

Letter to the Editor

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A preliminary study on serum proteomics in fibromyalgia syndrome

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To the Editor,

Fibromyalgia syndrome (FMS) is a complex illness to diagnose and treat, which presents symptoms that may be part of, or overlap with other diseases or syndromes.

The most widely used diagnostic criteria are those of the American College of Rheumatology [1]; no laboratory test has been validated for FMS diagnosis which remains primarily clinically based.

Oxidative stress has been proposed as a relevant event in the pathogenesis of FMS with an increase of lipid peroxidation (LPO) [2] and a decrease in vitamin A and E concentrations [3].

Although the etiology of FMS remains unclear, genetic predisposition is likely to be an important factor and transmission is thought to be polygenic [4, 5]. If FMS is a multi-genetic disease then it could be hypothesized that differences exist in the type of proteins or protein expression levels in sera of FMS patients compared with healthy controls.

Proteomic analysis is a powerful tool for the global evaluation of protein expression and plays a central role

in clinical diagnosis and monitoring. Some studies [6] reported interesting proteomic analysis data with an over-expression of transaldolase and phosphoglycerate mutase I in salivary fluid of FMS patients and focused on the role of oxidative stress in the pathogenesis of the disease.

The aim of this preliminary study was to evaluate, using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS), changes in protein profiles of FMS patients with respect to control subjects to identify potential serum biomarkers useful for disease diagnosis and management.

Sixteen females (52±12 years) affected by FMS and 12 healthy females (48±13 years) were enrolled; all patients fulfilled new ACR diagnostic criteria [1] (Table 1). The presence of a major clinical condition other than fibromyalgia was excluded by physical examination and routine blood and urine screening. No patients suffered of thyroid dysfunction. Psychiatric examination data of patients were reported in a previous study [7].

Study was approved from our Local Ethical Committee and informed consent was obtained from all subjects.

Serum protein concentrations were quantified using the PlusOne 2D Quant Kit (GE Healthcare); first dimension isoelectric focusing (IEF) was carried out with 18 cm immobilized pH gradient strips of pH 3–10. The IPG strips were applied onto 10% acrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) slab gels (25.5 cm×20.5 cm×1.0 mm) and overlaid with a solution of 0.5% agarose with a trace of bromophenol blue. Gels were fixed, stained with Coomassie Brilliant Blue G-250 colloidal for 24 h and stored at room temperature. At least three sample replicates were performed.

2D data were processed with Progenesis SameSpots software, which computed multiplication fold, false discovery rate (FDR) q-value, p-values of all spots using one-way ANOVA analysis. A p-value <0.05 was considered statistically significant; no difference has been found in protein expression according to patient therapy.

After normalization, volume calculation and statistical analysis, same data differences were observed between

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Table 1 Characteristics and scores of the patients and healthy controls examined subjects.

	FMS (n=16)	HC (n=12)
Age, years (mean (SD), range)	52±12 (27–72)	48±13 (27–67)
Sex	Females	Females
Menopause	n=10	n=5
Body mass index, m ² /kg (mean±SD)	27±5	24±3
Duration, month from diagnosis of the disease, mean±SD	6.3±4.5	–
Pain duration, years (mean±SD)	4.9±0.5	–
Pain score patient VAS, mm (mean±SD)	57±31	–
Pain score physician VAS, mm (mean±SD)	34±18	–
FIQ total score, 0–80 (mean±SD)	61.8±21.9	–
Associated clinical distresses		
Tension type headache	n=12	–
Irritable colon	n=9	–
Oto vestibule syndrome	n=9	–
Paraesthesia	n=8	–
Sleep disturbance	n=8	–
Dysmenorrhea	n=2	–
Hemicrania	n=4	–
Urethral syndrome	n=5	–
Medication		
No therapy	n=5	n=12
Tricyclic, Amitriptyline <15 mg/day	n=6	–
Selective serotonin reuptake inhibitors (SSRIs) (Citalopram)	n=1	–
Selective Serotonin Noradrenaline Reuptake Inhibitors (SSNRIs) (Duloxetine)	n=4	–

FIQ, FMS impact questionnaire; VAS, visual analogue scale.

Table 2 Identification of 2D-separated proteins.

Spot ID	Method	Short name	Accession no.	MW, kDa	pI	Score	Coverage, %	p-Value	Fold-increase	Sequence
106	MS	AAT	P01009	46	5.37	91	51	3.228e-005	2.0	K.LVDFKLFEDVKK.L K.VFSNGADLSGVTEEAAPLK.L K.VFSNGADLSGVTEEAAPLKLSK.A K.GKWERPFEVKDTEEDFHVDQVTTVK.V
1065	MS	TTR	P02766	15	5.52	121	100	0.007	1.3	K.AADDTWEPFASGK.T R.KAADDTWEPFASGK.T K.ALGISPFHEAEVFTANDSGPR.R K.ALGISPFHEAEVFTANDSGPRR.Y K.TSESGELHGLTTEEFVEGIYKVEEIDTK.S
2334	MS/MS	RBP4	P02753	23	5.8	70	12	0.039	1.2	R.LIVHNGYCDGRSERNLL.N K.GNDDHWIVDIDYDYAVQYSCR.L

healthy subjects and patients. In particular, three statistically different spots (106, 1065 and 2334) were highlighted (Table 2) and the related proteins were subsequently identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and MS/MS.

Protein spots of interest were manually excised from 2-DE gels following the procedure described and adapted by Shevchenko et al. [8]. Identification of proteins was performed by peptide-mass fingerprint MALDI/MS measurements using a MALDI-TOF/TOF UltrafleXtreme, equipped

with 1 kHz smartbeam II laser ($\lambda=355$ nm) and operating in the positive reflectron ion modes. The instrumental conditions employed to analyze molecular weight in the m/z range 980–7000 were: Ion Source 1: 25.00 kV; Ion Source 2: 22.40 kV, Lens: 8.00 kV, Reflector: 26.45 kV, Reflector 2: 13.45 kV, Pulsed ion extraction: 120 ns. The matrix employed was α -cyano-4-hydroxycinnamic acid (HCCA) (saturated solution in H₂O/Acetonitrile (50:50; v/v) containing 0.1% trifluoroacetic acid (TFA). External mass calibration was performed using the Peptide Calibration Standard, based

on the monoisotopic values of $[M+H]^+$ of Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip (1-17), ACTH clip (18-39), Somatostatin 28 at m/z 1046.5420, 1296.6853, 1347.7361, 1619.8230, 2093.0868, 2465.1990 and 3147.4714, respectively. The peptide masses were searched against the National Center for Biotechnology Information non-redundant mammalian (Taxonomy homo sapiens) database using Mascot 2.3 searched from Matrix Science, selecting the Swiss-Prot database. For the present study, protein identification was based on measurements with a Mascot score higher than 55. Minimal expectation for valid identification was $p < 0.005$ and $p < 0.05$.

The spot ID106 is due to α_1 -antitrypsin (AAT), spot ID1065 to transthyretin (TTR) and spot ID2334 to retinol binding protein 4 (RBP4) (Mascot database). These identifications were confirmed by MS/MS experiments on selected peptides present in the tryptic digestion mixture of each spot.

Immunoprecipitation tests, carried out by automated immunochemistry analyzer, confirmed an increase of identified proteins. No correlation has been found between overexpressed proteins and duration of the disease, VAS or FIQ.

For the first time, in this preliminary study some differences between the human serum protein pattern of FMS patients with respect to healthy controls have been found.

The most relevant result is a significant over-expression of three proteins identified by a proteomic approach: TTR, RBP4 and AAT in FMS patients.

Oxidative stress has been proposed as a relevant event in the pathogenesis of FMS [2]. A complex defence system protects against oxidative stress including enzymes and non-enzymatic species, such as vitamins C, E and A; vitamin A-bound RBP4 is associated to TTR.

Akkus et al. [3] showed a reduction of vitamin A in FMS patients as a result of its role on radical inhibition and hypothesized an imbalance between ROS production and the antioxidant defence system in patients with fibromyalgia syndrome. These data might be consistent with a previous study [9] that showed unexpected normal levels of uric acid (despite high levels of inosine, hypoxanthine, xanthine) in FMS patients possibly as a result of its role as a ROS scavenger.

Our data show increased serum RBP4; upregulation of RBP4 may induce hypovitaminosis A [10].

High serum TTR and RBP4 might be a result of a flow out from the cells due to ROS-mediated alteration of membrane integrity or permeability; in fact, in vivo experiments have suggested that RBP4 also accumulates in vitamin A-deficient hepatocytes but is not secreted into the bloodstream [11].

In our study, an overexpression of serum AAT was also observed; Blanco and Coll reported high AAT plasma concentrations in a subset of FMS patients and a significantly increased number of mast cells (MCs) in the papillary dermis of all FMS patients with moderately high levels of AAT [12]. The mastocyte-released chemicals could cause local manifestations (e.g., skin tenderness or pain) that might be related to enhanced pain sensitivity of these patients.

A previous study [13] reported a slight increase of AAT associated with a decrease of vitamin A suggesting that genetic or environmental factors may influence AAT activity, which is dependent on the antioxidant capacity of liposoluble vitamins. AAT can be oxidized by free radicals released from oxidative stress, resulting in protein dysfunction that leads to the imbalance between AAT and proteases, which can cause tissue damage [14]. Then there may be high levels of AAT (as reported in our study) but with ineffective action as protease inhibitor.

Considering that the TTR and RBP4 are both retinoid transporters, retinoid dysfunction is related to oxidative stress as well as AAT. Furthermore, assessment of thiobarbituric acid reactive substances for the measurement of lipid peroxidation revealed values over two-fold of the normal range in FMS patients (data not shown), indicating a condition of oxidative stress. These data are in agreement with previous studies that underline the involvement of oxidative stress in the pathology of FMS. Additional studies are required to clarify their exact relationships.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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