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Insight into the biological pathways underlying fibromyalgia by a proteomic approach

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ABSTRACT

Fibromyalgia (FM) is a form of non-articular rheumatism difficult to diagnose and treat because its etiology remains still elusive. Proteomics makes possible the systematic analysis of hundreds of proteins in clinical samples. Consequently, it has become a key tool for finding altered molecular pathways in different diseases. In this context, the present study analyzes changes in the plasma proteome of patients with FM by nanoscale liquid chromatography coupled to tandem mass spectrometry. Deregulated proteins were studied using Ingenuity Pathways Analysis (IPA) and Kyoto Encyclopedia of Genes and Genomes. Conventional analytical methods were used to validate selected proteins. We found a total of 33 proteins differentially expressed in patients with FM. Haptoglobin and fibrinogen showed the highest FM/control ratio. IPA analysis revealed that the top enriched canonical pathways were acute-phase response signaling, Liver-X Receptor/Retinoid-X Receptor activation, Farnesoid-X Receptor/Retinoid-X Receptor activation, and coagulation and complement systems. The importance of inflammation in FM was corroborated by the increase in erythrocyte sedimentation rate. In conclusion, our results support the existence of a plasma protein signature of FM that involves different biological pathways all of them related to inflammation, and point to haptoglobin and fibrinogen as plausible biomarker-candidates for future studies.

Significance: The etiology of fibromyalgia (FM) remains elusive making its diagnosis and treatment difficult. The characterization of the proteome signature of this syndrome will improve its understanding. However, to date proteomic analyses in FM are scarce. The goal of the present work is to analyse, for the first time, changes in plasma protein profiles of patients with FM in comparison to control subjects, using label free relative protein quantification by nanoscale liquid chromatography coupled to tandem mass spectrometry. Our data demonstrate the existence of a common protein signature in the plasma of patients with FM that could explain some of the symptoms associated to this syndrome. The analysis of the 33 proteins differentially expressed corroborates the crucial role of inflammation in the pathogenesis of this syndrome. The interplay of the complement and coagulation cascades contributes to the inflammatory process, while the activation of Liver-X Receptor/Retinoid-X Receptor and Farnesoid-X Receptor/Retinoid-X Receptor could attempt to alleviate it. Finally, we have identified two proteins, haptoglobin and fibrinogen, as potential biomarker-candidates of FM for future studies.

1. Introduction

Fibromyalgia (FM) is a form of non-articular rheumatism, with an estimated prevalence in the general population of 1.7–5.4% and a ratio of females to males of 2.3–13.7 to 1, according to the different classification criteria sets available [1]. This syndrome is characterized by chronic musculoskeletal pain and fatigue with significantly impaired function and quality of life. Moreover, it is also frequently associated with sleep problems, morning stiffness, cognitive impairment, headache, depression, and anxiety [2] that, altogether, lead to activity limitations and impaired work ability.

Although the etiology of FM remains still elusive, several factors have been proposed to be involved. Patients' relatives have a higher risk of developing this syndrome compared to the general population [3], consequently there seem to be a genetic predisposition to FM. In this line, gene polymorphisms in the serotonin receptor 2A region of

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chromosome 13, the serotonin transporter gene regulatory region, the catecholamine methyltransferase, the dopamine-D-3 receptor and the adrenergic receptor have been related to an increased risk of FM [3]. The cognitive and behavioral responses observed in FM have also been linked to glutamatergic hyperactivity. In fact, a recent and thorough review in this field suggests that there is a significant association between increased cerebral glutamate levels and this syndrome [4]. FM and other related pathologies, such as Chronic Fatigue Syndrome, have also been associated with neuroendocrine disorders [5]. Particularly, an exacerbated hypothalamic-pituitary-adrenal axis and an alteration in cortisol levels have been proposed to be involved in FM [6]. However, among all the possible factors that could underlay FM pathophysiology, inflammation and oxidative stress are the most frequently reported. Our research group, among others, found correlations between a pro-oxidative status and a decreased antioxidant capacity in patients with FM [7–9]. Moreover, features related with a pro-oxidative and a pro-inflammatory status, like increased levels of inflammatory markers, lower zinc levels or an augmented lipid peroxidation, seem to be common in these patients. Based on these data, it seems unlikely that FM is caused exclusively by the dysregulation of a single factor. This multifactorial nature makes its treatment highly complex. Therefore, symptomatic medication is at present the main form of treatment, although it often causes adverse effects in these patients.

The lack of known etiology also makes the diagnosis of this syndrome difficult. In fact, nowadays FM takes up to 5 years to be diagnosed, making it imperious to find markers that help in its diagnosis and treatment. Proteomic is a key tool in health research because it makes possible the systematic analysis of hundreds of proteins in clinical samples, with the promise of discovering new biomarkers or altered molecular pathways for different disease conditions. To date, proteomic analyses in FM are scarce. Two studies performed in salivary fluid of patients with FM [10, 11] showed differentially expressed proteins related to oxidative stress, cytoskeletal arrangements and central sensitization in these patients compared to healthy controls. In a preliminary study on serum proteomics of patients with FM, Ruggiero et al. [12] found a significant overexpression of three proteins related to oxidative stress: α1-antitrypsin, transthyretin and retinol binding protein 4. A more recent study in muscle of women diagnosed of chronic widespread pain including FM, showed altered levels of stress and inflammation proteins that correlated with pain intensity [13]. With this background, the goal of the present work is to analyse, for the first time, changes in plasma protein profiles in patients with FM using label free relative protein quantification by nanoscale liquid chromatography coupled to tandem mass spectrometry (NanoLC-MS/MS). This approximation may help to gain insight into the biological pathways potentially related to the FM process and to set a common protein signature in order to improve the diagnosis and management of this syndrome.

2. Material and methods

2.1. Patients and samples

This study was carried out in two groups: 12 age-matched healthy women recruited from the University of Jaén (Spain), and 12 age-matched FM patients from AFIXA (Association of Fibromyalgia of Jaén). All subjects provided written informed consent. The study was approved by the Ethics and Research Committee of the Complejo Hospitalario de Jaén (Spain) and carried out in accordance with the Declaration of Helsinki of the World Medical Association. The inclusion criterion for the FM group was to meet the 1990 American College of Rheumatology (ACR) Criteria for classification of primary FM [14]. Exclusion criteria for the study included the presence of any other chronic disease (diabetes mellitus, hypertension, cancer, ischemic heart disease), pregnancy, lactation, and grade II obesity (with a body mass index (BMI) ≥ 35 kg/m^2). None of the participants were using any medicine that affects the antioxidative status, or were under the treatment of corticosteroids, estrogens, analgesics or anti-inflammatory drugs. None was consuming alcohol, and all of them were non-smokers. All the participants were sedentary living women. The clinical and demographic characteristics of each participant were acquired through interviews and questionnaires (Table 1, Supplementary Table 1). In order to avoid variations, all the procedures and tests were carried out by the same specialist. In patients with FM, the FM impact questionnaire (FIQ) was used to evaluate functional capacity in daily living activities. Musculoskeletal pain was assessed by a visual analogue scale (VAS; 10 cm). The mental (Mental Component Summary, MCS12) and physical (Physical Component Summary, PCS12) health status of the participants was determined by the Spanish version of SF-12 Health Survey [15]. The lower score between 0 and 100 meant worse health status. The mental (MCS-12) and physical (PCS-12) health status were significantly lower in the FM group compared to healthy volunteers ($p < 0.05$ and $p < 0.001$, respectively).

Table 1
Demographic and clinical data of patients with FM and healthy controls.

Variable	FM group	Control group	*p* value
Age (years)	50.58 ± 6.27	47.58 ± 7.94	0.316
FIQ score	52.38 ± 17.07	–	–
VAS score	5.62 ± 2.83	–	–
PCS-12 score	33.53 ± 9.16	55.08 ± 2.52	< 0.001
MCS-12 score	38.53 ± 13.99	50.48 ± 5.75	0.049

Values represent mean ± SD.

Blood samples from patients were extracted at the same time in the early morning to avoid daily variations of the parameters, and after an overnight fast. The blood was disposed in EDTA tubes and EDTA-free tubes to obtain plasma (for proteomic analysis and fibrinogen quantitation) and serum samples (for haptoglobin quantitation), respectively. After centrifugation for 10 min at 1300*g* and 4 °C, supernatants were harvested, aliquoted and stored at −80 °C until used. Whole blood samples were also recollected and immediately used to evaluate Erythrocyte Sedimentation Rate (ESR).

2.2. NanoLC-MS/MS analysis

Plasma was depleted using Pierce top 12 abundant protein depletion spin columns, following manufacturer's instructions. Depleted protein samples were tryptically digested following the filter-aided sample preparation (FASP) protocol described by Wisniewski et al. with minor variations [16]. The resulting peptides were dried and resuspended in 0.1% formic acid, and sonicated for 5 min prior to mass spectrometry analysis. Peptide mixtures were separated on a nanoACQUITY UPLC System (Waters) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron) or a Synapt G2 Si (Waters). An aliquot of each sample was loaded onto a Symmetry 300 C18 UPLC Trap column (180 µm × 20 mm, 5 µm; Waters). The precolumn was connected to a BEH130 C18 column, 75 µm × 200 mm, 1.7 µm (Waters), and equilibrated in 3% acetonitrile and 0.1% FA. Peptides were eluted directly into the nanoelectrospray capillary (Proxeon Biosystems) at 300 nl/min, using a 120 min linear gradient of 3–50% acetonitrile. The Orbitrap XL ETD mass spectrometer (Thermo) automatically switched between MS and MS/MS acquisition in data-dependent acquisition (DDA) mode, in an alternating fashion. Full MS survey spectra (m/z 400–2000) were acquired in the Orbitrap with 30,000 resolution at m/z 400, and two lock-masses were used for increased mass measurement accuracy (445.120024 and 462.146573). The six most intense ions were subjected to collision-induced dissociation (CID) in the linear ion trap. Precursors with charge states of 2 and 3 were specifically selected for fragmentation. Analyzed ions were excluded from further analysis during 30 s using dynamic exclusion lists.

Database searches were performed using the software Proteome Discoverer v.1.4 (Thermo Fisher Scientific).