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 (ESR). 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.

Keywords: fFibromyalgia; pPlasma protein signature; pPathogenesis; iInflammation

1.1 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 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 ~~analyze~~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.~~2 Material and methods

~~2.1.~~2.1 Patients and ~~S~~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).

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).

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

alt-text: Table 1

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.

~~2.2.~~2.2 NanoLC-MS/MS ~~A~~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~~x~~ × 20 mm, 5 µm; Waters). The precolumn was connected to a BEH130 C18 column, 75 µm~~x~~ × 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).

~~2.3.~~2.3 Differential ~~E~~expression ~~A~~analysis

Progenesis LC-MS software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) was used to analyse protein variation among samples. Raw files were directly loaded onto the program and ~~analysed~~analyzed following the workflow provided by the manufacturers. One of the samples was selected as a reference run to which the precursor masses in all the other samples were aligned to. Abundance ratios between the run to be aligned and the reference run, were calculated for all features at given retention times. Protein identifications were performed using Mascot search engine v2.1 (Matrix Science). Carbamidomethylation of cysteines was set as fixed modification, and oxidation of methionines as variable modification, and 2 missed cleavages were allowed. 10 ppm of peptide mass tolerance and 0.5 Da fragment mass tolerance were used. Spectra were searched against Uniprot/Swissprot database version 2016_02 restricted to *Homo sapiens*. A decoy search was carried out in order to estimate the false discovery rate (FDR). Only peptides with a false discovery rate of <1% were selected. Protein quantitation was performed using the information concerning to the three most intense peptides (when available), and only proteins with ANOVA $p<0.05$ and ratio >1.2 in either direction and at least two peptides at a FDR<1%, were considered as significantly deregulated. The normalized relative abundance of every protein was expressed as mean ± standard deviation (SD).

~~2.4.~~2.4 Network ~~A~~analysis

Deregulated proteins in FM were studied by using the Canonical Pathways, Diseases & Functions and Network-building tools, Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com). The Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper –Search pathway tool (http://www.genome.jp/kegg/tool/map_pathway1.html) was also used.

~~2.5.~~2.5 ESR, ~~F~~fibrinogen and haptoglobin ~~D~~determination

ESR was assessed with BD Seditainer~~TM~~™ tubes in a BDVacutainer® Sedi-15~~TM~~™ analyzer (Becton Dickinson). Fibrinogen was determined by the Multifibren®U reagent (Dade Behring Holdings, Inc.) in a BCS XP System analyzer (Siemens). Haptoglobin concentration was quantified by immunoturbidimetry (Tina-quant Haptoglobin test, Roche Diagnostics®) in a Roche/Hitachi MODULAR P analyzer.

~~2.6.~~2.6 Statistical ~~A~~analysis of ~~C~~clinical ~~D~~data

Data were expressed as mean ± standard deviation (SD). Management and data analysis were performed using the statistical package SPSS for Windows version 19.0 (SPSS Inc, Chicago, IL, USA). Kolmogorov-Smirnov test (α-

value = 0.05) and Levenne test (α-value = 0.05) were performed to test normality and homoscedasticity, respectively. Data which followed a normal distribution and the principle of homoscedasticity of variances were tested by an unpaired Student's *t*-test to compare differences between means. The degree of statistical significance in data which did not follow a normal distribution or the principle of homoscedasticity (ESR, fibrinogen, PCS-12, MCS-12) was established by applying the Mann-Whitney *U*-test. To assess the relation between variables, Pearson or Spearman correlation was used. The level of statistical significance was set at $p < 0.05$.

~~3.~~3 Results

~~3.1.~~3.1 Plasma proteome profiling in fibromyalgia

The proteomic profile of plasma samples from healthy controls and patients with FM was determined by NanoLC-MS/MS. A total of 266 proteins were identified. Using a cutoff value of 1% FDR for statistical significance, 33 of these proteins were found to be differentially expressed (Table 2): 25 significantly overexpressed (FM/control ratio ≥ 1.20) and 8 underexpressed (FM/control ratio ≤ 0.80).

Table 2 Proteins differentially expressed in the plasma of patients with FM.

alt-text: Table 2

Uniprot ID	Protein name	Abbreviation	Gene name	Ratio (FM/Healthy)	*p* value
P00738	Haptoglobin	HPT	HP	2.54	7.95E-05
P02679	Fibrinogen gamma chain	FIBG	FGG	2.30	5.49E-03
P60709	Actin, cytoplasmic 1	ACTB	ACTB	2.26	1.11E-03
P07996	Thrombospondin-1	TSP1	THBS1	2.21	3.33E-02
O14791	Apolipoprotein L1	APOL1	APOL1	1.91	3.77E-04
P35542	Serum amyloid A-4 protein	SAA4	SAA4	1.89	3.43E-03
P02655	Apolipoprotein C-II	APOC2	APOC2	1.83	1.15E-02
P02787	Serotransferrin	TRFE	TF	1.83	1.30E-03
P02675	Fibrinogen beta chain	FIBB	FGB	1.78	4.38E-02
P20742	Pregnancy zone protein	PZP	PZP	1.76	4.05E-03
P19652	Alpha-1-acid glycoprotein 2	A1AG2	ORM2	1.74	4.58E-03
P01876	Ig alpha-1 chain C region	IGHA1	IGHA1	1.74	3.09E-02
P00742	Coagulation factor X	FA10	F10	1.63	9.92E-03
P02763	Alpha-1-acid glycoprotein 1	A1AG1	ORM1	1.58	2.27E-02
P01023	Alpha-2-macroglobulin	A2M	A2M	1.52	1.20E-02
P01871	Ig mu chain C region	IGHM	IGHM	1.52	2.24E-02
P09871	Complement C1s subcomponent	C1S	C1S	1.49	1.23E-04
P02743	Serum amyloid P-component	SAMP	APCS	1.48	2.44E-02
P08603	Complement factor H	CFAH	CFH	1.43	4.20E-03
P10643	Complement component C7	CO7	C7	1.39	9.26E-03
P06681	Complement C2	CO2	C2	1.38	9.72E-03
P07225	Vitamin K-dependent protein S	PROS	PROS1	1.38	1.06E-02
P00740	Coagulation factor IX	FA9	F9	1.36	7.09E-03

P02747	Complement C1q subcomponent subunit C	C1QC	C1QC	1.35	1.55E-02
P02748	Complement component C9	CO9	C9	1.33	7.31E-03
P01019	Angiotensinogen	ANGT	AGT	0.73	3.98E-02
P04196	Histidine-rich glycoprotein	HRG	HRG	0.70	2.90E-02
P08571	Monocyte differentiation antigen CD14	CD14	CD14	0.67	5.95E-03
P08185	Corticosteroid-binding globulin	CBG	SERPINA6	0.66	2.78E-02
P05543	Thyroxine-binding globulin	THBG	SERPINA7	0.63	5.93E-03
Q9UK55	Protein *Z*-dependent protease inhibitor	ZPI	SERPINA10	0.63	1.29E-02
Q9UGM5	Fetuin-B	FETUB	FETUB	0.53	3.75E-03
P00915	Carbonic anhydrase 1	CA1	CA1	0.47	4.06E-03

~~3.2.~~3.2 Network and ~~P~~pathway ~~A~~analysis of ~~P~~proteins ~~D~~differentially ~~E~~expressed

We used an IPA analysis in order to assess whether the proteins with different abundance were related to specific molecular pathways and networks. In the pathway analysis, we found 25 statistically significant enriched canonical pathways associated with the 33 deregulated plasma proteins of FM patients (Fig. 1). Among these, the top 5 according to the *p*-value were acute-phase response signaling (14 proteins: HPT, FIBB, FIBG, SAA4, TRFE, A1AG1, A1AG2, C1S, CO2, CO9, A2M, SAMP, HRG and ANGT; Fig. 2A), Liver-X Receptor/Retinoid-X Receptor (LXR/RXR) activation (9 proteins: APOL1, TRFE, A1AG1, A1AG2, CO9, CD14, APOC2, SAA4 and ANGT), Farnesoid-X Receptor/Retinoid-X Receptor (FXR/RXR) activation (9 proteins: APOL1, TRFE, A1AG1, A1AG2, CO9, FETUB, APOC2, SAA4 and ANGT), coagulation system (7 proteins: FIBB, FIBG, FA10, FA9, A2M, PROS, ZPI) and complement system (6 proteins: C1S, CFAH, CO7, CO2, C1QC, CO9). The highest ratio corresponded to the extrinsic prothrombin activation pathway. The LXR/RXR activation and production of nitric oxide and reactive oxygen species in macrophages showed a Z-score > 2 ($Z = 2.333$; $p = 1.58E\text{-}13$ and $Z = 2.236$; $p = 1.20E\text{-}5$, respectively). Using the Disease & Biofunction tool we found that coagulation was activated in patients with FM, while bleeding was inhibited (Fig. 2B). These result was corroborated by the KEGG mapper –Search pathway tool (Fig. 3).

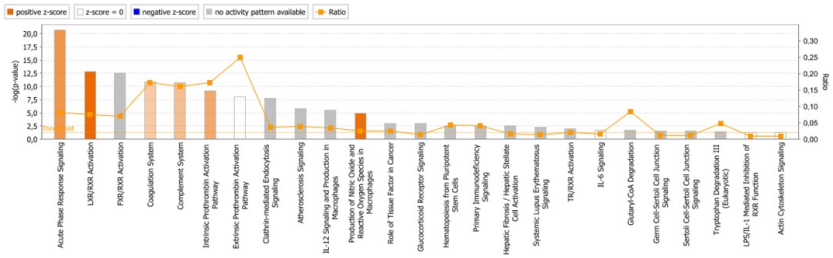

Fig. 1 Canonical pathways enriched in fibromyalgia sorted by statistical significance. Orange or blue colours mean positive or negative *Z*-score, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 1

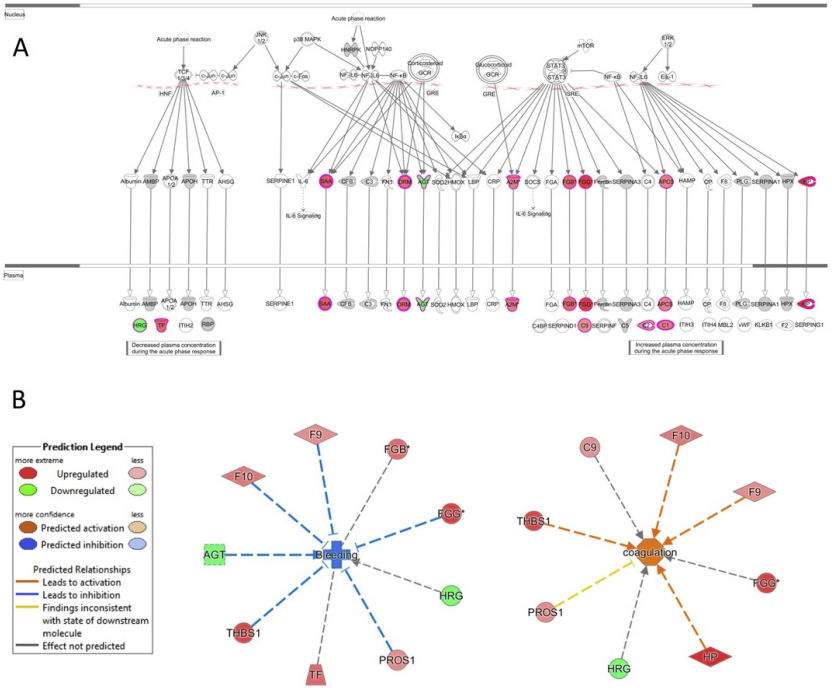

Fig. 2 Ingenuity Pathways Analysis representation of (A) Acute phase reaction signaling in fibromyalgia. Significant pathway nodes are shaded according to size of fold change (red >1.2; green <1.2), with white nodes indicating proteins that were not detected in the samples and grey indicating proteins that were detected, but not significantly. (B) Predicted state of coagulation and bleeding in fibromyalgia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 2

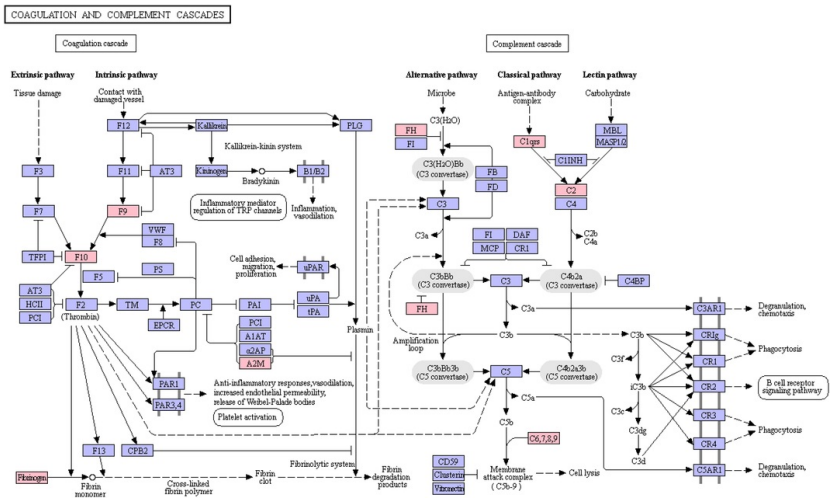

Fig. 3 KEGG representation of coagulation and complement cascades. Red coloured proteins are those with higher levels in patients with FM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 3

For the network analysis, proteins linked with developmental disorder, hereditary disorder and immunological disease were gathered, and the majority of the proteins resulted to be connected to pro-inflammatory cytokines and ERK-1/2 network (Fig. 4).

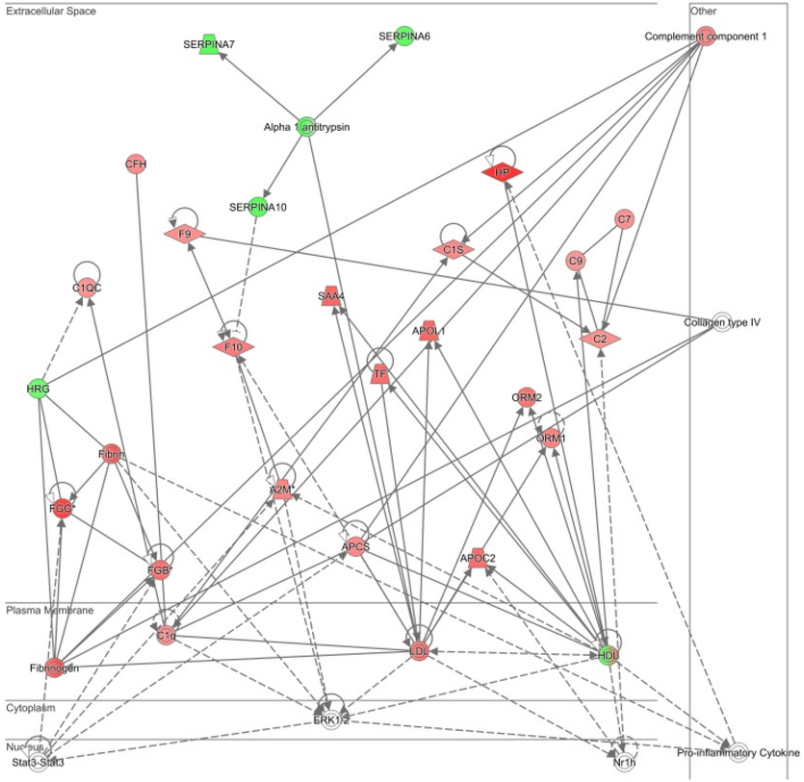

Fig. 4 "Developmental disorder, hereditary disorder, immunological disease", the most significant molecular network identified by Ingenuity Pathways Analysis of differentially expressed proteins (red fold change >1.2; green fold change <1.2) in fibromyalgia. White nodes indicate proteins that were not detected in the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 4

~~3.3.~~3.3 Haptoglobin, ~~F~~fibrinogen and ESR ~~D~~determination

Haptoglobin and fibrinogen are the two proteins with the highest FM/control ratio. Given their involvement in the acute-phase response and in the coagulation cascade, two of the canonical pathways enriched in patients with FM, we next confirmed their overexpression. In agreement with the NanoLC-MS/MS results, the levels of haptoglobin and fibrinogen in patients with FM were significantly increased ($p < 0.02$ and $p < 0.05$, respectively; Table 3).

Table 3 Haptoglobin, fibrinogen and ESR levels in patients with FM and healthy controls.

alt-text: Table 3

Variable	FM group	Control group	*p* value
Haptoglobin (mg/dL)	154.86 ± 62.26	100.55 ± 22.41	0.021
Fibrinogen (g/L)	3.51 ± 0.77	2.77 ± 0.40	0.009
ESR (mm)	20.33 ± 9.17	7.86 ± 3.34	0.002

Values represent mean ± SD.

ESR can indirectly measure the activation of acute-phase response. As shown in Table 3, ESR was also significantly increased in patients with FM compared to controls ($p < 0.05$). These inflammatory clinical data (haptoglobin, fibrinogen, and ESR) positively correlated with the proteomic results (Table 4).

Table 4 Correlations between inflammatory clinical data and proteomics.

alt-text: Table 4

		Clinical data			Proteomic data		
		Haptoglobin (mg/dl)	Fibrinogen (g/l)	ESR (mm)	HPT	FIBB	FIBG
Clinical data	Haptoglobin (mg/dl)	1	0.648**	0.667**	0.755***	0.506*	-0.548**
	Fibrinogen (g/l)		1	-0.791***	0.720***	0.568*	0.582*
	ESR (mm)			1	0.729***	0.484*	0.504*
Proteomic data	HPT				1	-0.668***	-0.670***
	FIBB					1	-0.932***
	FIBG						1

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

4.4 Discussion

We performed, for the first time, NanoLC-MS/MS analysis in the plasma from FM patients and age-matched healthy volunteers. We found a total of 33 proteins with different abundance in patients with FM. Most of the proteins were related to inflammation processes and, among others, five dominant pathways according to its *p*-value were identified as enriched: the acute phase response signaling, LXR/RXR activation, FXR/RXR activation, coagulation system and complement system.

Our comparative IPA analysis showed 14 proteins related with the acute-phase response signaling, 12 significantly more abundant (HPT, FIBB/FIBG, SAA4, TRFE, A1AG1, A1AG2, C1S, CO2, CO9, A2M, SAMP) and 2 significantly less abundant (HRG, ANGT) in FM. The acute-phase response is a complex mechanism from the organism against local or systemic disturbances that occurs, among others, during inflammatory processes [17]. In these processes, the tissular damage leads to the release of pro-inflammatory cytokines which, together with nitric oxide and glucocorticoids, control the hepatic synthesis of the acute-phase reactants [18–20]. The acute-phase reactants include proteins whose plasma concentrations increase (positive reactants) or decrease (negative reactants) significantly. In our study, we identified 11 positive acute-phase reactants upregulated and 1 negative acute-phase reactant downregulated. The activation of this response can be indirectly measured by ESR, and we have also detected a statistically significant increase in ESR in FM. Altogether, these results suggest the involvement of an inflammatory response in FM, even though TRFE, a negative reactant, was increased and ANGT, a positive reactant, was decreased. Similar discordances have been previously reported, as not all the acute-phase reactants increase uniformly [21]. In fact, as proposed by Baralla et al. in the inflammatory context of chronic pulmonary disease [22], the increase of TRFE may reflect an attempt to preserve from the deleterious effects of free iron. Acute-phase response is traditionally associated with a high level of C-reactive Protein (CRP) and several interleukins. However, our results do not show changes in any of these proteins. In this sense, Bazzichi et al. [23] reported that only the 25% of the FM patients exhibited high levels of CRP, and no correlation with CRP level has been described in other typical inflammatory diseases, such as Systemic Lupus Erythematosus (SLE) [24]. Similarly, published data do not show a consistent interleukin pattern in FM either. Some authors found higher levels of IL-10, IL-8 and TNF-α in this condition [23]. Contrary and supporting our findings, Wallace et al. [25] were unable to describe higher levels of IL-6 and IL-8 in FM patients compared to healthy controls. Likewise, Kasiphaz et al. [26] highlighted that the alteration in cytokine production was not a dominant factor in the pathogenesis of FM. These conflicting results may underlie the existence of a chronic inflammatory state. This situation associates with moderate elevations of CRP and IL-6 that may even be masked by the influence of other factors. In fact, it has recently been demonstrated, in women with rheumatoid arthritis, that CRP levels are correlated with fat mass but not with disease activity, suggesting that CRP should be used cautiously in certain pathologies [27]. In line with those results, although Feinberg et al. [28] demonstrated a positive

correlation between CRP and FM, the CRP values found in FM patients were nearly within the reference range and the addition of BMI weakened this relationship. Consequently, the heterogeneity in the findings from small case-control studies may reflect differing selection criteria among the controls. The exclusion criteria stablished in our study could explain the lack of correlation between CRP and FM. However, the modest influence of obesity on ESR [27] could account for our increased ESR values in FM patients, and would support its use as a more accurate disease activity biomarker.

During the inflammatory processes, complement and coagulation systems play crucial roles [29]. In fact, complement system has been associated to other rheumatic disorders such as Rheumatoid Arthritis [30] and SLE [25]. We found 6 proteins of the complement system (C1S, CFAH, CO7, CO2, C1QC, CO9) overexpressed in FM patients. Except CFAH, an inhibitory protein of the alternative pathway, all these proteins are involved in the formation of the Membrane Attack Complex ~~(MAC)~~ through the classical pathway. This pathway is mainly triggered by IgM/IgG-antigen complexes [31] and leads to chemotaxis and plasma protein exudation at inflammatory sites, facilitating the opsonization of damaged cells [21]. In accordance, our data showed increased levels of IgM, pointing out that the classical pathway of complement system is another factor to be considered in FM. Moreover, the high abundance of IgM could also contribute to the increased ESR values observed in our cohort of patients and previously reported in other inflammatory pathologies [24]. The complement cascade can also be activated by some coagulation proteins [29]. Our results showed higher levels of FIBB, FIBG, FA10, FA9, A2M and PROS, and a lower amount of ZPI, all of them involved in coagulation. Among these, FA10 and FA9 have been previously described as complement cascade activators. Therefore, these results could indicate the interplay of both pathways in FM. In fact, the extrinsic and the intrinsic prothrombin activation pathways showed the two highest ratios among the 25 statistically significant enriched canonical pathways, emphasizing the crucial role of coagulation in the FM condition. The scarce literature in this field explains the pro-coagulant state in terms of platelet activation [32]. However, Berg et al. [33] described an increased level of fibrinogen in chronic fatigue syndrome and/or FM, which not always associates with platelet activation. In our study, the quantitative determination of fibrinogen by a standard analytical method confirmed the higher levels of fibrinogen observed by proteomics in patients with FM.

The proteomic analysis also identified significantly enriched the LXR/RXR activation pathway (APOL1, TRFE, A1AG1, A1AG2, CO9, CD14, APOC2, SAA4 and ANGT), predicted as activated with a z-score of 2.33, and the FXR/RXR pathway (APOL1, TRFE, A1AG1, A1AG2, CO9, FETUB, APOC2, SAA4 and ANGT). The RXR is a nuclear hormone receptor of the retinoid receptor family and the common partner for several others nuclear receptors such as LXR and FXR [34]. These receptors are known to be involved in many biological and pathological pathways associated with lipid metabolism and inflammation [35]. Particularly, LXR once bound to oxysterols activates sterol regulatory element binding protein 1c (SREBP-1c), a central lipogenic transcription factor [36, 37]. This activation results in the upregulation of different genes involved in lipogenesis and lipoprotein metabolism such as the apolipoprotein C-I/C-IV/C-II gene cluster [38], increased in our cohort of FM patients. Besides, LXR also activates reverse cholesterol transport in macrophages preventing foam cell formation and inhibits different pro-inflammatory transcription factors. Similarly, FXR activation regulates triacylglyceride metabolism, cholesterol homeostasis, and suppresses inflammatory pathways [35]. The anti-inflammatory activity of both pathways suggests that the activation of LXR and FXR pathways could attempt to alleviate the inflammatory environment mentioned above in FM patients. Glucocorticoids also exert a modulatory effect on inflammation and, in our study, CBG was found to be downregulated in FM patients, according to other authors [39]. CBG transports the 90% of the total cortisol in blood and is cleavaged by leukocyte elastase at sites of inflammation, inducing a typical S-to-R transition that results in hormone release [40]. Although there is no consensus about the effect of FM on cortisol levels [41–43] our group, among others, has not found differences in total cortisol level in this pathology [9, 44]. In this context, the CBG downregulation in our cohort could involve a higher bioavailability of free cortisol. Nevertheless, it is plausible that this cortisol may be unable to exert its modulatory effect on inflammation due to abnormalities in glucocorticoid receptor. In fact, therapeutic interventions with glucocorticoids have resulted ineffective in FM [45].

Neuroinflammatory mechanisms underlie physical and psychological distress [46], typical symptoms of FM condition. Consequently, the activation of acute phase response described in our proteomic study could be related to the statistically significant differences in the PCS-12 and MCS-12 results observed between FM patients and controls. Moreover, inflammation and stress physiology are reportedly associated to a high production of oxygen/nitrogen reactive species particularly in macrophages, and one of the pathways with a highest z-score found in our IPA analysis was precisely the production of nitric oxide and reactive oxygen species in macrophages. In this sense, our group has already described that patients with FM exhibit an imbalance between oxidants and antioxidants that results in higher oxidative DNA damage [9]. Haptoglobin is an acute phase reactant with antioxidant activity through its capability to bind hemoglobin and prevent the toxicity of heme iron. Consequently, its increase in chronic processes aims to counteract excessive oxidative stress preventing, among others, muscle atrophy [47]. Moreover, its higher levels in patients with FM were confirmed by conventional analytical methods. Plasma haptoglobin concentrations are known to be positively related to vegetative symptoms of depression, such ~~u~~as psychomotor retardation, energy, fatigue, hyperalgesia, and loss of interest or insomnia [48]. In a recent study, haptoglobin was also increased in plasma from women with chronic widespread pain [49] suggesting the existence of a low-grade systemic inflammation that contributes to central nervous system alterations. In fact, these authors describe a proteomic pattern similar to the one in our study that also involves the complement system, coagulation and inflammatory processes. Therefore, in line with them, our results also point to the importance of inflammation in the pathogenesis of FM.

5.5 Conclusions

As a whole, our study supports 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. Since all the different biological pathways involved this protein profile are related to inflammation, we point to this event as crucial in the pathogenesis of FM. The interplay of the complement and coagulation cascades probably contributes to the inflammatory process, while

the activation of LXR/RXR and FXR/RXR could attempt to alleviate it. Moreover, we suggest that further studies should be carried out to assess whether haptoglobin and fibrinogen could be useful biomarkers in the diagnosis and management of this syndrome.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.07.009.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCESReferences

[1] GTG.T. Jones, FF. Atzeni, MM. Beasley, EE. Flüß, PP. Sarzi-Puttini and GJG.J. Macfarlane, The prevalence of fibromyalgia in the general population: a comparison of the American College of Rheumatology 1990, 2010, and modified 2010 classification criteria, *Arthritis RheumArthritis Rheum.* **67**, 2015, 568–575.

[2] RAR.A. Hawkins, Fibromyalgia: a clinical update, *J. Am. Osteopath Assoc.* **113** (9), 2013, 680–689.

[3] LML.M. Arnold, HJ.I. Hudson, EVE.V. Hess, AEA.E. Ware, DAD.A. Fritz, MBM.B. Auchenbach, et al., Family study of fibromyalgia, *Arthritis Rheum.* **50**, 2004, 944–952.

[4] TLT.L. Pyke, PGP.G. Osmotherly and SS. Baines, Measuring glutamate levels in the brains of fibromyalgia patients and a potential role for glutamate in the pathophysiology of fibromyalgia symptoms: a systematic review, *Clin J Pain.Clin. J. Pain* **33**, 2017, 944–953.

[5] GFG.F. Romano, SS. Tomassi, AA. Russell, VV. Mondelli and CMC.M. Pariante, Fibromyalgia and chronic fatigue: the underlying biology and related theoretical issues, *Adv Psychosom Med.Adv. Psychosom. Med.* **34**, 2015, 61–77.

[6] AJA.J. Cleare, The HPA axis and the génesis of chronic fatigue síndrome, *Trends Endocrinol Metab.Trends Endocrinol. Metab.* **15**, 2004, 55–59.

[7] SS. Chinn, WW. Caldwell and KK. Gritsenko, Fibromyalgia Ppathogenesis and Ttreatment Ooptions Uupdate, *Curr Pain Headache RepCurr. Pain Headache Rep.* **20**, 2016, 25.

[8] AA. Rus, FF. Molina, MM. Gassó, MVM.V. Camacho, MAM.A. Peinado and MLM.L. Del Moral, Nitric Ooxide, Iinflammation, Llipid profile and Ccortisol in Nnormal and Ooverweight Wwomen with Ffibromyalgia, *Biol. Res. Nurs* **18**, 2016, 138–146.

[9] MM. La Rubia, AA. Rus and FF. Molina, Del Mmoral ML. Is fibromyalgia-related oxidative stress implicated in the decline of physical and mental health status?, *Clin Exp RheumatolClin. Exp. Rheumatol.* **31**, 2013, S121–S127

[10] LL. Bazzichi, FF. Ciregia, LL. Giusti, CC. Baldini, GG. Giannaccini, CC. Giacomelli, et al., Detection of potential markers of primary fibromyalgia syndrome in human saliva, *Proteomics Clin. Appl.* **3**, 2009, 1296–1304.

[11] CC. Giacomelli, LL. Bazzichi, LL. Giusti, FF. Ciregia, CC. Baldini, YY. Da Valle, et al., MALDI-TOF and SELDI-TOF analysis: "tandem" techniques to identify potential biomarker in fibromyalgia, *Reumatismo* **63**, 2011, 165–170.

[12] VV. Ruggiero, BB. Era, EE. Cacace, LL. Molin, MM. Corda, AA. Fais, et al., A preliminary study on serum proteomics in fibromyalgia syndrome, *Clin Chem Lab MedClin. Chem. Lab. Med.* **52**, 2014, e207–e210.

[13] PP. Olausson, BB. Ghafouri, NN. Ghafouri and BB. Gerdle, Specific proteins of the trapezius muscle correlate with pain intensity and sensitivity - an explorative multivariate proteomic study of the trapezius muscle in women with chronic widespread pain, *J Pain ResJ. Pain Res.* **9**, 2016, 345–356.

[14] FF. Wolfe, HAH.A. Smythe, MBM.B. Yunus, RMR.M. Bennett, CC. Bombardier, DLD.L. Goldenberg, et al., The American College of Rheumatology 1990 criteria for the classification of fibromyalgia: report of the multicenter criteria committee, *Arthritis RheumArthritis Rheum.* **33**, 1990, 160–172.

[15] GG. Vilagut, MM. Ferrer, LL. Rajmil, PP. Rebollo, GG. Permanyer-Miralda, JMJ.M. Quintana, et al., The Spanish version of the Sshort Fform 36 Hhealth Ssurvey: a decade of experience and new developments, *Gac SanitGac.*

Sanit. **19**, 2005, 135–150.

[16] JR.J.R. Wiśniewski, A.A. Zougman, N.N. Nagaraj and M.M. Mann, Universal sample preparation method for proteome analysis, *Nat Methods**Nat. Methods* **6**, 2009, 359–362.

[17] F.F. Ceciliani, A.A. Giordano and V.V. Spagnolo, The systemic reaction during inflammation: the acute-phase proteins, *Protein Pept. Lett.* **9**, 2002, 221–223.

[18] PC.P.C. Heinrich, TA.T.A. Castell and T.T. Andus, Interleukin-6 and the acute phase response, *Biochem JBiochem. J.* **265**, 1990, 621–636.

[19] AS.A.S. Van Miert, Pro-inflammatory cytokines in a rummiant model: pathophysiological, pharmacological, and therapeutic aspects, *Vet Q* **17**, 1995, 41–50.

[20] PC.P.C. Heinrich, I.I. Behrmann, G.G. Müller-nNewmen, F.F. Schaper and L.L. Graeve, Interlukin-6-type cytokine signalling through the gp130/Jak/STAT pathway, *Biochem JBiochem. J.* **334**, 1998, 297–314.

[21] C.C. Gabay and I.I. Kushner, Acute-phase proteins and other systemic responses to inflammation, *N Engl J MedN. Engl. J. Med.* **340**, 1999, 448–454.

[22] A.A. Baralla, AG.A.G. Fois, E.E. Sotgiu, E.E. Zinellu, AA.A.A. Mangoni, S.S. Sotgia, et al., Plasma Pproteomic Ssignatures in Eearly Cchronic Oobstructive Ppulmonary Ddisease, *Proteomics Clin. Appl.* 2018, https://doi.org/10.1002/prca.201700088.

[23] L.L. Bazzichi, A.A. Rossi, G.G. Massimetti, G.G. Giannaccini, T.T. Giuliano, F.F. De Feo, et al., Cytokine patterns in fibromyalgia and their correlation with clinical manifestations, *Clin Exp RheumatolClin. Exp. Rheumatol.* **25**, 2007, 225–230.

[24] A.A. Wiik and M.M. Fritzler, Laboratory tests in rheumatic disorders, In: M.M. Hochenberg, A.A. Silman, J.J. Smolen, M.M. Winblatt and M.M. Weisman, (Eds.), *Rheumatology. Philadelphia (PA),* 2008, Elsevier, 219–232.

[25] DJ.D.J. Wallace, SL.S.L. Silverman, J.J. Conklin and D.D. Barken, Systemic lupus erythematosus and primary fibromyalgia can be distinguished by testing for cell-bound complement activation products, *Lupus Sci. Med.* **3**, 2016.

[26] MA.M.A. Kashipaz, D.D. Swinden, I.I. Todd and R.R. Powell, Normal production of inflammatory cytokines in chronic fatigue and fibromyalgia syndromes determined by intracellular cytokine staining in short-term cultured blood mononuclear cells, *Clin Exp ImmunolClin. Exp. Immunol.* **132**, 2003, 360–365.

[27] MD.M.D. George, JF.J.F. Giles, PP.P.P. Katz, BR.B.R. England, TR.T.R. Mikuls, K.K. Michaud, et al., Impact of Oobesity and Aadiposity on Iinflammatory Mmarkers in Ppatients Wwith Rrheumatoid Aarthritis, *Arthritis Care ResArthritis Care Res.* **69**, 2017, 1789–1798.

[28] T.T. Feinberg, U.U. Sambamoorthi, C.C. Lilly and KK.K.K. Innes, Potential Mmediators between Ffibromyalgia and C-Rreactive protein: Rresults from a Llarge U.S. Ccommunity Ssurvey, *BMC Musculoskelet DisoreBMC Musculoskelet. Disord.* **18**, 2017, 294.

[29] K.K. Oikonomopoulou, D.D. Ricklin, PA.P.A. Ward and JD.J.D. Lambris, Interactions between coagulation and complement-their role in inflammation, *Semin Immunopathol Semin. Immunopathol.* **34**, 2012, 151–165.

[30] M.M. Okroj, D.D. Heinegård, R.R. Holmdahl and AM.A.M. Blom, Rheumatoid arthritis and the complement system, *Ann MedAnn. Med.* **39**, 2007, 517–530.

[31] D.D. Ricklin, G.G. Hajishengallis, K.K. Yang and JD.J.D. Lambris, Complement - a key system for immune surveillance and homeostasis, *Nature ImmunologyNat. Immunol.* **11**, 2010, 785–797.

[32] M.M. Milovanovic, S.S. Nilsson, PI.P.I. Haakara, C.C. Post and B.B. Gerdle, High Iin vivo platelet activity in female fibromyalgia patients, *J. Biomed. Sci.* **5**, 2016, 3.

[33] D.D. Berg, LH.L.H. Berg, J.J. Couvaras and H.H. Harrison, Chronic fatigue syndrome and/or fibromyalgia as a variation of antiphospholipid antibody syndrome: an explanatory model and approach to laboratory diagnosis *Blood Coagul FibrinolysisBlood Coagul. Fibrinolysis* **10**, 1999, 435–438.

[34] TH.T.H. Bugge, J.J. Pohl, O.O. Ionnoy and HG.H.G. Stunnenberg, RXR alpha: a promiscuous partner of retinoic acid and thyroid hormone receptors, *EMBO JEMBO J.* **11**, 1992, 1406–1418.

[35] JA.J.A. van Diepen, JF.J.F. Berbée, LM.L.M. Havekes and PC.P.C. Rensen, Interactions between inflammation and lipid metabolism: relevance for efficacy of anti-inflammatory drugs in the treatment of atherosclerosis, *Atherosclerosis* **228**, 2013, 306–315.

[36] JR.J.R. Schultz, H.H. Tu, A.A. Luk, J.J. Repa, JC.J.C. Medina, L.L. Lil, et al., Role of LXRs in control of lipogenesis, *Genes DevGenes Dev.* **14**, 2000, 2831–2838.

[37] TT. Yoshikawa, HH. Shimano, MM. Amemiya-Kudo, NN. Yahagi, AHA.H. Hasty, TT. Matsuzaka, et al., Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1 c promoter, *Mol Cell Biol Mol. Cell. Biol.* **21**, 2001, 2991–3000.

[38] PAP.A. Mark, BAB.A. Laffitte, CC. Desrumaux, SBS.B. Joseph, LKL.K. Curtiss, DJD.J. Mangelsdorf, et al., Regulated expression of the apolipoprotein E/C-I/C-IV/C-II gene cluster in murine and human macrophages. A critical role for nuclear liver X receptors alpha and beta, *J. Biol. Chem.* **277**, 2002, 31900–31908.

[39] EGE.G. Lentjes, ENE.N. Griep, JWJ.W. Boersma, FPF.P. Romijn and ERE.R. de Kloet, Glucocorticoid receptors, fibromyalgia and low back pain, *Psychoneuroendocrinology* **22**, 1997, 603–614.

[40] JGJ.G. Lewis, CJC.J. Bagley, PAP.A. Elder, AWA.W. Bachmann and DJD.J. Torpy, Plasma free cortisol fraction reflects levels of functioning corticosteroid-binding globulin, *Clin. Chim. Acta Int. J. Clin. Chem.* **359**, 2005, 189–194.

[41] AA. Gur, RR. Cevik, AJA.J. Sarac, LL. Colpan and SS. Em, Hypothalamic-pituitary-gonadal axis and cortisol in young women with primary fibromyalgia: the potential roles of depression, fatigue, and sleep disturbance ir the occurrence of hypocortisolism, *Ann Rheum Dis Ann. Rheum. Dis.* **63**, 2004, 1504–1506.

[42] LJL.J. Crofford, EAE.A. Young, NCN.C. Engleberg, AA. Korszun, CBC.B. Brucksch, LAL.A. McClure, et al., Basal circadian and pulsatile ACTH and cortisol secretion in patients with fibromyalgia and/or chronic fatigue syndrome, *Brain Behav Immun Brain Behav. Immun.* **18**, 2004, 314–325.

[43] MEM.E. Bote, JJJ.J. García, MDM.D. Hinchado and EE. Ortega, Inflammatory/stress feedback dysregulation in women with fibromyalgia, *Neuroimmunomodulation* **19**, 2012, 343–351.

[44] RPR.P. Freitas, TMT.M. Lemos, MHM.H. Spyrides and MBM.B. Sousa, Influence of cortisol and DHEA-S on pain and other symptoms in post menopausal women with fibromyalgia, *J. Back Musculoskelet. Rehabil.* **25**, 2012, 245–252.

[45] GG. Littlejohn, Neurogenic neuroinflammation in fibromyalgia and complex regional pain syndrome, *Nat Rev Rheumatol Nat. Rev. Rheumatol.* **11**, 2015, 639–648.

[46] AHA.H. Miller and CLC.L. Raison, The role of inflammation in depression: from evolutionary imperative to modern treatment target, *Nat. Rev. Immunol.* **16**, 2016, 22–34.

[47] EE. Bertaggia, GG. Scabia, SS. Dalise, FF. Lo Verso, FF. Santini, PP. Vitti, et al., Haptoglobin is required to prevent oxidative stress and muscle atrophy, *PLoS ONE* **9**, 2014.

[48] MM. Maes, SS. Scharpé, HYH.Y. Meltzer and PP. Cosyns, Relationships between increased haptoglobin plasma levels and activation of cell-mediated immunity in depression, *Biol Psychiatry Biol. Psychiatry* **34**, 1993, 690–701.

[49] KK. Wåhlén, PP. Olausson, AA. Carlsson, NN. Ghafouri, BB. Gerdle and BB. Ghafouri, Systemic alterations in plasma proteins from women with chronic widespread pain compared to healthy controls: a proteomic study, *J Pain Res J. Pain Res.* **10**, 2017, 797–809.

E-Extra

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) $\geq 35\ kg/m^2$). 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).

E-component

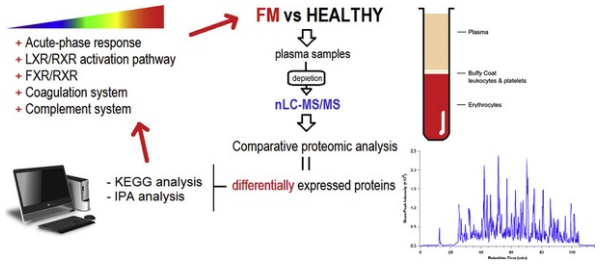
The following are the supplementary data related to this article.

Multimedia Component 1

Supplementary Table 1 Individual demographic and clinical data of patients with FM and healthy controls.

alt-text: Supplementary Table 1

Graphical abstract



alt-text: Unlabelled Image

Highlights

- Comprehensive analysis of changes in the plasma proteome of patients with FM by NanoLC-MS/MS/Characterization of the proteome signature of FM by NanoLC-MS/MS.
- Patients with FM exhibit changes in the plasma proteomic profile.
- The biological pathways involved in fibromyalgia are related to inflammation.
- Complement and coagulation cascades, and LXR and FXR pathways are activated in FM.
- High levels of haptoglobin and fibrinogen, biomarker-candidates for future studies.

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