 proteins to be visualized. The development of special software and the use of Internet have allowed multiple genes and databases to be compared. When being combined with mass spectrometry, a separation appears which allows efficient identification of proteins of interest, including many of their PTM. This analysis can be applied in comparative studies of expression profiles during different stages of the illness or healthy tissue compared to affected tissue, thus being able to identify the different modifications in the protein characteristics of clinical interest in different illnesses (8).

Clinical proteomics is aimed at identifying proteins involved in pathological processes, as well as evaluating changes in their expression during different stages of an illness.

Proteomics in clinical practice offers the technical skill for identifying biomarkers for diagnosis and therapeutic intervention. Potential biomarkers developed from proteomic analysis will have further specificity and sensitivity in clinical trials, since they measure protein alteration involved in an illness (9). A good understanding of data management, correlation, interpretation, and validation is crucial in obtaining precise results contributing towards understanding cellular alterations which could be involved in developing SSc.

Only two proteomic studies were found in the current literature. Rottoli has analyzed the type of immune response and protein composition in pulmonary fibrosis patients' LBA medium associated with SSc, sarcoidosis and idiopathic pulmonary fibrosis. Proteomic analysis revealed quantitative differences between the three illnesses, finding increased SSc in plasmatic proteins such as alpha1-beta glycoprotein, C3 complement, alpha 1-antitrypsin, beta- haptoglobin, and prothrombin (10,11). Czubaty has used a commercial cell line (HeLa S3) for proteomic analysis of Topoisomerase I protein patterns by comparing co-immunoprecipitation with mass spectrometry and identified 36 new proteins which were associated with Topoisomerase I and their possible interaction site in the RRM domain (12). However, these studies have been carried out in a not very specific medium, such as LBA.

A two-phase fibroblast proteomic study was thus proposed (pre-treatment and post-treatment) in fibroblasts, these being the cells initially involved in SSc physiopathology in one of its most important expressions: fibrosis. Fibrosis is one of the pathognomonic pathological findings for SSc, representing one of the most exemplary phenotypes.

Characteristically, there is uncontrollable collagen production and that of other extracellular matrix proteins due to resident fibroblasts in the skin, lungs and other vital organs leading to an excessive accumulation of connective tissue. As the illness progresses, this increased deposit of connective tissue alters the tissues' normal architecture, ending in a functional alteration of the latter and determining a very significant involvement in morbidity-mortality of patients suffering fibrosis-related SSc (13).

Protein expression pattern was observed when carrying out a proteomic analysis on SSc patients' fibroblasts during different stages of the illness and comparing them to healthy individuals' fibroblasts. Their appearance was analyzed and thus an increase, decrease, or absence of their profiles was determined, looking for an association of the proteins found with phases and serological and clinical characteristics. Proteins involved in the illness' etiopathology during its different stages were isolated as this could have therapeutic implications in an illness in which current treatment is very limited and not very efficient.

2. Materials and methods

2.1 Patients

This was a cases and controls study in which 11 patients who fulfilled with American College of Rheumatology SSc criteria were included during different phases of the illness (14,15) and subdivided into two groups: limited SSc and diffuse SSc, according to the parameters proposed by Le Roy (1). Table 1.

The cutaneous involvement of the skin was evaluated according to the modified Rodnan index (16) which ranges from 0 (normal) to 3 (severe), measured in 17 different body areas (maximum possible score is 51).

Clinical features	SSc Patients (n=11)
Age at onset/yrs	44.75 ±10
Female/male ratio	3:01
Disease duration yrs	9,65 ± 4
SSc subtype	
ISSc	7
dSSc	3
Morphea	1
Raynaud phenomenon %	90
Raynaud duration, yrs	9.1 ± 4
Rodnan Score	22.1 ± 9
Calcinosis %	45
Telangiectasias %	64
Renal Crisis %	0
Digital ulcers %	0
Gastrointestinal involvement %	20
Pulmonary involvement %	0
Antibodies Anticentromere %	60
Antibodies Anti SL-70 %	30
Antibodies Antinuclear %	90

Table 1. General characteristics of SSc patients

Patients had no treatment or had suspended 4 weeks before taking the biopsy (a treatment scheme was defined as involving any of the following medications, alone or combined: prednisone, D-penicillamine, colchicine, micophenolate mofetil, methotrexate, cyclophosphamide).

Healthy controls were individuals without an autoimmune illness or who had not undergone previous immunodepressor treatment.

Registration forms were completed; they then contained SSc patients' demographic data, clinical characteristics and antibody levels.

All individuals involved in the study signed the participation consent form according to established ethical norms.

2.2 Skin biopsy

Following the cutaneous biopsy technique's guidelines by means of punch (17), two skin biopsies were taken from each SSc patient: a skin sample with SSc involvement obtained from the body area having the maximum Rodan score and another clinically healthy skin sample having a zero Rodan score. The same technique was used for a skin biopsy of healthy individuals taken from a non-esthetic non-visible area. The material was prepared for cell culture.

2.3 Obtaining fibroblasts from skin biopsies from healthy controls and SSc patients

This stage of the study, as well as the rest of the procedures, had been previously agreed on by the interdisciplinary team for which the critical route in each process was determined. Clear coordination of activities was needed to guarantee that:

- The patients were appropriately and conveniently informed about the investigation and the lab procedures that would be carried out for analyzing samples;
- The biopsies would arrive at the lab immediately after samples had been taken to ensure rapid processing; and
- Serum taken from patients was convenient and suitable for lab procedures.
- According to previously-defined protocols, the sample should have arrived at the lab on the day the sample was taken as follows (18):
- Tubes marked with the names of the patients, indicating whether the fragment of skin had been taken from a clinically healthy area or from a clinically sick one; and
- Dry tubes to take the blood sample from the same patient.

The following procedures had been previously carried out in the lab:

- Preparation of the means of transport for the biopsy: A DMEM medium was used with a F-12 medium supplemented with an antibiotic solution (100ug/ml streptomycin, 0.25ug/ml B anphotericin) at 3% in sealed sterile glasses; and
- Preparation of the material and supplies for the culture: a culture medium was prepared to be supplemented with autologous human serum.

The skin biopsies immersed in the transport medium and the serums were transported at 4°C and taken to a lab specializing in human fibroblast cultures. Once in the lab, the samples were processed in the white zone, cell culture room, in the safety cabin following management protocols for these areas, according to the Lab Quality Manual.

- Each sample was washed three times with HANK's saline solution supplemented at 3% with antibiotic and antimycotic solution (100mg/ml penicillin, 100ug/ml streptomycin, 0.25ug/ml B anphotericine);
- The samples were cut by a scalpel into small explants (half millimeter maximum size). The fragments so obtained were planted as explants in six-well culture plates;
- A total blood sample was taken from each patient in a dry tube to obtain serum by centrifuging at 2,500 rpm for 20 minutes at room temperature, with which the fibroblast culture medium would be autologously supplemented;
- 2ml DNEM culture medium with F-12 supplemented at 20% with autologous serum and 1% antimycotic antibiotic solution was added to each well; and
- Each sample was identified with a number for each patient, followed by whether the sample was healthy or unhealthy.

The cultures were monitored daily under an inverted microscope at 10X by 40X enlargement:

- Observations and photographs were registered;
- The culture medium was changed every third day; and
- The first cells began to be observed during the second week after culture.

Confluence was obtained around the fourth week.

Cell preparation for obtaining the proteins was carried out, following the following steps:

- Cells were previously washed with 1X PBS solution;
- Once the washing solution had been removed, 600 ul extraction protein buffer was added to each well as described in the protocol for 2D electrophoresis or Trizol study for obtaining NRA; and
- The fibroblasts were incubated for 10 minutes and then homogenized with the help of a rake. Cell separation was confirmed with an inverted microscope and each well's content was placed in a 1.5 ml Eppendorf tube and stored at -80°C until proteins were analyzed.

3. 2D electrophoresis for analyzing human fibroblast proteins in SSc patients and controls

14 fibroblast culture samples were studied by 2D-SD PAGE obtained from skin biopsies from three healthy controls and skin from a healthy and unhealthy region in 11 SSc patients. A recognition code was assigned. All individuals involved in the study signed the participation consent form, according to the ethical standards for such protocol.

The fibroblasts were lysed in a 600 μ l protein extraction buffer made up of 7M thiourea, 2M ABS-14 detergent (1%), 40 mM Tris base and 0.001% bromophenol, all of which form part of BIO RAD protein extraction kit (cat. 163-2086). Anpholites (pH 3-10) were added at 200 mM final concentration before starting the lysis for reducing cysteine disulphide links.

The samples suspended in lysis buffer were initially sonified on ice to break up the genomic DNA cells and fragments (10%). The product was spun at 16,000 g for 20 minutes, separating proteins from the remains of cells and other macromolecules. These samples were stored at -80°C until subsequent analysis.

The Lowry method (RC DC, assay protein, Bio-Rad) was used for protein quantification; uni-dimensional electrophoresis was carried out by means of Laemmli's method to obtain an electrophoretic map and thus guarantee the integrity of proteins from fibroblast lysates. Once protein concentration and integrity had been verified, 2D PAG SDS electrophoresis trials were carried out. Electrophoresis was carried out on 10% and 12% acrylamide gels (30%/0.8v/v acrylamide/bisacrylamide), best results being obtained at 12%.

Isoelectrofocusing (IEF) followed BIO RAD's recommended method (cat.163-2105); 7 cm IPG strips, pH 3-10 and pH 4-7 ranks were selected. The latter were placed on trays to hold samples of interest suspended in rehydration buffer (125 μ l total volume); this buffer contained (m urea, 2% CHAPS, 50 Mm dithiethreitol (DTT), 3-10 anpholites (0.2%) and blue bromophenol traces. The fibroblast lysates were left (for one or two hours) and it was verified that the gel was totally covered by the previous solution, after which mineral oil was placed on the strip to avoid evaporation. The samples were covered and incubated for sixteen hours. Two functions were fulfilled in this step: the strips were hydrated and the samples were absorbed by the pH strip gel (which is why time taken and conditions for this procedure were so important).

Human fibroblast culture protein IEF was carried out on Protean IEF Cell equipment (BIO RAD), initially on a linear gradient until reaching 250V for 30 minutes, then at 4,000V for 2 hours on a linear gradient and finally on a fast ramp until the equipment reached 12,000V when the IEF finished. Small wicks of filter paper were placed before passing the strip from the hydration tray to the IEF equipment; they were moistened with ultrapure water and the strip was then placed. However, everything had to be covered with mineral oil so as to avoid evaporation before starting the process.

The 2D in which the proteins were separated according to weight was developed on 12% gels according to the preliminary analysis. Once the IEF was finished, the strips were separated from the electrode and placed in the equilibrium solution trays again, with 2% of equilibrium buffer I (6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol and 2% DTT). They were incubated for 10 minutes, the disulphur groups thus being reduced. The strips were then incubated for 10 minutes in equilibrium II buffer (6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol and 0.5g iodoacetamide). The sulphidryl groups were

removed to avoid reduction reversibility. This step was repeated, but this time the strips were placed in the 2D running buffer (tri/glycine/SDS at pH 8.8).

Meanwhile the 2D gel was placed in low fusion point agarose solution which was dissolved in SDS_PAGE running buffer. The proteins were separated in BIO RAD chambers, whether with Mini-Protean 3 cell (cat165-3301/02) or Mini-Protean Tetra cell (CAT 165-8000/01); the procedure began with a 40V voltage and was slowly increased to 60V voltage.

Once the proteins had been separated in 2D, they were silver stained according to manufacturer's recommendations (Invitrogen, Silver Express staining kit, cat.LC 61000).

The gels were documented with Quantity One 1-D Analysis Software and differential expression points were found with PDQuest 2-D Analysis Software.

The spots or differential expression points between controls and patients were analyzed with MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems). Four points were split and sent to the Córdoba University's central research support service (SCAI) proteomics unit in Spain.

It should be pointed out that the best results obtained in separating proteins from fibroblasts in patients and controls by means of 2D electrophoresis were on 4-7 pH strips and 12% gels. All 2D electrophoresis trials were carried out from the same human fibroblast culture lysate for both patients' samples and triplicate controls.

4. Statistical study

The results were presented descriptively with measurements, medians and interquartile ranges expressed according to expected variables. Association measurements having binominal categorical variables were presented in the analysis, depending on population distribution. A Wilcoxon or Mann-Whitney chi square test was used and association was measured by odds ratio (OR). Controls having similar conditions to the chosen patient cases regarding age and gender were sought to avoid differential expression which could have been explained by a physiological condition associated with these two variables and which could have increased or decreased potential associations.

5. Results

Proteins in cells from silver stained fibroblast cultures were observed in representative 2D SD-Page electrophoresis. The trials were carried out on 12% gel and IPG strips having pH4-7. Standardization studies were carried out on strips having pH 3-10 but most proteins were located in the pH 4-7 range where better resolution appeared. Each fibroblast sample was analyzed by 2D electrophoresis in triplicate.

2D electrophoresis images of human fibroblast proteins from controls and SSc patients were analyzed by PDQuest software allowing the gels to be normalized. Proteins (spots) which were differentially expressed in controls and scleroderma patients (marked with arrows and numbers on each gel in Figure 1) were isolated, digested with trypsin and the peptides so produced were analyzed by mass spectrometry (peptide mass fingerprints) (MALDI-TOF). The analyzed spots from silver stained gels as well as isolated ones stained with Coomassie blue were mainly from different haptoglobin protein isoforms (Table 2), having greater than

SPOT	PROTEIN	SPECIE	WEIGHT MOLECULAR (KDA)	PROTEIN SCORE	PROTEIN SCORE CI %	NUMBER OF MATCHED PEPTIDES	ION SCORE TOTAL	ION SCORE CI %	ACCESSION NO.
1	haptoglobin precursor [Homo sapiens]	Homo sapiens	45859.8	91	99.997	8	44	99.611	gi 306882
1	haptoglobin, isoform CRA_a [Homo sapiens]	Homo sapiens	47377.6	90	99.997	8	44	99.611	gi 119579598
1	haptoglobin [Homo sapiens]	Homo sapiens	38722.4	88	99.995	8	44	99.611	gi 3337390
1	haptoglobin isoform 2 preproprotein [Homo sapiens]	Homo sapiens	38940.5	88	99.995	8	44	99.611	gi 186910296
1	haptoglobin, isoform CRA_d [Homo sapiens]	Homo sapiens	40457.3	87	99.993	8	44	99.611	gi 119579601
1	haptoglobin, isoform CRA_c [Homo sapiens]	Homo sapiens	40528.3	87	99.993	8	44	99.611	gi 119579600

SPOT	PROTEIN	SPECIE	WEIGHT MOLECULAR (KDA)	PROTEIN SCORE	PROTEIN SCORE CI %	NUMBER OF MATCHED PEPTIDES	ION SCORE TOTAL	ION SCORE CI %	ACCESSION NO.
2	haptoglobin precursor [Homo sapiens]	Homo sapiens	45859.8	159	100	8	109	100	gi 306882
2	haptoglobin, isoform CRA_a [Homo sapiens]	Homo sapiens	47377.6	158	100	8	109	100	gi 119579598
2	haptoglobin isoform 2 preproprotein [Homo sapiens]	Homo sapiens	38940.5	156	100	8	109	100	gi 186910296
2	haptoglobin, isoform CRA_c [Homo sapiens]	Homo sapiens	40528.3	155	100	8	109	100	gi 119579600
2	haptoglobin Hp2	Homo sapiens	42344.1	153	100	7	109	100	gi 223976
2	haptoglobin, isoform CRA_b [Homo sapiens]	Homo sapiens	51387.5	152	100	8	108	100	gi 119579599
3	haptoglobin precursor [Homo sapiens]	Homo sapiens	45859.8	116	100	9	61	99.988	gi 306882

SPOT	PROTEIN	SPECIE	WEIGHT MOLECULAR (KDA)	PROTEIN SCORE	PROTEIN SCORE CI %	NUMBER OF MATCHED PEPTIDES	ION SCORE TOTAL	ION SCORE CI %	ACCESSION NO.
3	haptoglobin, isoform CRA_a [Homo sapiens]	Homo sapiens	47377.6	115	100	9	61	99.988	gi 119579598
3	haptoglobin [Homo sapiens]	Homo sapiens	38722.4	114	100	9	61	99.988	gi 3337390
3	haptoglobin isoform 2 preproprotein [Homo sapiens]	Homo sapiens	38940.5	113	100	9	61	99.988	gi 186910296
3	haptoglobin, isoform CRA_c [Homo sapiens]	Homo sapiens	40528.3	112	100	9	61	99.988	gi 119579600
3	haptoglobin Hp2	Homo sapiens	42344.1	110	100	8	61	99.988	gi 223976
3	haptoglobin, isoform CRA_b [Homo sapiens]	Homo sapiens	51387.5	109	100	9	60	99.985	gi 119579599
3	HP protein [Homo sapiens]	Homo sapiens	38868.5	104	100	8	61	99.988	gi 78174390

SPOT	PROTEIN	SPECIE	WEIGHT MOLECULAR (KDA)	PROTEIN SCORE	PROTEIN SCORE CI %	NUMBER OF MATCHED PEPTIDES	ION SCORE TOTAL	ION SCORE CI %	ACCESSION NO.
4	haptoglobin [Homo sapiens]	Homo sapiens	38722.4	79	99.957	7	42	99.676	gi 3337390
4	haptoglobin [Homo sapiens]	Homo sapiens	38940.5	79	99.956	7	42	99.676	gi 1212947
4	haptoglobin, isoform CRA_c [Homo sapiens] 42	Homo sapiens	40528.3	78	99.943	7	42	99.676	gi 119579600
4	haptoglobin isoform 1 preproprotein [Homo sapiens]	Homo sapiens	45860.8	75	99.892	7	42	99.676	gi 4826762
4	haptoglobin, isoform CRA_a [Homo sapiens]	Homo sapiens	47377.6	74	99.867	7	42	99.676	gi 119579598

Table 2. Proteins identified by mass spectrometry (MALDI/TOF- TOF) which were separated by 2 D electrophoresis and obtained from cultures of human fibroblasts from SSc patients and healthy subjects. The proteins corresponding to isolated spots in Figure 1, which differ in expression profile between controls subjects and patients.

99% protein score confidence interval (the search will be more credible the nearer this is to 100 but confirmation must be above 99%). The protein score is a score given by a search engine (MASCOT) to each identified peptide, according to the probabilistic system based on peptide mass distribution, depending on the mass of the protein to which they belong (Mowse System). These were identified by comparing the MALDI-TOF peptide map to the theoretic value calculated for peptides from all SWISS-PROT database proteins and the TrEMBL database for human sequences and by applying Mascot software. Proteins corresponding to spots identified by MALDI-TOF (Table 2) were correlated with their molecular weights and isoelectric points when located on the 2D gels (Figure 1 and 2)

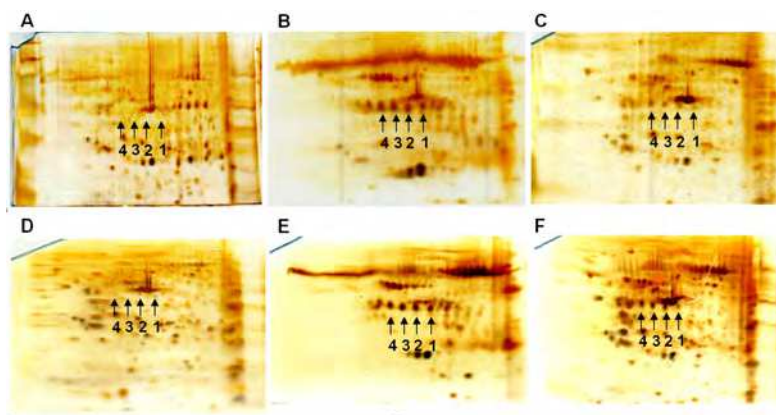


Fig. 1. Representative electrophoresis of proteins obtained from isolated lysates human fibroblasts cultures from skin biopsies of healthy and Scleroderma patients using 2D SDS - PAGE (12%). Healthy controls (A, D) SSc patient F11, healthy skin (B) and diseased skin (C) SSc patient F7, healthy skin (E) and sick skin (F). IPGs strips were used (pH 4-) and staining of the gels were developed with silver reagent.

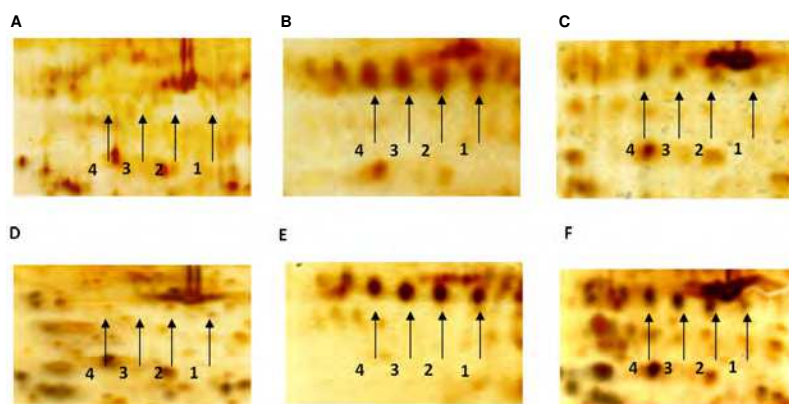


Fig. 2. Amplified region noted in Figure 1. 2D electrophoresis of human fibroblast proteins corresponding to differentially expressed spots were identified by MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems). Healthy controls (A, D) SSc patient F11, healthy region (B) and diseased region (C) SSc patient F7, healthy region (E) and sick region (F).

Table 3. Main clinical and serological variables and intensity of expression of the spots in Colombian patients suffering SSC

#	Age	Disease	Raynaud	Raynaud	Antibodies	Pattern	Levels	Rodnan	Microsomy	Calcinosis	Sclerodactyl	Hyperpigmenta	Hypopigmenta	Angiectas
Patient	at onset, yrs	Duration, yrs		Duration, yrs	Antinuclears			Score						
1	29	7	Absent	0	Negative	Negative	0	0	Absent	Absent	Absent	Absent	Absent	Absent
2	42	3	Present	6	Positive	Centromere	40	16	Present	Absent	Present	Present	Present	Present
3	27	1	Present	8	Positive	Centromere	1260	26	Present	Absent	Present	Present	Present	Present
4	48	7	Present	10	Positive	Centromere	1260	18	Present	Present	Present	Present	Present	Present
5	39	9	Present	5	Positive	Centromere	1260	12	Present	Present	Present	Absent	Absent	Present
6	50	10	Present	12	Positive	Homogeneous	1260	9	Present	Absent	Absent	Absent	Absent	Absent
7	49	9	Present	8	Positive	Nucleolar	640	12	Absent	Absent	Absent	Absent	Absent	Absent
8	41	4	Present	18	Positive	Nucleolar	40	8	Absent	Absent	Absent	Present	Absent	Absent
9	45	3	Present	3	Positive	Nucleolar	1260	20	Present	Present	Present	Present	Present	Present
10	20	20	Present	20	Positive	Centromere	1240	23	Present	Present	Present	Present	Present	Present
11	48	14	Present	15	Positive	Centromere	160	23	Present	Present	Present	Absent	Absent	Present

presenting approximately identical molecular weight but having a different isoelectric point, thus reflecting different protein processing mechanisms as previously described in scleroderma patients. Table 2 shows the identity of haptoglobin isoforms in scleroderma-derived human fibroblasts in scleroderma patients.

It was determined whether there were any associations between the clinical and serologic variables and the intensity of the spots' expression. Gender, initiation age, Raynaud's disease duration, pulmonary hypertension, antinuclear antibodies' pattern and dilution, modified Rodnan index, microstomy, calcinosis, telangiectasia and classification of the illness were then categorically and quantitatively evaluated with dominant spots' expression intensity, without finding any type of association (Table 3 and 4).

Characteristic	Health Skin	Sick Skin
Microstomy	0,72	0,821
Antibodies antinuclear	0,465	0,602
Calcinosis	0,97	0,821
Sclerodactilia	0,43	0,502
Hyperpigmentation	0,152	0,821
Hypopigmentation	0,233	0,821
Telangiectasia	0,437	0,502
SSc Subtype	0,151	0,119
PAH	0,181	0,978

Table 4. Association between clinical characteristics and expression of the spots. Measured by the intensity of protein electrophoresis. Determined by Chi square - p value

6. Discussion

Despite recent advances in understanding some molecular paths involved in SSc, its etiopathogenesis still remains unknown. Treating these patients has very limited effectiveness and the disease's natural course inevitably leads to a fatal outcome. A better understanding of its physiopathology is required to orientate suitable therapeutic treatment for efficiently monitoring its response and determining severity criteria indicating a poor prognosis for the illness. Genomics, micro-array analysis and proteomics thus appear as valuable diagnostic and therapeutic tools.

Proteomic analysis uses many methodologies orientated towards identifying and characterizing altered proteins as a result of illness. Millions of proteins are evaluated in one trial in these studies, leading to the detection of expression profiles as a consequence of abnormal function or cell interaction. The traditionally-used methods in proteomic analysis include 2D electrophoresis on polyacrylamide gel where proteins are separated first depending on their electric charge and then by their mass in the second direction and finally stained, visualizing 1,000 to 3,000 proteins. Special software having been developed and the use of internet have led to many genes and databases being compared. Separation is achieved when combined with mass spectrometry leading to the efficient identification of proteins of interest, including many of their PTMs. Such analysis can be applied to comparative expression profile studies during different stages of the illness or comparing

healthy tissues to unhealthy tissues, thereby identifying modifications in the characteristics of t proteins of clinical interest in different illnesses.

Haptoglobin was identified in the current study after proteomic analysis in fibroblasts from SSc patients during different stages of the illness as being a protein which expressed itself in a different but constant way in all SSc patients by contrast with healthy individuals.

Haptoglobin is an acute phase protein, indicative of different pathological conditions such as forms of cancer, hepatic cirrhosis and hepatitis C. This protein appears with around 6 phenotypes, besides combinations in PTM, such as glycolization and deamination, thus increasing the number of presentation forms (19).

Recent studies have demonstrated that idiopathic pulmonary fibrosis is caused by alteration of protein expression involved in different processes such as matrix remodeling, inflammation and tissue damage and repair. Similar studies to the current study (carried out in LBA in pulmonary fibrosis by proteomics) have demonstrated that this protein's expression significantly increased (20). However, the advantage of this study was the identification of this protein in a fibroblast culture, cells directly involved in the illness's physiopathology and whose increase did not correlate to the illness' severity but to its presence, thereby assuming that high haptoglobin values can predict SSc development.

Once haptoglobin has been identified as a protein present in untreated SSc patients, proteomic studies must be carried out to analyze this protein's behavior when influenced by different therapeutic schemes. Such study is currently taking place.

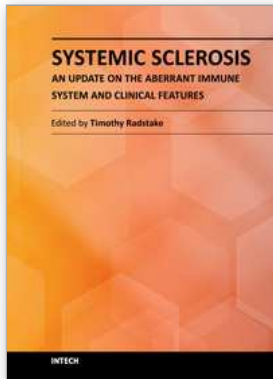
7. Conclusion

Identifying haptoglobin in a fibroblast culture in untreated SSc patients did not correlate with the severity of the illness but with its presence. It could thus become a predictive tool for SSc development. However, it is worth studying its behavior in the same patients using different therapeutic schemes and prospective studies are needed including a bigger population to verify these observations.

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Systemic sclerosis (SSc), or often referred to as Scleroderma (tight skin), is characterized by an exaggerated formation of collagen fibers in the skin, which leads to fibrosis. Accumulating evidence now points toward three pathological hallmarks that are implicated in Ssc, the order of which has yet to be determined: endothelial dysfunction, autoantibody formation, and activation of fibroblasts. This current book provides up-to-date information on the pathogenesis and clinical features of this severe syndrome. It is our hope that this book will aid both clinicians and researchers in dealing with patients with this clinical syndrome. In addition, we hope to shed more light on this rare and severely disabling syndrome, ultimately leading to better research and successful therapeutic targeting.

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