REVIEW ARTICLE

Proteomics and Fibromyalgia: A Perspective on the Study of the Inflammatory Response in Fibromyalgia

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Abstract
Fibromyalgia (FM) is a chronic, non-degenerative disease characterized by widespread and sustained pain, sleep disturbances, physical exhaustion, and cognitive difficulties. The FM pathophysiology has not been completely clarified, and several theories have been postulated, among which is the dysregulation of the inflammatory response as a mediator of the painful phenomenon. In addition, it has been reported that FM patients present a rise of IL-6 and IL-8 serum levels; this fact has clinical relevance since these inflammatory molecules induce symptoms such as pain, fatigue, hyperalgesia, and allodynia.
Additionally, some studies have been carried out on the participation of leukocytes in the physiopathology of fibromyalgia; The evidence suggests that mast cells are the most relevant leukocytes in the pathophysiology of FM since they promote the release of pro-inflammatory cytokines in response to stimuli such as substance P or corticotropin-releasing hormone, which were elevated in patients.
The number of FM patients is increasing year after year around the world. For this reason, it is essential to study the proteins involved in the inflammatory response in fibromyalgia. Proteomic analysis techniques such as tandem mass tag (TMT) with isobaric labeling offer a hope to find biological markers that allow the study simultaneously the participation of multiple inflammatory proteins in FM patients, allowing the identification of biomarkers. Thus, the use of isobaric tagging will allow shortly to expand the knowledge of pathophysiology in fibromyalgia, helping to identify biomarkers to improve the diagnosis of the FM disease and increase the quality of life of patients and their families.

Keywords: Fibromyalgia, leucocytes, cytokines, proteomics, tandem mass tag, isobaric label

1. Introduction

Fibromyalgia (FM) is an orphan disease that has not been thoroughly studied. A search in PubMed with the terms "fibromyalgia" and "human" in the last 20 years shows only 9,711 results. Meanwhile, a search with the terms "depression" and "human" for the same period gives 270,920 results.
A chronologic evolution of the concepts, descriptions, and the efforts to established diagnostic criteria of FM throughout the past 150 years. There are descriptions in the literature of musculoskeletal pain problems dating back to the 16th century in the "*Liber de reumatismo*". The french physician Guillaume de Baillou introduced the term rheumatism to describe clinical manifestations of muscular pain and acute rheumatic fever in 1592. In 18th century, physicians started to distinguish articular rheumatism to muscular rheumatism. Since the 19th century, various forms of muscular rheumatism suggesting that muscular rheumatism could be a form of neuralgia. Literature on muscular rheumatism was published by German, Scandinavian, and British physicians from the beginning of 1800's. Tender points and fibrositis nodules were reported to be associated with rheumatism by Balfour in 1824. Beard described the association of fatigue, widespread pain, and psychological disorders and coined the term neurasthenia. By the early 20th century, the pain on muscular disorders was proposed to result from hyperactivity of nerve endings; the role of tender points or muscular nodules in FM was popularized by Stockman in 1904; but

the term fibrositis was introduced by Gowers latter. In 1976, Hench was the first to use the term fibromyalgia. The initial preliminary criteria for the diagnosis of primary fibromyalgia were proposed by Yunus and cols in 1981. In 1990 Wolfe *et al*. published in The American College of Rheumatology (ACR) criteria for classification and diagnosis of FMS in a well-designed and blinded study, which have been modified in 2010, 2011, and 2016[1–4].

FM is a clinical syndrome characterized by dysregulation of neuroendocrine function or nociceptive processing. Clinically it is characterized by generalized pain sustained over time, sleep disturbances, physical exhaustion, and cognitive difficulties[5]. The ACR defines FM as widespread chronic pain (≤ 3 months) and pain on palpation of at least 11 of 18 tender points throughout the body (areas of tenderness occurring in muscles and muscle-tendon junctions)[6]. In addition, FM has been recognized as the most prevalent member of the central sensitivity syndromes (CSS) and the medically unexplained symptoms[7].

Its prevalence is estimated to be between 2% and 4% depending on the study population, and it affects more women than men[6]. In Mexico, the prevalence of FM was 0.7% according to the study by Peláez-Ballestas and cols[8]. A factor that may influence the differences in prevalence is the difficulty in making the diagnosis, causing a considerable number of physicians to fail to diagnose this syndrome[6,7]. In addition, it has been estimated that the average time to receive a diagnosis is 6.5 years[9]. According to Núñez-Narvaez et al, a delay was observed in the 9.5 year diagnosis[13]; however, in our experience, we have had patients who have waited 10 ten years or more to receive a diagnosis, which generates frustration in patients and negatively affects their health[10]. A delay in diagnosis may be influenced by multiple variables, such as the age of symptom onset, presence of comorbidities, physician experience, and geographic location[10].

In addition, it should be noted that patients with FM have a poor quality of life due to the high costs involved in treating the disease[5]. One of the main problems that afflict patients with this disease is the difficulty in making a diagnosis that excludes other conditions, causing uncertainty and impact on the patient's economy[11]. It is estimated that the health services costs for a patient with FM can be up to three times more than that of a patient with another disease[11,12]. In addition, the fact that it is a disabling disease also indirectly affects the economy because patients diagnosed with FM stop working around five years after diagnosis, although it may be in a shorter period of time[13]. It is highlighted that patients who present more problems at work have more severe symptoms due to difficulties carrying out their activities, causing a higher rate of dismissals and resignations[14]. In addition, patients with FM suffer from social isolation due to family problems and emotional relationships, and reduced activities of daily living and leisure, which negatively impacts the quality of life[15].

2. The inflammatory response in FM

The first report using the term "fibromyalgia" was published in 1987; since this first report and despite the efforts of doctors and researchers, the etiopathogenic aspects of this disease are still under discussion. There are several theories involving dysautonomia or the presence of inflammatory disorders, among others[16,17].

Van West and Maes proposed that inflammatory disorders accompanied by changes in the neuroendocrine-immune system might underlie the FM syndrome[18]. In 2012 Bote et al. concluded that FM syndrome could be categorized as an inflammatory and stress-related disease. The mild chronic increase in cortisol found in FM patients with a chronic low-grade

inflammatory status indicates that the hypothalamic-pituitary-adrenal (HPA) axis failed to control the increase of pro-inflammatory cytokines. The increased release of pro-inflammatory cytokines in patients corroborates this information[19].

Several studies have associated the inflammatory response with FM symptoms. Cytokine elevation in cerebrospinal fluid (CSF) and serum has been reported[20,21]. In the same way, the participation of inflammatory molecules in the development of pain and sensitization phenomena has been described[22].

On the other hand, it has been proposed that FM occurs with alterations of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the inflammatory response and may influence sensitization processes[23]. Additionally, it has also been described that in pain syndromes, there may be hyper- or hypocortisolism and an infiltrate of mast cells that release mediators that cause sensitization of nociceptors and an increase in the concentration of pro-inflammatory cytokines[22,24]. It has been reported that patients with FM have higher concentrations of the corticotropin-releasing hormone (CRH) in their serum and cerebrospinal fluid, which correlates with increased symptomatology[22]. Likewise, stress has been reported to increase the severity of FM symptoms, which may be explained by the overactivation of HPA and the increased release of CRH and inflammatory molecules[16,19,25].

2.1 Cytokines on fibromyalgia

Patients with FM have increased serum levels of inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor–alpha (TNF-α), and chemokines, such as CCL11 (eotaxin) and CCL17 (thymus- and activation-regulated chemokine or TARC) in patients with FM[16,26–28].

Two cytokines, IL-6 and IL-8, are strongly associated with the clinical symptoms of FM. They have clinical relevance in FM and are secreted by the stimulation of substance P (SP)[27,28]. Previously, our group observed that IL-6 and IL-8 are present at high levels in the circulatory system of FM patients despite pharmacological treatment. This pair of cytokines are the most reported inflammatory mediators, and they correlate directly with symptom severity[29].

IL-6 is a pro-inflammatory cytokine with diverse functions such as leukocyte activation to increase antibody production, Th17 and T follicular cell differentiation, regulation of metabolism, production of acute-phase proteins, platelet production, osteoclast differentiation, angiogenesis, and mesangial cell and keratinocyte proliferation[30]. IL-6 promotes pain generation through central sensitization and the sensitization of nociceptive neurons. Additionally, it has been linked to nociceptive plasticity[31]. IL-6 is widely distributed in the central nervous system (CNS), especially in the hypothalamus. High levels of gp130, IL-6, and IL-6R expressions have been described in the dorsal root ganglion and the spinal cord in studies on pathological pain[32]. In a murine model, it has been reported that IL-6 may modify the current threshold for action potential firing by causing hyperexcitability in neurons secondary to the phosphorylation of the sodium channel Nav1.7. This generates an increase in the number of action potentials and a decrease in latency, causing prolonged migraine-related pain behavior in animals through the activation of the ERK pathway[33].

IL-8 is a chemokine that promotes the recruitment and activation of neutrophils and granulocytes. It is also crucial in inflammatory processes and has been described as being involved in the pain that occurs in chronic inflammatory disorders[16]. Increased levels of IL-8 have been reported in both blood and CSF of FM patients[20].

These effects of IL-8 are the result of increased sympathetic system activity due to binding to β-receptors[34]. It should be emphasized that patients with FM may present elevated sympathetic tone, which favors the appearance of fatigue, hyperalgesia, and allodynia[35].

Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine released mainly by macrophages[36]. It can modulate pain signaling by binding to the TNFR1 receptor[37]. It has been reported that in periods of emotional and physical stress, high levels of SP and CRH are released; they are involved in the induction of inflammation and the activation of mast cells, which release TNF-α[23,25].

IL-1β has been implicated in the pathophysiology of FM. Some studies have demonstrated the presence of increased levels of IL- 1β in patients with FM[36,38,39]. It has been stated that IL-1 produces somnolence and promotes hyperalgesia by vagal afferents, which can be blocked by the administration of IL-1Ra. Additionally, it regulates substance P expression and is associated with fever[40].

IL-17 is another cytokine that has been reported to have increased levels in patients with FM; however, it is unclear how it influences the pathophysiology of FM[41]. IL-17 dysregulation is implicated in the pathogenesis of autoimmune diseases and chronic inflammatory diseases[21]. In murine models, it has been stated that the elevation of this cytokine contributes to the development of neuropathic pain[42]; one of the proposed mechanisms is that IL-17 promotes astrocyte proliferation and pro-inflammatory cytokine release in rat models of spinal cord injury[43].

The overexpression of IL-10, IL-25, and IL-36 cytokine genes was reported in the peripheral blood mononuclear cells (PBMC) of FM patients[44], and some TNF-α polymorphisms have been associated with anxiety, depressive symptoms, pain, cognitive impairment, fatigue, and sleep disturbances[45–47]. At the same time, an IL-6 polymorphism has been associated with fatigue, pain, anxiety, and depressive symptoms[46].

It has been proposed that the cytokines IL-6, IL-8, and TNF-α could be a follow-up marker for FM patients[7,48]. Some trials have been conducted with complementary treatments such as meditation or exercise that have not shown promising results[48–50]. In this context, clinical trials that implemented the use of naltrexone for two months showed a decrease in the severity of symptoms and pro-inflammatory cytokines levels; however, this study has limitations, such as the short duration, the small sample used, and the lack of a control group, so this study's application is not recommended[51]. On the other hand, IL-37 (a member of the IL-1 family) has been proposed as a treatment since it down-regulates cJun, MAP kinase, p38α, STAT, and p53 transcription factors, which are involved in inflammation. However, there are no reports of clinical protocols that are employing this cytokine[52,53].

2.2 Leukocytes on fibromyalgia

2.2.1 T cells

T lymphocytes participate in the adaptive immune response. It has been reported that T lymphocytes obtained from FM patients suggest that these cells have functional alterations that limit their capacity for activation and proliferation in response to stimuli such as PMA or cytokines[17,54]; it has also been reported that there are no alterations in the CD4/CD8 T lymphocyte ratio in patients with FM[55]. The number of T lymphocytes in patients with FM has been analyzed, and the information is controversial since some authors have reported an increase in the T lymphocytes number, while others reported a decrease[17,56,57].

2.2.2 Mast cells

Mast cells are located at connective tissues in the perivascular zone and participate in the inflammatory response[23]; these cells maintain close communication with nociceptors, permitting a bidirectional communication that regulates the inflammatory response and pain[52]. It is worth mentioning that high plasma levels of IL-6, TNF-α, SP, and CRH were reported in FM patients in comparison with controls[19,58]; previously it has been reported that CRH and SP promote mast cells degranulation and release of TNF-α and IL-6 from mast cells[25].

3. Protein study by tandem mass tagging in FM

The study of the immune system in FM has generated relevant information, suggesting that inflammatory molecules and some leukocytes are involved in the pathophysiology. Currently, a broad range of techniques has been developed to allow us to perform more comprehensive and more exhaustive analyses in less time. Proteomics is not an exception, being a technique that will enable us to identify numerous potential biomarkers in a *single shot*, which can help us improve on such issues as diagnosis, prognosis, and clinical follow-up in the future[59]. Moreover, proteomics can be applied to numerous samples such as biopsies, blood, urine, or saliva[60].

A tandem mass tag (TMT) is a technique that employs a chemical label to facilitate the multiplexing of samples through mass spectrometry (MS). It allows the identification and quantification of biomolecules, such as proteins, peptides, and nucleic acids. Reagents called isobaric mass labels are used; these are molecules with the same mass that, after fragmentation, generate reporter ions with different mass[61]. The most common isobaric labels are amine-reactive labels[62]. These amine-reactive groups undergo N-hydroxysuccinimide (NHS) reactions based on three types of functional groups[63,64]. Tandem mass labels have a mass indicator region, a cleavable binding region, a mass normalization region, and a reactive protein group and have the same total mass[62]. The use of isobaric labels has the advantage of enabling the analysis of multiple samples in one run without dramatically affecting MS complexity because quantification is performed at the MS/MS level. However, one of the drawbacks of this method is the simultaneous isolation of multiple precursor ions in the MS sweep, termed isobaric interference, which can underestimate protein/peptide fold changes[65].

We used this technique to analyze samples from patients with FM and perform proteomic studies to identify proteins involved in the inflammatory response in this disease. For this purpose, we recruited patients with FM and healthy volunteers to obtain peripheral blood mononuclear cells (PBMC) and subsequently proteins for pooling and generating peptides labeled with isobaric tags for analysis by mass spectrometry. Finally, we performed bioinformatic analysis and validation in patient samples (Figure 1).

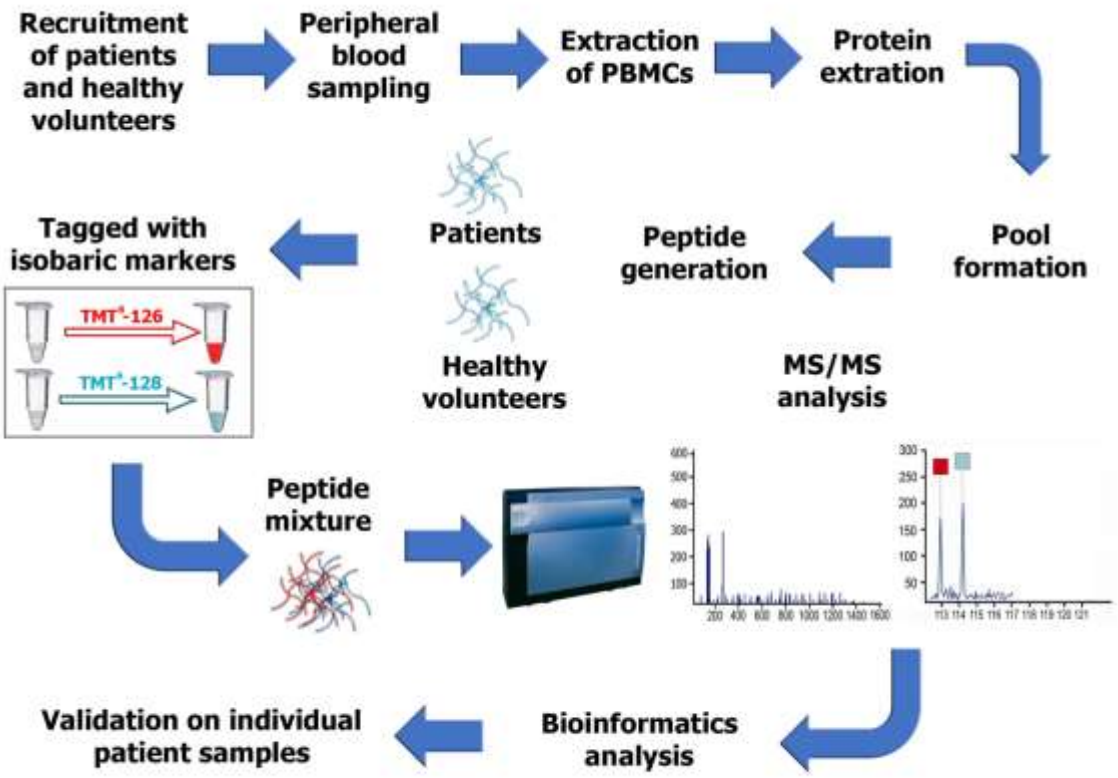

Figure 1. Methodology used for the analysis of samples from fibromyalgia patients. Fibromyalgia patients and healthy individuals were recruited. Subsequently, blood samples were obtained to isolate peripheral blood mononuclear cells, from which proteins were extracted. Finally, pools were performed with samples from patients with fibromyalgia and healthy individuals, which were labeled with isobaric markers to be analyzed by mass spectrometry. The results were evaluated by bioinformatic analysis, and the results were validated on individual patient samples.

Our results confirm orthogonally some alterations reported in previous work; however, it also provides information on alterations in other proteins. Below, we show some examples of the proteins we found in FM patients participating in acute phase response, the coagulation cascade and lipid metabolism, all involved in fibromyalgia inflammation (Table 1).

A. Liver X Receptor-Retinoid X Receptor (LXR/RXR) (proteins involved APOL1, TRFE, A1AG1, A1AG2, CO9, CD14, APOC2, SAA4, and ANGT) **and Farnesoid X Receptor- Retinoid X Receptor (FXR/RXR)** (proteins involved APOL1, TRFE, A1AG1, A1AG2, CO9, FETUB, APOC2, SAA4, and ANGT) pathway. The RXR is a nuclear hormone receptor of the retinoid receptor family and the joint partner for several other nuclear receptors such as LXR and FXR. These receptors are known to be involved in many biological and pathological pathways associated with lipid metabolism and inflammation. LXR activates reverse cholesterol transport in macrophages. It is an important modulator

of macrophage cholesterol efflux, preventing foam cell formation, inhibits different pro-inflammatory transcription factors, and can inhibit T-cell proliferation. Liver X receptors (LXRs) are another class of intracellular lipid sensors that are activated by oxysterols in response to elevated intracellular cholesterol levels in multiple cell types and are a key regulator of hepatic lipogenesis. Glucose metabolism is also impacted by LXR activity. Similarly, the farnesoid X receptor (FXR) is another metabolic receptor activated by endogenous bile acids, which has a dual role in the regulation of lipid metabolism and inflammation. Activation of FXR regulates bile acid, lipid, and glucose metabolism and, similar to LXRs, suppresses inflammatory pathways via transrepression[66–69].

Table 1. Proteins obtained by isobaric labeling analysis

Proteins	Function	Role in fibromyalgia
Liver X Receptor-Retinoid X Receptor (LXR/RXR) and Farnesoid X Receptor-Retinoid X Receptor (FXR/RXR)	These receptors regulate lipid metabolism and inflammation.	These proteins regulate the expression of proinflammatory cytokine genes; loss of these mechanisms could trigger inflammation.
Haptoglobin (HP)	Is an acute phase reactant with antioxidant, it binds hemoglobin and prevent iron toxicity	HP elevation is associated with vegetative symptoms of depression, such as psychomotor retardation, energy, fatigue, hyperalgesia, and loss of interest or insomnia.
Fibrinogen	Is a protein involved in acute systemic responses to inflammation, stress, and coagulation	It can be used as a surrogate marker to assess the level of dysautonomia in patients with FM.
Heat shock protein β-1	Is involved in stress resistance and actin organization.	Their decrease may reflect insufficient capacity or stress in the processes of promoting muscle recovery.
α-crystallin B chain	Functions as stabilizers of the altered myofibrillar structures, so it could be related to muscle recovery	Could be an inflammatory marker
Pyruvate, ATP and PCr	Mitochondrial proteins	They could be an indicator of mitochondrial dysfunction in muscle.

B. Haptoglobin (HP) and **fibrinogen** are two proteins involved in acute-phase response and coagulation cascade. Consequently, the increase of HP and fibrinogen in chronic processes aims to counteract excessive oxidative stress, preventing, among other things, muscle atrophy. HP is an acute phase reactant with antioxidant activity due to its capability to bind hemoglobin and avoid heme iron toxicity. Plasma HP concentrations are known to be positively related to vegetative symptoms of depression, such as psychomotor retardation, energy, fatigue, hyperalgesia, and loss of interest or insomnia. FM has a plasma protein signature, is upregulated, and suggests the existence of inflammation. Fibrinogen is a protein involved in acute systemic responses to inflammation, stress, and coagulation. Therefore, fibrinogen levels can act as a surrogate marker to evaluate the level of dysautonomia in patients with FM.[66,67,70].

C. Heat shock proteins β-1 and **α-crystallin B** belong to the small heat shock protein (HSP20) family. The α-crystallin B chain has chaperone-like activity, preventing aggregation of various proteins under a wide range of stress conditions[71,72]. The up-regulation and accumulation of the heat shock proteins in the muscle may result in myofibrils being more resilient to both oxidative and mechanical stresses and protect the cytoskeleton of the myofibrils. It has also been suggested that by binding to cytoskeletal and myofibrillar proteins, the α-crystallin B chain functions as a stabilizer of disrupted myofibrillar structures, indicating a role in muscle recovery[73,74]. The heat shock protein β-1 is found in various tissues, including striated muscle, and is involved in stress resistance and actin organization[75]. A lowered abundance may reflect an insufficient capacity or strain upon the processes promoting muscle recovery. Furthermore, prostaglandins are reported to play a role in the induction of the HSP20 family response. The increased levels of α-crystallin B chain in chronic pain in FM could result from increased levels of prostaglandins, known as nociceptive/pain mediators. Glutathione S-transferase *Mu*2 is involved in the detoxification of products from oxidative stress, for example. Taken together, they indicate alterations in oxidative stress and inflammation, metabolic pathways, and processes associated with muscle damage and recovery in chronic pain in FM[73,74].

D. Pyruvate, **ATP,** and **PCr** suggest mitochondrial muscle dysfunction in FM. The increased pyruvate levels may reflect insufficient pyruvate transportation into the mitochondria, insufficient reduction of pyruvate to lactate, or defective transport of lactate into the mitochondria. All of these conditions would result in a decrease in ATP and PCr concentrations. In addition, increased pyruvate levels may be the result of an inflammatory environment that switches metabolism from the mitochondrial oxidative phosphorylation system (OXPHOS) to glycolysis, a condition that would prevent pyruvate from being transported into mitochondria. In this scenario, lactate dehydrogenase (LDH) in the cytosol converts two pyruvate molecules back into lactate, resulting in only two ATP molecules rather than the 36 molecules generated by OXPHOS, a decrease in energy production. Dysfunctional mitochondria are responsible, interacting with immune and nociceptive systems, inflammatory conditions, and the regulation of immune cells and immune responses, and releasing damage-associated molecular patterns recognized by the immune system, thereby triggering an inflammatory response[76–79].

4. Conclusions

The study of the impact of the inflammatory response in fibromyalgia has focused mainly on pro-inflammatory cytokines and some cell populations such as mast cells or T

lymphocytes. However, several proteins involved in the inflammatory response that has not been considered in fibromyalgia may help understand the pathophysiology. The use of techniques such as isobaric labeling for the study of fibromyalgia will allow the identification of these proteins that are associated with inflammation and related to other functions, such as lipid metabolism or mitochondrial dysfunction in muscle. We obtained orthogonal results that coincide with the proteins already described. Additionally, our results have allowed us to identify other immune system components, such as proteins of the complement system and proteins associated with the coagulation cascade, that still need to be validated. Thus, in the medium and long term, these results will generate new opportunities for developing biomarkers or therapeutic strategies to improve the quality of life of fibromyalgia patients.

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