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BIOMARKERS IDENTIFICATION IN FIBROMYALGIA SYNDROME

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LE DONNE CHE HANNO CAMBIATO IL MONDO,

NON HANNO MAI AVUTO BISOGNO DI MOSTRARE NULLA,

SE NON LA LORO INTELLIGENZA.

(RITA LEVI MONTALCINI)

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ABSTRACT

Fibromyalgia Syndrome (FMS) is a chronic syndrome characterized by widespread pain. FMS is a collection of other symptoms and overlapping conditions contribute to complicate the diagnosis, the assessment and the treatment. Unknown etiology and none laboratory tests have been appropriately validated for the diagnosis of the disease. The comparison of protein patterns in body fluids of diseased and healthy subjects has the potential to identify new disease-specific biomarkers. Some purine nucleotide metabolism disorders such as myoadenylate deaminase (MAD) deficiency report symptoms similar to those seen in FMS.

In consideration of what described above, we carried out a serum proteomic analysis of FMS patients with respect to control subjects searching potentially useful biomarkers for the disease. In addition, we evaluated serum purine metabolite concentrations in patients affected by FMS and the relationships between their levels and FMS clinical parameters.

Twenty-two females affected by FMS (according to the American College of Rheumatology, 1990) and twenty-two healthy women were recruited as controls for analysis of purine metabolite. Sixteen females FMS and twelve controls were enrolled in the study for the analyses of the proteome. Proteomic analysis was performed by combining two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) and serum purine levels were quantified using reverse phase high performance liquid chromatographic (RP-HPLC).

In our study, using the proteomic approach, we have identified differentially expressed proteins, such as Transthyretin (TTR), Alpha-1 Antitrypsin (A1AT) and Retinol Binding Protein 4 (RBP4). The serum

concentrations of these proteins were significantly higher in FMS patients compared with healthy controls. TTR and RBP4 are retinoid transporters, moreover retinoid dysfunction is related to oxidative stress as well as A1AT. These results support the hypothesis that oxidative stress could be implicated in the pathophysiology of FMS.

Moreover, considerably higher serum concentration of inosine, hypoxanthine and xanthine levels ($p<0.001$) and lower serum adenosine ($p<0.05$) were detected in the FMS patients when compared to healthy controls. Our data show a negative correlation between adenosine and the Fibromyalgia Impact Questionnaire (FIQ).

Our results suggest that purines, in particular adenosine and inosine, may be involved in pain transmission in fibromyalgia.

SOMMARIO

La sindrome fibromialgica (FMS) è caratterizzata da dolore diffuso cronico associato a un insieme di sintomi e condizioni sovrapposte che potrebbero contribuire a complicare la diagnosi, la valutazione e il trattamento della malattia. L'eziologia è sconosciuta e nessun esame di laboratorio è stato adeguatamente validato per la diagnosi e la gestione della malattia. Il confronto di pattern proteici nei fluidi biologici di pazienti e controlli potrebbe portare all'identificazione di specifici biomarcatori. Alcuni autori hanno riportato che alcune disfunzioni del metabolismo delle purine, come la carenza della mioadenilato deaminasi (MAD), determinano sintomi simili a quelli osservati nei pazienti fibromialgici.

A seguito di queste considerazioni abbiamo effettuato un analisi del proteoma del siero di 16 pazienti affetti da FMS (secondo i criteri dell'American College of Rheumatology, 1990), confrontato con quello di 12 soggetti sani, alla ricerca di potenziali biomarcatori. Inoltre, abbiamo determinato le concentrazioni sieriche di alcuni metaboliti purinici in 22 pazienti affetti da FMS e 22 controlli e abbiamo valutato le relazioni tra i loro livelli e i sintomi della patologia. L'analisi proteomica è stata eseguita combinando l'elettroforesi bi-dimensionale (2-DE) e la spettrometria di massa (MS), mentre l'analisi dei metaboliti purinici è stata effettuata mediante una cromatografia liquida ad alta prestazione in fase inversa (RP-HPLC).

L'analisi proteomica ha evidenziato la differente espressione di proteine come la Transtiretina (TTR), l'Alfa-1-Antitripsina (A1AT) e la proteina legante il retinolo (RBP4), nei soggetti affetti da FMS rispetto ai controlli. TTR e RBP4 sono trasportatori dei retinoidi; disfunzioni dei retinoidi e

dell'A1AT sono correlate allo stress ossidativo. I risultati ottenuti ci consentono di ipotizzare che lo stress ossidativo potrebbe essere coinvolto nella patofisiologia della FMS.

L'analisi, mediante RP-HPLC, del siero di pazienti con FMS ha rilevato un aumento delle concentrazioni di inosina, ipoxantina e xantina ($p<0.001$) e valori significativamente bassi di adenosina ($p<0.05$) nei pazienti affetti da FMS rispetto ai controlli. I dati ottenuti mostrano una correlazione negativa tra adenosina e FIQ che potrebbe essere coerente con una maggiore sensibilità al dolore nei pazienti affetti da FMS.

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ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
A1AT	Alpha-1 antitrypsin
ACN	Acetonitrile
ACR	American College of Rheumatology
ACTH	Adrenocorticotropic hormone
ADA	Adenosine deaminase
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CTS	Carpal tunnel syndrome
ddH_2O	Double distilled water
DIGE	Differential gel electrophoresis

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Ionization
FDR	False discovery rate
FIQ	Fibromyalgia Impact Questionnaire
FIQR	Revised Fibromyalgia Impact Questionnaire
FMS	Fibromyalgia Syndrome
GH	Growth hormone
GTP	Guanosine-5'-triphosphate
HCCA	α-cyano-4-hydroxycinnamic acid
HPLC	High performance liquid chromatographic
IAA	Iodoacetamide
IEF	Isoelectric focusing
IGF-1	Insulin-like growth factor 1
IMP	Inosine monophosphate
IPG	Immobilized pH gradient
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MAD	Myoadenylate Deaminase

MALDI	Matrix-Assisted Laser Desorption Ionization
Mr	Molecular weights
MS	Mass spectrometry
MW	Molecular weight
NH_4HCO_3	Ammonium bicarbonate
NSAIDs	Non-steroidal anti-inflammatory drugs
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PTMs	Post- translational modifications
RBP4	Retinol Binding Protein 4
ROS	Reactive oxygen species
RPC	Reversed-Phase Chromatography
RSD	Relative standard deviation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SS	Symptom severity
SSNRIs	Selective serotonin noradrenaline reuptake inhibitors
SSRIs	Selective serotonin reuptake inhibitors
T4	Thyroxine

TFA	Trifluoroacetic acid
TNF	Tumor necrosis factor
TOF	Time of flight
TRH	Thyrotropin-releasing hormone
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TTR	Transthyretin
VAS	Visual analog scale
WPI	Widespread Pain Index

1. INTRODUCTION

1.1. Fibromyalgia

1.1.1. Definition and epidemiology

Fibromyalgia Syndrome (FMS) or fibromyalgia is the most common cause of widespread musculoskeletal pain (Wolfe F et al, 1990) and is a chronic condition causing pain, stiffness, and tenderness of the muscles, tendons, and joints. It is also characterized by restless sleep, tiredness, fatigue, anxiety, depression and disturbances in bowel function.

In the past different terms were used to define what today is indicated as fibromyalgia. Fibromyalgia was previously referred as *fibrositis*, as suggested by William Gowers (Gowers WR, 1904) referring to regional pain syndromes associated with profound fatigue and sleep disturbance.

Graham W in 1950 (Graham W, 1953) introduced the modern concept of fibromyalgia as "pain syndrome" in the absence of a specific organic disease. The term "fibromyalgia" was coined in the mid-1970s with the identification of extreme tenderness regions , the so-called "tender points".

The prevalence of FMS is estimated to range between 1.3% and 4.8% in the general population (Biewer W et al, 2004).

Fibromyalgia affects approximately 6 million people in the United States (Sprott H, 2003), 85% to 92% are women (Lawrence RC et al, 1998; Wolfe F et al, 1990), many of whom are sedentary (Gowans SE et al, 2004) and overweight or obese (Yunus MB et al, 2002).

White KP et al (1999), in the London Fibromyalgia Epidemiology Study in Ontario, Canada, estimated the prevalence of FMS at 3.3% (4.9% in women versus 1.6% in men), and Branco JC, et al (2010) reported on a

multinational study of the prevalence of FMS in 5 European countries, estimating it at 4.7%.

Häuser W and coworkers estimated the prevalence in Germany at 3.8%, with similar rates in men and women (Häuser W et al, 2009).

In Italy affects at least 2% of the adult population (Salaffi F et al, 2005). However, it is still a poorly understood condition that is difficult to diagnose.

1.1.2. Symptoms

FMS is a complex illness to diagnose and treat, with symptoms that may be part of, or overlap with other diseases or syndromes.

FMS is one of the most common diseases affecting the muscles manifested with pain, stiffness and tenderness of the muscles, tendons and joints. Despite potentially disabling body pain, patients with fibromyalgia do not develop tissue damage or deformity (Mease P et al, 2007; Schmidt-Wilcke T et al, 2011). The pain in fibromyalgia is generally widespread, involving both sides of the body. Pain usually affects neck, buttocks, shoulders, arms, upper back and chest. "Tender points" are localized tender areas of the body that can bring on widespread pain and muscle spasm when touched (Perrot S, 2008; Culpepper L, 2010).

FMS typically presents in young or middle-aged females as persistent widespread pain, stiffness, fatigue and exhaustion, disrupted unrefreshing sleep, sleep disorders, restless sleep, awakening from sleep, difficulty in falling asleep and cognitive difficulties, often accompanied by multiple other unexplained symptoms, anxiety and/or depression, panic attacks, migraine/headache, gastrointestinal problems and functional impairment of daily living activities (Gerdle B et al, 2008; Wolfe F et al, 1995; Clauw DJ et al, 2003; Karakuş İ et al, 2004). A recent study reported that 88,9% of FMS patients have pain, 70.4% restless sleep, 88.9% fatigue and similar symptoms (Demirbağ B et al, 2012).

Diagnosis is difficult and frequently overlooked because symptoms are vague and generalized. Despite this, three main symptoms are referred by almost every patient: pain, fatigue and sleep disturbance (Aaron LA et al, 2000; Aaron LA et al, 2001).

Pain is the main symptom of FMS and it is known that many factors can influence soreness perception; there are discrepant findings related to vitamin D levels and pain intensity (De Rezende Pena C et al, 2010; Heidari B et al, 2010).

Fatigue represents one of the most disabling symptoms associated with FMS, greatly impacting patients quality of life; sleep disturbance, such as difficulty falling asleep, frequent awakening during the night and early awakening with difficulty returning to sleep, are very commonly reported in persons with FMS.

Recent years, two studies evaluated the concordance between subjective and objective methods of sleep assessment reporting that women with objective sleep deficits had significantly higher pain scores on the tender point index, perceived their sleep as significantly shoddier and reported significantly more depressive symptoms and more negative impact of FMS on daily life activities than those without deficit (Stuifbergen AK et al, 2010; Okifuji A et al, 2011). It is well known that FMS is frequently associated with psychiatric symptoms such as anxiety and depression; indeed some authors have argued the possibility of classifying this syndrome into affective spectrum disorders.

Thyroid hormone levels are usually normal, even if the patients often show symptoms of hypothyroidism and there is some evidence suggesting an association with abnormal thyrotropin-releasing hormone (TRH) stimulation tests (Garrison L et al, 2003).

Many conditions may be associated with fibromyalgia (Table 1):

Musculoskeletal	Genitourinary	Gastro intestinal	Miscellaneous
• Nondermatomal paresthesia • Temporo mandibular joint syndrome • Hyper mobility syndrome • Restless legs syndrome • Rheumatoid arthritis • Systemic lupus erythematosus • Sjögren syndrome • Osteoarthritis • Chronic fatigue syndrome • Carpal tunnel syndrome • Myofascialpain syndrome	• Dysmenorrhea • Interstitial cystitis • Vulvodynia • Female urethral syndrome • Vulvar vestibulitis • Premenstrual syndrome	• Irritable bowel syndrome • Esophageal dysmotility	• Tension/migraine headaches • Mitral valve prolapsed • Allergy • Vestibular disorders • Ocular disturbances • Anxiety disorders • Reynaud phenomenon • Thyroid dysfunction • Lyme disease • Hyperventilation • Cognitive dysfunction

Table 1. Conditions associated with fibromyalgia (Tanriverdi F et al, 2007; Bradley LA, 2005; Woolf AD, 2000).

1.1.3. Etiopathogenesis

The etiology and pathogenesis of fibromyalgia are still not fully understood but the current hypothesis is that FMS arises from interactions between the autonomic central nervous system, the hypothalamic-pituitary-adrenal axis and the immune system (Clauw DJ et al 2011; Arnold LM, 2010; DeLeao JA et al, 2001; Buskila D, 2001).

Central sensitization is considered the main mechanism involved and it is defined by the increased response to stimulation mediated by central nervous system (CNS) signaling (Yunus MB, 1992). Metabolic and pharmacologic findings suggest involvement of the CNS in FMS, such as alterations of N-methyl-D-aspartate receptors or monoaminergic modulation in the spinal cord (Russell IJ et al, 1994; Sörensen J et al, 1997; Sörensen J et al, 1998; Graven–Nielsen T et al, 2000).

Another proposed mechanism involves the well-known descending inhibitory pain pathways, which modulate spinal cord responses to painful stimuli. They seem to be impaired in patients with fibromyalgia, helping to exacerbate the central sensitization (Staud R et al, 2002; Staud R et al, 2003; Kosek E et al, 1997). Glial cells activation appears to play an important role in the pathogenesis of fibromyalgia because they help to modulate pain transmission in the spinal cord. Activated by various painful stimuli, they release proinflammatory cytokines, nitric oxide, prostaglandins, and reactive oxygen species that stimulate and prolong spinal cord hyperexcitability (Watkins LR et al, 2001; Watkins LR et al, 2005). As early as 1988, it was noted that aberrant expressions of immune mediators such as cytokines may contribute to the onset of disease symptoms (Wallace DJ et al, 1988). Wallace et al have been observed the

correlation between cytokines and several symptoms common in FMS (Wallace DJ et al, 2001).

However, reports on changes in serum cytokine levels in FM patients have revealed conflicting results (Menzies V et al, 2010; Űceyler N et al, 2011). Recently Cordero MD et al (2013) showed in FMS high levels of serum TNF-alpha as a consequence of high cytokine levels, these data underline an important role of oxidative stress and mitochondrial dysfunction in the inflammatory process in several FMS patients, thus identifying a new subgroup of patients in FMS.

Many evidence point out alterations in neurotransmitter systems in fibromyalgia, something which is interesting given that the main symptoms of fibromyalgia (that is, heightened pain perception, fatigue, sleep disorders and depressive- as well as anxiety-related symptoms) are closely related to neurotransmitters.

In FMS patients, serotonin, dopamine and noradrenalin levels appear to be decreased, possibly contributing to dysfunctional descending pathways and resulting in attenuated descending inhibition (Becker S et al, 2012). Cerebrospinal fluid (CSF) concentrations of substance P and glutamate have been repeatedly found to be increased in fibromyalgia patients (Liu Z et al, 2000; Russell IJ et al, 1994; Vaerøy H et al, 1988).

There is evidence for both a hypercortisolemic and hypocortisolemic state in FMS. In more detail, FMS patients have been reported to exhibit an elevated cortisol profile (Catley D et al, 2000), but also to display an attenuated cortisol response during the first hour after awakening (Weissbecker I et al, 2006; Klingmann PO et al, 2008).

FM patients showed high values of adrenocorticotropic hormone (ACTH) both basally (Neeck G, 2000; Geenen R, 2002) and in response to stress, most likely as a consequence of a chronic hyposecretion of corticotropin-

releasing hormone (CRH) (Griep EN et al, 1993). Growth hormone (GH) levels tend to be normal during the day, reduced during sleep because GH is mainly secreted during stage 4 of sleep and this phase is disrupted in patients affected by fibromyalgia; another these patients have high levels of somatostatin, a GH inhibitor, induced by ACTH whose levels are high as previously mentioned (Jones KD et al, 2007) but, recently, Kadetoff D et al (2010) found normal baseline ACTH and cortisol levels in patients with FMS.

Thyroid hormone levels are usually normal, even if the patients often show symptoms of hypothyroidism and there is some evidence suggesting an association with abnormal thyrotropin-releasing hormone (TRH) stimulation tests (Garrison RL et al, 2003). Various studies (Cohen H et al, 2001; Kooh M et al, 2003) seem to confirm that in fibromyalgia the sympathetic nervous system is persistently hyperactive, but hyporeactive to stress. This could explain some clinical symptoms such as fatigue, morning stiffness, sleep disorders, anxiety, pseudo-Raynaud's phenomenon and bowel irritability (Stisi S et al, 2008). Furthermore patients with FMS often complain of sleep disorders. As revealed by electroencephalographic examinations the fourth phase of sleep is the most disturbed and a direct consequence should be a deficit of GH and insulin-like growth factor 1 (IGF-1) (Van Cauter E et al, 1998; Prinz PN et al, 1995), given that these hormones are involved in muscle microtrauma repair, the healing of this tissue could be affected by sleep disturbances (Bennett RM et al, 1992). Fibromyalgia is common in patients affected by autoimmune disease (Middleton GD et al, 1994; Wolfe F et al, 2004). Different studies in the literature deal with autoantibodies in fibromyalgia (Kötter I et al, 2007; Bazzichi L et al, 2007) with equivocal results.

The chronicity of FMS affects patients quality of life actions on their social relations, habits and routines, causing an increase in the psychological abnormalities common to FMS, especially depressive states and psychiatric disorders (Payne TC et al, 1982.). The prevalence of psychiatric conditions among patients affected by fibromyalgia is higher than among subjects complaining of other rheumatic diseases (Giesecke T et al, 2003). The most common disorders associated are anxiety, somatization, dysthymia, panic disorders, post-traumatic stress and overall depression (Epstein SA et al, 1999). Peripheral tissues such as skin, muscles, and microvessels are coming under closer investigation. Inadequate response to oxidative stress (Ozgocmen S et al, 2006), increased IL-1 in cutaneous tissues, increased substance P in muscles are all suspected to possibly play a role in this condition.

Although the etiology of fibromyalgia remains unclear, it is believed that genetic and environmental factors may play significant roles in the development of fibromyalgia

Genetic predisposition is likely to be an important factor as suggested by several familial studies (Buskila D et al, 2006) and transmission is thought to be polygenic (Buskila D et al, 2007). Among the various genes investigated, the most important are associated with neurotransmitters involved in pain transmission. A number of associations have been identified that including the serotonin 5-HT2A receptor polymorphism T/T phenotype, serotonin transporter, dopamine 4 receptor, and COMT (catechol-O-methyltransferase) polymorphism. Lee YH et al, (2012) demonstrates that the serotonin 2A (5-HT2A) receptor 102T/C polymorphism confers susceptibility to fibromyalgia, in contrast, no association was found between the serotonin transporter (5-HTT) gene-

linked polymorphic region (5-HTTLPR) S/L allele, catechol-O-methyltransferase (COMT) val158Met, and susceptibility to fibromyalgia.

Recently, Arnold LM et al (2012) identify loci for fibromyalgia. This is the first study to report genome-wide suggestive linkage of fibromyalgia to the chromosome 17p11.2-q11.2 region. It is notable that chromosome 17p11.2-q11.2 region coincides with the map coordinate for two potential candidate genes for fibromyalgia: the serotonin transporter gene (SLC6A4) and the transient receptor potential (TRP) vanilloid 2 (TRPV2) genes.

FMS is multifactorial disorders and there is increasing evidence that it may be triggered by environmental factors and many authors have highlighted a relationship with various infectious agents and some have suggested that vaccinations may play a role (Cassisi G et al, 2011). There is no precise evidence of FMS due to infections or vaccinations and in particular, viruses such as HCV, HIV, and *Parvovirus* (Rivera J et al, 1997; Leventhal LJ et al, 1991; Buskila D et al, 1990) could be involved.

1.1.4. Diagnosis

Although fibromyalgia is the most common chronic widespread pain condition, it is often under diagnosed and recognition of FMS may not always be straightforward, because FMS symptoms may be part of- or overlap with - other diseases or syndromes. Furthermore the laboratory tests are limited to a complete blood count, erythrocyte sedimentation rate, rheumatoid factor, antinuclear antibody, thyroid-stimulating hormone, T3, T4, creatinine phosphokinase, serum muscle enzymes, vitamin D, C-reactive protein (CRP) and typically there are no abnormalities specifically associated with this condition. As such, diagnosis is principally based on the two criteria defined by the American College of Rheumatology, (ACR) 1990 (Wolfe F et al, 1990): 1) a history of widespread pain that has been present for at least three months, and 2) Pain in 11 of 18 tender point sites on digital palpation (both side of the body): Occiput (2), Low cervical (2), Trapezius (2), Supraspinatus (2), Second rib (2), Lateral epicondyle (2), Gluteal (2), Greater trochanter (2), Knee (2) (Figure 1).

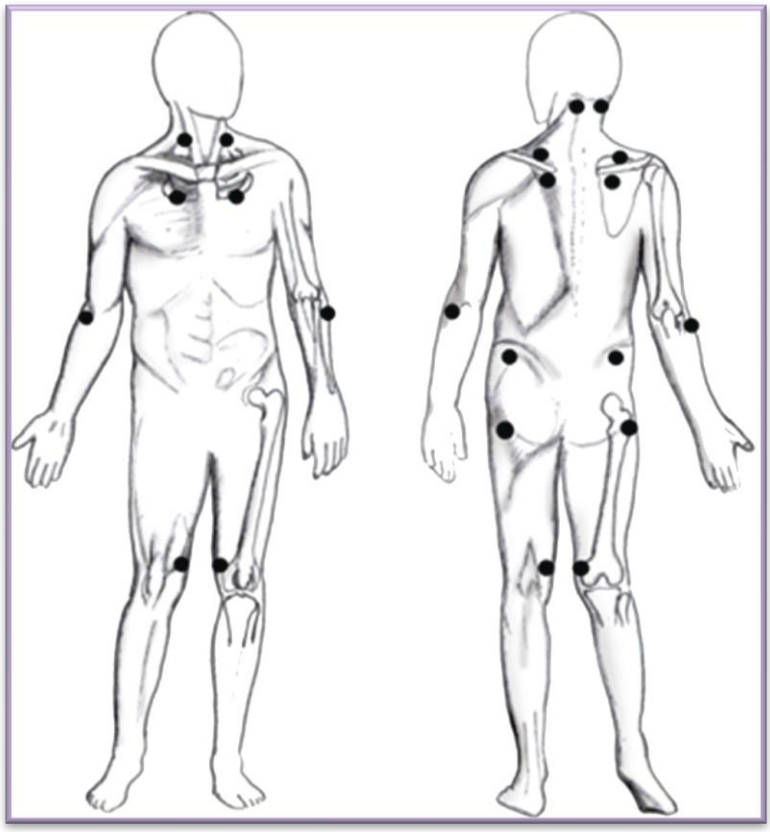


Figure 1. The black dots indicate the 18 tenderness points.

The tender points are specific places on the body that are painful when a standard amount of pressure (about 4 Kg) is applied. Although not essential for diagnosis, sleep disturbance, fatigue and morning stiffness are present in the vast majority of patients; other quite common symptoms include cognitive problems, paresthesias, headache, irritable bowel or bladder and anxiety. Recently the ACR published modified preliminary diagnostic criteria which enable diagnosis without the tender point examination and includes the severity of fatigue, waking unrefreshed and cognitive symptoms as core diagnostic assessments. Two factors best discriminate between patients with FMS and those with other disorders: the Widespread Pain Index (WPI) and the Symptom Severity (SS) score (Wolfe F et al, 2010). The WPI is a count of number of painful body

regions and patients (or their physician) may endorse 19 body regions in which pain has been experienced during the past week and one point is given for each area, so the score is between 0-19. The SS score is a measure of cognitive symptoms, sleep, fatigue, and additional somatic symptoms (such as headache, weakness, bowel problems, nausea, dizziness, numbness/tingling, hair loss) and the patient ranks specific symptoms on a scale of 0-3. However, Toda K (2011) suggested that SS scale introduces ambiguity into the clinical diagnosis and this issue has begun be addressed in the modification of the 2010 ACR criteria. In their modified 2010 diagnostic criteria Wolfe et al retain the 19-site WPI and the self-reported specific symptoms, but eliminate the physician estimation of SS score and replace it with three dichotomous "yes/no" answers regarding the presence of abdominal pain, depression, and headaches in the past 6 months (Wolfe F et al, 2011).

1.1.4.1. Fibromyalgia Impact Questionnaire (FIQ)

The Fibromyalgia Impact Questionnaire (FIQ) was developed by members of the fibromyalgia treatment team at Oregon Health & Sciences University (OHSU) in an effort to capture this total spectrum of fibromyalgia related symptoms (Burckhardt CS et al, 1991). This instrument measures physical functioning, work status (missed days of work and job difficulty), depression, anxiety, morning tiredness, pain, stiffness, fatigue, and well-being over the past week. The original FIQ contained 10 items (10 sub-items in the physical scale and consisted of 10 items with 10 sub-items in the physical function scale (total items + sub-items = 19). The original questionnaire used a visual analog scale (VAS) that required patients to slash a 100 mm line and was scored with a ruler. The scoring was further complicated by the need to reverse scores in one questions and the use of constants to convert the first 13 questions to a standardized scale of 0 to 10. Moreover, questions that now are considered relevant, such dyscognition (memory, executive function, concentration and attention; Ambrose KR et al, 2012), tenderness, balance, and environment sensitivity, were not part of the original FIQ. Modifications were made in 1997, 2002, and 2009, each with different scoring mechanisms. The 1997 modifications were the additions of an item to the physical function scale about stair climbing, hash marks to the visual analog scales, and the words "including housework" to the two work-related items and consists of 20 items with 11 physical function items (total items + sub-items = 30). The scoring was modified in 2002 for FIQs that contained crossed-out questions or other incomplete data (Bennett RM et al, 2005). The final score was to be adjusted to reflect a final maximum score of 100 to maintain homogeneity on a 0 to 100 continuum,

both within and between studies. The FIQR (Revised Fibromyalgia Impact Questionnaire) published in 2009 has the same 3 domains as the FIQ (i.e. Function, Overall Impact, and Symptoms) and consists of 21 items across the 3 domains of Function (n = 9), Overall Impact (n = 2), and Symptoms (n = 10) (Bennett MR et al, 2009). It differs from the FIQ in having modified function questions and the inclusion of questions on memory, tenderness, balance, and environmental sensitivity. It has comparable scoring characteristics to the original FIQ, making it possible to compare past FIQ results with future FIQR results. The FIQ has been translated into several 14 languages (Appendix: Italian FIQ).

1.1.5. Treatment

Since there is no definitive cure for FMS, treatment focuses on managing symptoms and improving overall quality of life. The aim of treating FMS is to decrease pain and increase function by means of a multimodal therapeutic strategy which, in most cases, includes pharmacological and non-pharmacologic interventions (Burckhardt CS et al, 2006) and has the main goal in symptom management (Barkhuizen A et al, 2002).

The European League Against Rheumatism (EULAR) suggested guidelines for treatment in FMS (Carville SF et al, 2008). The most efficacious treatment of FMS requires a multidisciplinary approach combining pharmacological treatment, exercise and cognitive behavioral therapy (Sarzi-Puttini P et al, 2011). Both pharmacological and non-pharmacological treatments have been shown to have an effect on symptom severity and physical function. The treatments need to be tailored with consideration to pain, fatigue, function and other features associated with FMS (Busch AJ et al, 2007; Carville SF et al, 2008; Mannerkorpi K et al, 2007).

A pharmacological therapy is often necessary to mitigate the symptoms of fibromyalgia, particularly as symptom profiles vary between patients. Such a combination may involve antidepressants, non-steroidal anti-inflammatory drugs (NSAIDs), sedatives, muscle relaxants, analgesics, sedatives, anticonvulsants, sleep aids and corticosteroids, depending on each patient's clinical history, target symptoms and functional impairments (Han C et al, 2011; Moldofsky H et al, 2010; Russell IJ et al, 2009; Di Franco M et al, 2010). In addition, adjunctive therapies may be prescribed to treat specific symptom domains such as sleep, and co-

morbid conditions the patient may have, such as irritable bowel syndrome and rheumatoid arthritis (Mease PJ et al, 2011).

1.2. Proteomics

The term ‘proteomics’ was first coined in 1995, and was defined as the large-scale characterization of the entire set of proteins expressed by a given cell, tissue, or organism. At present, proteomics is defined as large scale studies of the proteomes that encompass protein expression, folding, and localization.

The protein expression analysis in body fluid, tissue and cells of different origin is became one of the main focus after completion of the human genome sequence (International Human Genome Sequencing Consortium, 2004; Lander ES et al, 2001; Venter JC et al, 2001).

There are several reasons for focusing on the analysis of proteins: the level of mRNA expression frequently does not represent the amount of active protein in a cell (Anderson L et al, 1997); the gene sequence does not describe post-translational modifications, which may be essential for protein function and activity; and the study of the genome does not describe dynamic cellular processes (Humphrey-Smith I et al, 1997).

Many types of information cannot be obtained only from the study of gene, for example, proteins, not genes, are responsible for the phenotypes of cells. It is impossible to elucidate mechanism of disease, aging, effects of the environment solely by studying the genome.

Currently, 4543 genomes are being sequenced in ongoing projects, of which 3271 are bacterial, 110 archaeal and 1162 are eukaryotic (Liolios K et al, 2010). The available genome sequences provide useful information to the proteomics. From the DNA sequence it is possible to theoretically say what proteins may be present in a sample. Furthermore, a known gene sequence is commonly used to identify and verify proteins detected by

various proteomics methods. Actually, a significant portion of all sequenced genomes today encodes proteins remaining to be characterized.

While the set of genes in a cell, its genome, is essentially static over time, the composition and levels of mRNAs and expressed proteins is fluctuating as the cell adapts to environmental and life cycle situations at every single time point.

If a gene or protein is shown to be involved in a pathway or mechanism responsible for a diseased cell fate, it may be a good target for diagnosis, treatment or even prevention of that fate.

Proteomes of higher eukaryotes are very complex and several properties make the proteomic exploration enormously demanding. For instance, a factor to deal with in proteomics is the huge dynamic range in relative abundance of different proteins in a biological sample (Anderson NL et al, 2002).

This makes complicated detect rare protein species without extensive preparatory work as fractionation or depletion of several abundant protein species. Other critical factors are, alternative promoter usage and a number of post-transcriptional alterations of proteins including, alternative splicing and post-translational modifications (PTMs) (Godovac-Zimmermann J et al, 2005). Proteomics is supremely well suited to the analysis of human body fluids. Proteins in these fluids are typically soluble in nature, making them highly suitable to analysis using a variety of proteomics techniques. Whereas body fluids are well suited to analysis using proteomics, the application of m-RNA-based techniques such as polymerase chain reaction or hybridization array is largely irrelevant for the study of such fluids. This is because the cells that produce proteins in body fluids are either not found in the fluids (e.g. urine) or are some distance away from where the fluids are usually sampled (e.g., cerebrospinal fluid).

Additionally, proteins in some body fluids (e.g. serum or plasma) originate from cells that are present in multiple tissues. For these reasons, there is enormous interest in the use of proteomics for the study of human body fluids, in an attempt to understand better the normal physiology and pathophysiology of disease and to discover biomarkers. A number of proteomic technologies are commonly used for analysis of human body fluids. Typically, these technologies combine one or more means of high-resolution protein separation with high-sensitivity detection techniques. There are numerous techniques used to separate proteins derived from a tissue or biological fluid, but the current state of art is the 2-D Gel Electrophoresis (2-DE) followed by staining and mass spectrometric identification of protein spots. 2-DE not only generates information regarding protein modification and/or expression level changes, but also allows for the isolation of proteins in milligram amounts for further structural analyses, using mass spectrometry. The quantitative analysis of the images generated by digitization of 2D gels is critical for the identification of alterations in protein expression within a given biological system.

1.2.1. Proteomics of serum for biomarker identification

One of the important goals of biomedical research is to identify biomarkers that reflect an individual's health or disease state. Biomarkers of disease can be used for early diagnosis, therapeutic monitoring, and detecting the relapse of disease (Adkins JN et al, 2002; Verrills NM, 2006).

Blood is the most common source of biomarkers for use in the diagnosis and prognosis of disease (Anderson NL, et al, 2002). Blood constantly perfuse most tissues of the human body and the serum carries not only blood-specific proteins but also proteins that are synthesized, secreted, or shed from cell throughout the body (Anderson NL et al, 2004). Since serum is readily accessible, biomarkers that are identified in serum can serve as diagnostic tests and as targets for therapeutic intervention. The application of proteomic techniques to human serum may also have particular relevance to inflammatory vascular conditions such as systemic vasculitis, a group of disorders in which the site of pathology – the blood vessel wall – is in direct contact with the serum. The ability to make accurate inferences about the state of pathology (or health) within organ systems by examining the fluid that perfuse them has several major potential advantages. First, because traces of the molecular footprints of disease are expected to equilibrate (even at minute quantities) in the serum. Second, findings in the serum represent the sum of disease processes in organs, even those in which clinical involvement is unrecognized.

In addition, serum may be advantageous in searches for new peptide and protein markers, because the proteins and peptides that persist after blood

clotting are sufficiently stable to be exploited for in vitro diagnostics (Rai AJ et al, 2006).

Recently, the discovery of biomarkers from blood (serum or plasma) has become the subject of intensive attention, and the analysis of large numbers of proteins via proteomic techniques has greatly accelerated our ability to identify new biomarkers for disease including cancer (Li J et al, 2005), diabetes (Allard L et al, 2005), stroke (Zimmermann-Ivol CG et al, 2004) and kidney diseases (Merchant ML et al, 2005). As plasma contains such a variety of proteins in a wide and dynamic concentration range, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) are currently the principal techniques for the separation of such complex protein mixtures (O'Farrell PH et al, 1975;. Anderson L et al, 1977; Cristea IM et al, 2004).

Owing to these advantages, 2-DE has been used extensively for the discovery of new biomarkers (Jacobs JM et al, 2005; Nedelkov D et al, 2005; Thadikkaran L et al 2005). Examples of biomarkers identified via 2-DE are provided in Table 2 (Kim MR et al, 2007).

Context	Biomarker(s) Identified
Benzen exposure	T cell receptor β-chain, FK506-binding protein, matrix metalloproteinase-13
Polycyclic hydrocarbon exposure	Putative capacitative calcium entry channel
Strock	Fatty acid binding protein (FABP)
Ovarian cancer	Haptoglobin-α
Gastric carcinoma	Annexin V, carbonic anhydrase, prohibitin, fibrin beta, fibrinogen fragment D
Cardiovascular	Mature Cathepsin D
Severe acute respiratory syndrome	Peroxiredoxin II
Severe acute respiratory syndrome	Truncated α1-antitrypsin
Lung cancer	Haptoglobin, HGF
Pregnancy	Clusterin
Acute myeloid leukemia	α -2-HS glycoprotein, complement-associated SP-40, RBP4, lipoprotein C-III, haptoglobin, immunoglobulin heavy-chain variant, proteosome 26S ATPase subunit 1, haptoglobin-1
Myocardial infarction	Antitrypsin isoform 1
Cardiac allograft rejection	α Crystallin, tropomyosin
Relapsing polychondritis	Tubulin α ubiquitous, vimentin, alpha enolase, calreticulin, colligin-1

Table 2. Examples of biomarkers identified by 2-DE

1.2.2. Techniques in proteomics

Ever since its introduction in the mid-1970s, two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) has been a workhorse for separation of proteins in complex mixtures (Klose J et al, 1975; O'Farrell, 1975).

The technique separates proteins both in terms of their isoelectric points (pI) and molecular weights (Mr), and it is essentially a stepwise separation tool that combines isoelectric focusing and SDS-PAGE.

Using the current 2D PAGE technologies it is possible to separate complex protein mixtures into their individual polypeptide components and compare the protein expression profiles of sample pairs (normal versus transformed cells, cells at different stages of growth or differentiation, etc).

Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications.

Protein profiles can be scanned and quantitated to search for protein differences and interesting targets or molecular signatures can be identified using additional proteomic technologies such as mass spectrometry and Western blotting.

In combination with MALDI-TOF MS, 2D-PAGE constitutes the standard method in expression proteomics. Prior to MS-analysis, the proteins are enzymatically digested in the gel and thereafter extracted into liquid phase. The masses of the resulting peptides are determined by MS. As the genomes of many organisms are known, theoretical masses of translated proteins can be calculated. Bioinformatics tools, e.g. Mascot (www.matrixscience.com) and ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe), are used to match

the measured masses to theoretical masses from protein databases, and to estimate the probability that the ‘‘peptide mass fingerprint’’ originates from a certain protein. 2D-PAGE is a robust method that separates intact proteins, and reflects the presence of isoforms, post-translational modifications and the expression levels. At present, 2D-PAGE can resolve more than 5000 protein simultaneously (2000 proteins routinely) and can detect <1 ng protein per spot (Görg A et al, 2004). However, it is difficult to discriminate the proteins of extreme size and very hydrophobic, and also the method is rather labor intensive, difficult to automate and time consuming.

The major steps of the 2-D electrophoresis –MS workflow include: (i) Sample preparation and protein solubilization; (ii) Protein separation 2-D PAGE; (iii) Protein detection and quantitation; (iv) Computer assisted analysis of 2-D patterns; (v) Protein identifications and characterizations (Figure 2).

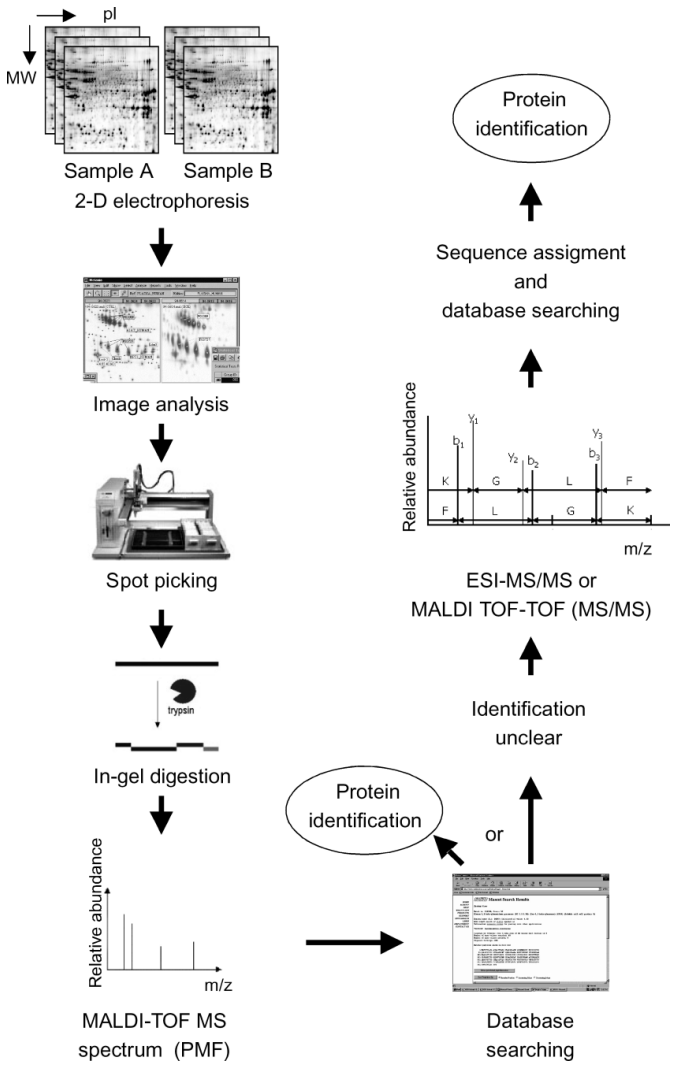


Figure 2. Classical proteomics approaches (2-DE followed by MS).

1.2.2.1. Two-dimensional Polyacrylamide Gel Electrophoresis

This technique combines isoelectric focusing, as a first dimension of separation, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as a second dimension.

1.2.2.2. Isoelectric Focusing

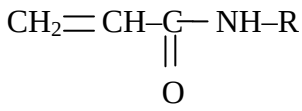
To take advantage of the high resolution of 2-DE, proteins of the sample have to be denaturated, disaggregated, reduced and solubilized to achieve complete disruption of molecular interactions and to ensure that each spot represent an individual polypeptide.

The isoelectric focusing first involves the extraction and solubilization of proteins using a buffer containing a variety of solubilizing agents such as:

- *Urea or Thiourea*: detergents; the high concentration is needed to convert proteins into single conformation by cancelling the secondary and tertiary structures, to get and keep hydrophobic proteins in solution, and to avoid protein-protein interaction. Recently, the use of thiourea in addition to urea has been found to further improve solubilization, particularly of membrane proteins.
- *3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS)*: a zwitterionic detergent increases the solubility of hydrophobic proteins
- *Dithiothreitol (DTT)*: reductant, prevent different oxidation steps of the proteins.
- *Carrier ampholytes*: which had been designed for generating pH gradient, improve the solubility of proteins considerably by substituting ionic buffers.

Isoelectric Focusing (IEF) is an electrophoretic method that separates proteins according to their isoelectric points (pI). The first-dimension IEF depended on carrier-ampholyte-generated pH gradients, Görg A et al. (1988; 2000) pioneered the development and use of immobilized pH gradients (IPG) IEF for the first-dimension of 2-D electrophoresis. An immobilized pH gradient is created by covalently incorporating a gradient of acidic and basic buffering groups (Immobilines) into a polyacrylamide gel at the time it is cast. Immobiline buffers are a set of well-characterized molecules, each with a single acidic or basic buffering group linked to an acrylamide monomer.

The general structure of Immobiline reagents is:



R = weakly acidic or basic buffering group

After IEF, the Immobiline DryStrip gels are equilibrated in equilibration solution and applied onto vertical or flatbed SDS-polyacrylamide gels for the second-dimension separation. The equilibration solution contains buffer, urea, glycerol, reductant, SDS, and dye. An additional equilibration step replaces the reducing agent with iodoacetamide.

Equilibration buffer (75 mM Tris-HCl, pH 8.8) maintains the Immobiline DryStrip gel in a pH range appropriate for electrophoresis.

Urea together with glycerol reduces the effects of electroendosmosis by increasing the viscosity of the buffer.

Glycerol (30%) together with urea reduces electroendosmosis and improves transfer of proteins from the first to the second dimension.

Dithiothreitol (DTT) preserves the fully reduced state of denatured, unalkylated proteins.

Sodium dodecyl sulfate (SDS) denatures proteins and forms negatively charged protein-SDS complexes.

Iodoacetamide alkylates thiol groups on proteins, preventing their reoxidation during electrophoresis.

Tracking dye (bromophenol blue) allows monitoring of the progress of electrophoresis.

1.2.2.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS-PAGE is an electrophoretic method for separating polypeptides according to their molecular weights. The technique is performed in polyacrylamide gels containing sodium dodecyl sulfate (SDS). SDS masks the charge of the proteins themselves and the formed anionic complexes have a roughly constant net negative charge per unit mass. Besides SDS, a reducing agent such as DTT is also added to break any disulfide bonds present in the proteins. When proteins are treated with both SDS and a reducing agent, the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular weight of the protein. The effective molecular weight range for proteins on SDS-PAGE can be tailored by adjusting the pore size of the gel. This is done by changing the concentration of acrylamide and cross-linking bis-acrylamide prior to polymerization. Typical concentrations are 12.5% T with 3% C for most SDS-PAGE separations with effective molecular weight separation between 14 and 100 kDa. The pH of the resolving gel is about 8.8, while the pH of the stacking gel is a lower at 6.8, buffered using Tris-HCl. The most commonly used buffer system for second-dimension SDS-PAGE is the Tris-Glycine system described by Laemmli UK, (1970). This buffer system separates proteins at high pH, which confers the advantage of minimal protein aggregation and clean separation even at relatively heavy protein loads.

1.2.2.4. In-gel Visualization

Following separation using 2D PAGE, proteins are visualized through staining techniques: Silver staining (Shevchenko A et al, 1996), Coomassie staining (Neuhoff V et al, 1988), fluorescent (Lopez MF et al, 2000; Berggren K et al, 2000) and autoradiography (Patton WF et al, 2002). Of these, silver staining is the most common used technique, as it detects as small a quantity as a single nanogram but its dynamic range is also restricted to a single order magnitude scale. Coomassie staining is an easy to use, low- cost staining agent, but a small linear dynamic range. Finally, despite of its high cost, fluorescent detection is gaining in popularity because of its sensitivity and wider linear dynamic range (Patton WF et al, 2002). Other methods for improving gel staining include differential gel electrophoresis (DIGE) (Unlu M et al, 1997), which substantially reduces variability by displaying two or more complex protein mixtures labeled with different fluorescent dyes in a single 2D gel. Fluorescent labeling also renders 2-D DIGE much more quantitative than colorimetric methods. It has a linear dynamic range of four or five orders of magnitude, by contrast with the approximately one- or two- order range of colloidal Coomassie or Silver stains (Tonge R et al, 2001). After protein separation and staining, further computer-based analysis is needed to detect differentially expression proteins. Computer programs are continuously being developed and improved - eg Progenesis (Nonlinear Dynamic), Image Master 2D Platinum and Melanie Software (GE Healthcare) or PDQuest (Biorad) - but image analysis remains a time-consuming process. Protein identification is based on matching peptide experimental masses of digested protein (PMF), using MS or tandem mass spectrometry (MS/MS) (Corthals, GL et al, 1999), versus the theoretical

masses obtained in the *in silico* digestion of all protein in a specific database. The result is a list of candidate proteins with different confidence levels.

1.2.2.5. Mass Spectrometry

Current mass spectrometers can detect and identify peptides in the femtomole (10^{-15}) to attomole (10^{-18}) range (Moyer SC et al, 2003). There are many types of mass spectrometers that can be used for proteomic studies, and each accomplishes the task of peptide identification in a slightly different way. All mass spectrometers consist of three basic components: ion source, mass analyser and ion detector. The role of the ion source is to introduce molecules into the mass spectrometer and convert them to a charged or ionized from. The ion source like the rest of the mass spectrometer is usually, though not always, held at a low pressure. Mass spectrometers are operated under vacuum to prevent the collision of ions with residual gas molecules during their flight from the ion source to the detector. This is because the ion are formed with excess energy and this, together with their charged character, can result in their reaction with other gaseous material present. The ideal operating pressure is that in which the average distance an ion travels before colliding with a gas molecule is longer than the distance from the source to the detector. After ions are formed in the source, they are accelerated into the mass analyser where they are separated in vacuum according to their mass and charge through the use of electric and/or magnetic field. Finally, the ions are passed onto an ion detector producing an electrical current that is amplified and detected (Figure 3).

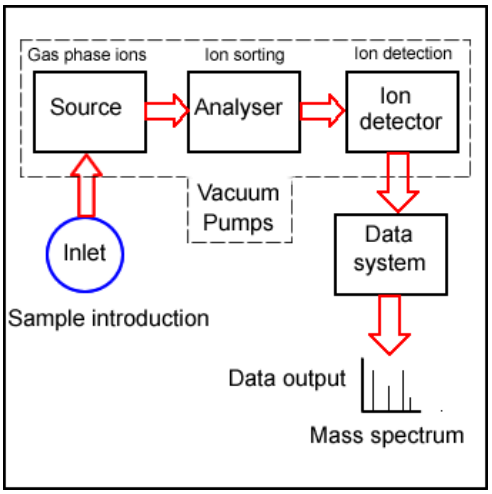


Figure 3. Schematics of a simple mass spectrometer with source, analyser and ion detector.

In the last twenty years, thanks to the development of soft ionization technique like Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption Ionization (MALDI), mass spectrometry found a considerable employment in the study of proteins. After initial protein digestion typically with trypsin, the obtained peptides are ionized in the ion source of the mass spectrometer. Peptides are then detected by the analyser of the mass spectrometer and in some cases they can be isolated and fragmented in order to obtain information on their aminoacidic sequence. Ionization of peptides is the first step in mass spectrometry of proteomes. The most frequently used ionization methods is matrix-assisted laser desorption ionization (MALDI; Yates, 1998) that is soft ionization technique. In MALDI, the analyte of interest is mixed with a large mole excess of (ca. 1,000-fold) a matrix compound that absorbs efficiently at the laser wavelength. The matrix allows the energy from laser to be dissipated and also assists with the ionization sample molecules through electron transfer and chemical processes. Common MALDI matrices are 2,5-dihydroxybenzoic acid (2,5-DHB), 2,4,6-trihydroxyacetophenone

(THAP), α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA) and 2,4-dihydroxyacetophenone (DHAP) (Figure 4). Where a UV laser is used MALDI matrices include sinapinic acid (SA) for proteins and α-cyano-4-hydroxycinnamic acid (CHCA) for peptides.

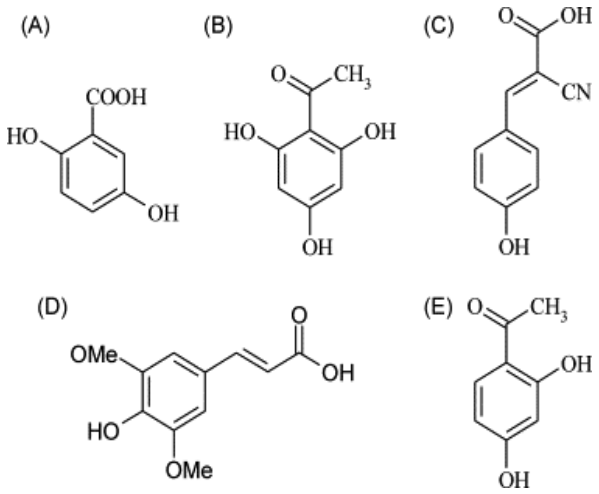


Figure 4. Structural formulas of used MALDI matrices: (A) 2,5-dihydroxybenzoic acid (2,5-DHB), (B) 2,4,6-trihydroxyacetophenone (THAP), (C) α-cyano-4-hydroxycinnamic acid (CHCA), (D) sinapinic acid (SA) and (E) 2,4-dihydroxyacetophenone (DHAP).

MALDI is now a firmly established technique, particularly for the study of polar, high molecular weight compounds such as proteins, glycoconjugates and nucleic acid. The peptide's mass is typically expressed as the mass to charge ratio (m/z). MALDI allows the protonation of analyte. The same peptide population may gain a different number of protons. The analyser is the part of the mass spectrometer where electric and/or magnetic fields are used for separating ions in gas phase. Mass spectrometer may be constructed with one or more analysers, depending on the task they will be used for. Instruments composed of two or more mass analysers coupled together are known as tandem mass

spectrometers. Until the spurt in interest in proteomics during the 1990s analyser like time-of-flight (TOF) (Figure 5) and the particularities and usefulness of the most used tandem TOF (TOF/TOF). The TOF is the simplest mass analyser and it consists essentially of a flight tube in high vacuum. Ions, accelerated with equal energies, fly along the tube with different velocities, which are inversely proportional to their mass.

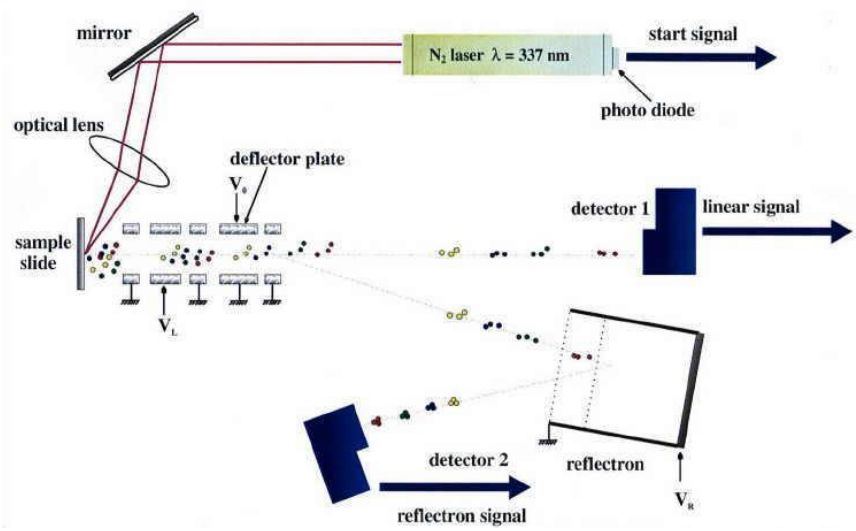


Figure 5. Schematic layout of a MALDI-TOF instrument

The time-of-flight (TOF) mass spectrometers separate ions and measure their m/z based on the time they take to pass (‘fly’) from the ion source to detector. The flight tube is usually 1-2 m in length and the basis of the separation makes no use of either electric or magnetic fields. Ions are separated in the field-free region of the flight tube before reaching the detector. The TOF spectrum is a recording of the signal produced by an ion detector at the end of the flight tube upon the impact of each ion group. A conventional mass spectrum, displaying intensity over m/z ratio is achieved by taking into account the relationship of the time of arrival to the detector (t) with the square root of m/z ratio value of the ion. The

coupling of a MALDI source with instruments capable of an efficient peptide isolation and fragmentation has proved to be very useful. When a protein cannot be identified by peptide mass fingerprinting (PMF), it would be very interesting to obtain peptide fragmentation data using the same sample without further purification. In this way, ambiguous PMF matches could be transformed into significant hits when enriched with the fragmentation data from at least one peptide. TOF/TOF has been coupled to MALDI sources and constitutes today workhorses for high throughput proteomics (Baldwin MA et al, 2001). The interpretation of the resulting MS/MS spectrum allows for the sequencing of the peptide. Each spectrum must be analyzed and a sequence of the peptide determined. Various software has been developed to either de novo sequence or match the spectra to known peptides. These peptides sequences are associated to proteins, and the proteins are identified with variable confidences based on the number and quality of the peptides sequenced. Most of the protein identification software (e.g. MASCOT, Seaquest) starts with the assumption that the peptide is being sequenced is in the protein database. If this assumption is not true, these programs will find the best matching peptide in the database and in the case of highly homologous proteins, sufficient identity may exist to correctly sequence a peptide and make a protein match in another closely related species. In addition, these protein sequencing software packages allow for several mismatches between the mass spectrometry data and the database; this allows identification of closely related proteins but can affect the confidence scoring of the protein. Thus, the quantity and quality of protein identification is affected by the quality of the protein databases.

1.2.3. Proteomics in fibromyalgia

In the last few years the proteomic approach has been widely used in order to identify new diagnostic biomarkers and therapeutical targets for a plethora of diseases, including FMS. Recently Giacomelli C et al, (2011) identify potential salivary FMS biomarker through a salivary proteomic tandem analysis based on MALDI-TOF and SELDI-TOF techniques. The peaks observed were likely to belong to the calgranulin family, and they are involved in cellular proliferation and migration, calcium homeostasis, inflammation and cellular protection against oxidative stress. Another peak observed with both techniques corresponded to the protein called Rho GDP-dissociation inhibitor2. This protein is involved in the RhoGTPasi activity, which controls cellular morphology and motility. Thus the peaks observed allow the research to focus on some of the particular pathogenic aspects of FMS: the oxidative stress, which contradistinguishes this condition, the involvement of proteins related to the cytoskeleton arrangements, and finally the central sensitization.

1.3. Purine

Purine metabolism involves synthesis and degradation of purine nucleotides and determines adenylate and guanylate pool values; consequently it is responsible for intracellular adenosine-5'-triphosphate (ATP) and guanosine-5'-triphosphate (GTP) concentrations. In addition to their roles within cells, purine nucleotides, nucleosides and bases and their metabolic products are released into the extracellular space where they act as signaling molecules. Receptor subtypes for purines have been identified in a variety of tissues, increasing interest in the role of purine-mediated signaling in pathophysiological processes and in the role of purine nucleotides and nucleosides in pain pathways (Burnstock G, 2006). Prompt effects are mediated by purines in the nervous system some of which are consequent to binding to purinergic surface receptors whereas others require purine uptake by cells. Extracellular purine concentrations depend on several factors which include the amount of purines released, local extracellular volume, uptake mechanisms and the presence of extracellular enzymes which metabolize purines. Once released, purine nucleotides and nucleosides undergo a complex extracellular metabolism catalyzed by many cell surface-located enzymes. Moreover, Yegutkin and co-workers (2003) revealed the existence in human serum of soluble enzymes capable of both inactivating and transphosphorylating circulating purines. Purine nucleosides, such as adenosine and its primary metabolite inosine, are low-molecular-weight molecules that participate in a wide variety of intracellular biochemical processes. Although both adenosine and inosine are present at low levels in the extracellular space, metabolically stressful conditions lead to an increase in their extracellular concentrations. Inosine is formed from adenosine both intracellularly and

extracellularly; inosine monophosphate (IMP) is dephosphorylated to inosine inside the cell and when inosine reaches high intracellular concentrations, it is shunted into the extracellular space by bidirectional equilibrative nucleoside transporters (Haskó G, 2004); inosine is degraded to hypoxanthine and then xanthine.

Inflammation, hypoxia and tissue injury cause adenosine degradation and the generation of its metabolite inosine (Eltzschig HK, 2009), a process mediated by adenosine deaminase (Spicuzza L et al 2006; Trams EG et al, 1974).

Some purine nucleotide metabolism disorders such as myoadenylate deaminase (MAD) deficiency report symptoms similar to those seen in FMS; the spectrum of symptoms of MAD deficiency ranges from permanent muscular hypotonia to excessive muscle weakness, fatigue, soreness, stiffness or cramps, and pain after exercise (Zöllner N et al, 1986) and these symptoms are consequences of an interruption of the purine nucleotide cycle.

1.4. High performance liquid chromatographic (HPLC)

High-performance liquid chromatography (HPLC) is a versatile analytical technology widely used for the analysis of pharmaceuticals, biomolecules, polymers, and many organic and ionic compounds. The first generation of high-performance liquid chromatographs was developed by researchers in the 1960s, including Horvath, Kirkland, and Huber. HPLC is a modern form of Liquid chromatography (LC) that uses small-particle columns through which the mobile phase is pumped at high pressure. The principle of LC is a separation process based on distribution between two phases, where the sample components is propelled by liquid which percolates a solid stationary phase. A sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). A variety of liquids and stationary phases can be used in liquid chromatographic systems. HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of stationary phase. Separation of a mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase. In HPLC this partitioning is affected by the relative solute/stationary phase and solute/mobile phase interactions. Since the compounds have different mobilities, they exit the column at different times; i.e., they have different retention times, t_R. The retention time is the time between injection and detection.

There are numerous detectors which can be used in liquid chromatography. It is a device that senses the presence of components different from the liquid mobile phase and converts that information to an electrical signal (Figure 6).

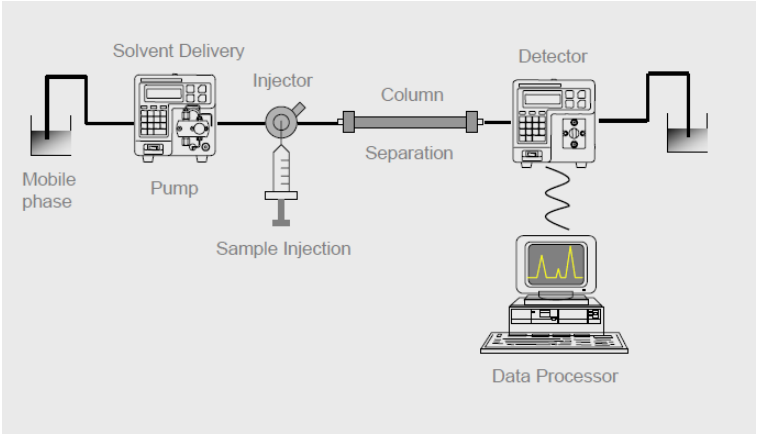


Figure 6. Schematic of an HPLC system. A high-pressure pump delivers a constant stream of mobile phase to the HPLC column. A plug of the sample to be analyzed is injected into this stream. At the outlet of the column, the separated components of the sample are detected and appear as peaks on the chromatogram.

For qualitative identification one must rely on matching retention times of known compounds with the retention times of components in the unknown mixture. Thus HPLC is most often used when one is performing a target compound analysis, where one has a good idea of the compounds present in a mixture so reference standards can be used for determining retention times. Today, HPLC continues to evolve rapidly toward higher speed, efficiency, and sensitivity, driven by the emerging needs of life sciences and pharmaceutical applications. HPLC is a premier separation technique capable of multicomponent analysis of real-life samples and complex mixtures. Few techniques can match its versatility and precision of <0.5% relative standard deviation (RSD). A host of highly sensitive and specific

detectors extend detection limits to nanogram, picogram, and even femtogram levels. As a preparative technique, it provides quantitative recovery of many labile components in milligram to kilogram quantities.

In **Reversed-Phase Chromatography (RPC)** the separation is based on analytes' partition coefficients between a polar mobile phase and a hydrophobic (nonpolar) stationary phase. The earliest stationary phases were solid particles coated with nonpolar liquids. These were quickly replaced by more permanently bonding hydrophobic groups, such as octadecyl (C18) bonded groups, on silica support. A simplified view of RPC is shown in Figure, where polar analytes elute first while nonpolar analytes interact more strongly with the hydrophobic C18 groups that form a "liquidlike" layer around the solid silica support. RPC typically uses a polar mobile phase such as a mixture of methanol or acetonitrile with water. The mechanism of separation is primarily attributed to solvophobic or hydrophobic interaction. It is suitable for the analysis of polar (water-soluble), medium-polarity, and some nonpolar analytes. Ionic analytes can be separated using ion-suppression or ion-pairing techniques. Separation of a mixture into its components depends on different degrees of retention of each component in the column.

Each purine bases its own characteristic UV absorbance spectrum which can be used for identification purpose. Single-wavelength UV detection in the HPLC analysis is usually set a 254 nm, where virtually all substance have an absorbance maximum.

2. AIMS OF THE STUDY

Currently no validated biomarkers are available for FMS and the diagnosis of the disease remain exclusively clinical. The aim of this study was to find biomarkers to be used to identify individuals susceptible to the FMS and to obtain the targeted diagnosis of FMS. Also monitoring and clinical assessment might be improved by useful serum biomarker.

We conducted experiments through the use of two different methods to identify potential biomarkers for FMS:

1 2-DE in combination with MS to obtain the serum protein map of FMS patients and controls.

2 RP-HPLC to determine levels of some purine metabolites in serum samples.

3. MATERIAL AND METHODS

3.1. Human subjects

Twenty-two females (53.5 ± 2.3 years; mean age ± S.D.) affected by FMS were studied for analysis of purine metabolite and sixteen females (52 ± 12 years; mean age ± S.D.) for analyses of proteome were enrolled; diagnosis was based on a history of widespread pain, defined as bilateral, upper and lower body, as well as in the spine, and the presence of excessive tenderness on applying pressure to 11 of 18 specific muscle–tendon sites (Tender Points) according to the ACR classification criteria (Wolfe F et al, 1990). The presence of a major clinical condition other than fibromyalgia was excluded by physical examination and routine blood and urine screening. All FMS patients were examined by a qualified rheumatologist and were interviewed by the examining physician using the Fibromyalgia Impact Questionnaire (FIQ; see Appendix) (Bennet R et al, 2005). Clinical monitoring included the 100 mm non anchored horizontal Visual Analogue Scale for pain (patient and medical VAS pain). Twenty-two healthy women (49.7 ± 2.3 years; mean age ± S.D.) were recruited as controls for analysis of purine metabolite and twelve controls for analyses of proteome (48 ± 13 years; mean age ± S.D.) were enrolled.

The samples were provided by Department Medical Science "Mario Aresu", University Hospital of Cagliari and informed consent was obtained from all the individuals enrolled on the study.

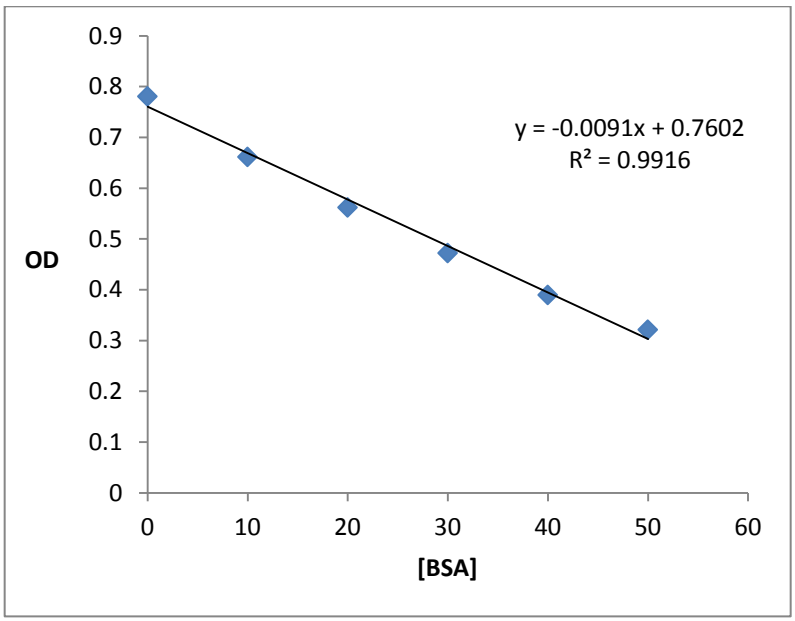
3.2. Blood collection

Blood sample were collected after overnight fasting. These were centrifuged (3000 rpm for 20 min) and serum were stored at -80°C until sample preparation. Only serum samples without hemolysis (proved by hemoglobin levels not detectable) were stored at -80°C until sample preparation.

3.3. Analysis of serum proteome

3.3.1. Protein quantification by 2-D Quant Kit

Serum protein concentrations were quantified using the PlusOne 2D Quant Kit (GE Healthcare, Uppsala, Sweden) according to manufacturer's instructions previously diluted 1:10 with rehydration solution (8 M Urea, 4% CHAPS, 1% dithiothreitol (DTT), 15mM Tris, 2% 3-10 ampholytes). The 2-D Quant kit quantitatively precipitates protein, leaving the interfering substances in solution. It is based on the specific binding of copper ions to proteins. The precipitated proteins are resuspended in a copper containing solution of which the unbound copper is then measured with a colorimetric agent at 480 nm. The color density is inversely related to the protein concentration. In short, a standard curve containing 5 dilution (0, 10, 20, 30, 40, 50 µg) was prepared using the 2 mg/ml BSA stock solution provided by kit (Graphic 1). Varying volumes of serum protein dilution (2, 4, 8 µl) were used to determine the protein concentration of each serum sample. 550µl precipitant were added to each tube and vortexed, followed by 500 µl of co-precipitant and mixed by inversion immediately upon addition. Samples were centrifuged at 10000 x g for 5 min at 20°C. The supernatants were decanted and centrifuged for 1 min at 10000 x g, 20°C**.** The remaining supernatant was removed, before the addition of 100 µl of copper containing solution followed by 400 µl MilliQ water and mixing each tube. This was followed by the addition of 1ml working solution to each tube, which was mixed immediately upon addition to ensure rapid mixing, before proceeding to the next tube. The tubes were then incubated for 15 min at room temperature, before the absorbance was measured at 480 nm by spectrophotometric techniques (Cary 50, Varian).



Graphic 1. Standard curve for albumin

3.3.2. Two-dimensional gel electrophoresis (2-DE)

3.3.2.1. Isoelectric focusing

For 2-DE 350 µl of rehydration buffer was pipetted along the length of the strip holder, avoiding any formation of bubbles. The Immobilized PH Gradient (IPG) strip was put onto the strip holder before layering mineral oil over the top of the strip, just enough to cover the strip and were started passive rehydration at room temperature overnight. The day after, two hundred micrograms of protein in rehydration buffer (8 M Urea, 4% CHAPS, 1% DTT, 15mM Tris and 2% 3-10 ampholytes) was applied to a 18 cm IPG, strip pH 3-10. First dimensional IEF was performed on an Multiphor II flatbed electrophoresis system (GE Healthcare) with a MultiTemp III thermostatic circulator (Amersham Biosciences) and an EPS 3501 XL power supply (GE Healthcare). Strips were focused using the protocol shown in Table 3 and was always allowed to proceed to a total of 55250 Volt-hours, that completed within 20 h at 20 °C. Strips were stored at −20 °C after focusing was completed.

STEP	VOLTAGE LIMIT (V)	HOUR (h)	VOLT HOUR (Vh)	GRADIENT
1	500V	0:01h	1Vh	Step and holder[a]
2	500V	3h	1500Vh	Step and holder
3	3500V	5h	10000Vh	Gradient[b]
4	3500V	12,5h	43750Vh	Step and holder
Total			55250Vh	

[a] Step and holder Vh = h x V

[b] Gradient $Vh = \frac{\text{h x Vh (V previous step+V new step)}}{2}$

Table 3. The IEF steps used for strips 18 cm IPG, pH 3-10.

3.3.2.2. SDS PAGE

Following IEF, After the IEF the strips were equilibrated for 15 min in a solution containing 1% (w/v) DTT, 6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl pH 8.8 and 30% (v/v) glycerol. A second equilibration step was carried out for 15 min in the same solution except for DTT, which was replaced by 2.5% (w/v) iodoacetamide and trace of bromophenol blue. Finally, the strip was placed in SDS electrophoresis running buffer (0.25 M Tris-HCl pH 8.3, 0.1% SDS, 192 mM glycine) for 2 min as a final equilibration step. Second dimensional separation was performed by placing the IPG strips on top of the 10% SDS PAGE gel (Ettan Daltsix, GE Healthcare), covered with 0.5% agarose dissolved in 1% SDS electrophoresis running buffer. Separation was performed at 25°C until the bromophenol blue front reached the bottom of the gel. Steps is given in table 4 .

Step	mA/gel	Voltage (V)	W/gel	Time (h:min)
1	10	80	1	1:00
2	40	500	13	4:30-6:00 [a]

[a] continue the electrophoresis until the bromophenol blue reaches the end of the gel

Table 4. Running condition using Ettan Daltsix electrophoresis system.

The gels were then fixed in the appropriate fixing solution for each specific stain.

3.3.3. Staining of 2-DE gels

3.3.3.1. Silver staining

After electrophoresis, gels were fixed in 40% ethanol, 10% acetic acid overnight, followed by sensitizing for 1h in sodium thiosulfate (2 g/L), sodium acetate (68 g/L), 30% ethanol, and rising with Milli-Q water cold forth. 0.2% silver nitrate, 0.04% formaldehyde was added and incubated for 1 h in dark, rinsed twice with Milli-Q water and develop in 2% sodium carbonate with 0.04% formaldehyde. The solution is prepared just before use. Development was stopped by adding 1% EDTA. All gels were stored in airtight containers.

3.3.3.2. Colloidal Coomassie Brillant Blue G-250 stain

After electrophoresis, gels were fixed in 30% ethanol, 2% phosphoric acid overnight, followed by 2% phosphoric acid for ten minutes for three time. Colloidal Coomassie Brillant Blue G250 stock solution (10% ammonium sulphate, 1% phosphoric acid, 18% ethanol and 2% Coomassie Brillant Blue G-250). The gels were immersed in the hot Colloidal Coomassie solution and left shaking overnight. Gels were then scanned on the Scanner Image III (GE Healthcare) and stored in cellophane until use for MS.

3.3.4. Image analysis

Coomassie-stained gels at high resolution (300 dpi) images were scanned with ImageScanner™ III (GE Healthcare) and were acquired using the LabScan 6.0 software; the image analysis was analyzed using Image Master 2D Platinum software III (GE Healthcare) of University of Cagliari and Progenesis SameSpots (Nonlinear Dynamics) of University of Vigo. In all experiments, two replicates from both the control and fibromialgic samples were used. A single gel was selected as a master gel and all the other gels were matched against this gel. The gels were normalized by the software based the total volume of a gel. The protein spots that visually appeared as technical artifacts (e.g., background areas of Coomassie staining, irregularly shaped dust particles, air bubbles), but were erroneously detected by the software, were manually removed from the analysis.

3.3.5. Statistical analysis

The optical density of the proteins was expressed as a percentage of the volume (mean±SD) with ImageMaster III (GE Healthcare) software. For each gel, the preliminary analysis included protein spot detection, editing, filtration and quantification. Spots were quantitated as a fraction of the total volume of protein spots on the gel. One typical control gel with the most protein spots was then set as reference gel, to which all the other gels were matched. Match rates of each group of gels were calculated automatically by the software. The non-parametric, Mann‑Whitney test was used for analysis of the mean intensity differences of the spots between normal and FMS gel groups. p values ≤ 0.05 were considered as significant differences.

Gels were processed also with Progenesis SameSpots software (Nonlinear Dynamics, Newcastle upon Tyne, UK), where quantitative analysis of protein spots was performed. Following automatic and subsequent manual editing, aligning and matching procedures as part of the Progenesis SameSpot workflow, ANOVA *p*-values between the samples were calculated within the Progenesis SameSpot software, which computed the fold change (the fold difference of logarithms of normalized spot volumes between the patient and control groups; the average values of normalized spot volume logarithms of a single spot in all samples within a group are compared) and *p*-values of all spots using one-way ANOVA analysis. One way to adjust for multiple comparisons is to consider the false discovery rate (FDR), which is the probability that a spot determined to be significantly different is, in fact, a false discovery. To account for multiple comparisons, p-values were adjusted by the FDR controlling method computed in automatic by software, obtaining the corresponding q-values. Principal Component Analysis (PCA) was performed to assess whether grouping of patients and healthy controls based on proteomic methods reflects their stratification using classical clinical diagnosis. PCA was performed making use of the same software, focusing only on the spots of statistical significance (based on 2D SDS-PAGE) employed for protein identification.

3.3.6. Mass spectrometry

3.3.6.1. In gel digestion and MS analysis

Protein spots of interest were identified by the National Council of Researches (CNR), Institute of Molecular Sciences and Technologies (ISTM) of Padova. The spots were manually excised from 2-DE gels following the procedure described and adapted from Shevchenko A et al, 1996. The spots were washed (25 mM ammonium bicarbonate), dehydrated (25 mM Ambioc/50% acetonitrile (ACN) followed by 100% ACN), reduction (10mM DTT/25mM NH_4HCO_3) and alkylation (55mM IAA/ 25mM NH_4HCO_3), dried, and enzymatically digested with sequence-grade modified porcine trypsin (Sigma) overnight. Peptides from gel-trypsin digestion were desalted and concentrated by Supel TipsU-C18 (Sigma), following the procedure described in the user's guide. Proteins identification was performed by peptide-mass fingerprint MALDI/MS measurements using a MALDI-TOF/TOF UltrafleXtreme (Bruker Daltonics, Bremen, Germany), equipped with 1 kHz smartbeam II laser (λ = 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 H_2O/Acetonitrile (50:50; v/v) containing 0.1% TFA). External mass calibration was done using the Peptide Calibration Standard, basing 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 instrumental conditions employed for the TOF/TOF experiments on the ion at m/z 2694 were: Ion Source 1: 7.5 kV, Ion Source 2: 6.75 kV, Lens: 3.5 kV, Lift Voltage 1: 19 kV, Lift Voltage 2: 2.9 kV, Reflector Voltage 1: 29.5 kV, Reflector Voltage 2: 13.95 kV. The peptide masses were searched against the National Center for Biotechnology Information nonredundant mammalian database using Mascot 2.3 searched from Matrix Science, selecting the Swiss-Prot database. For the present study, protein identification was based on the measurement with a Mascot score higher than 55. Minimal expectation for valid identification was $p < 0.005$ and $p < 0.05$.

3.4. Analysis of serum metabolites

3.4.1. HPLC

The samples were centrifuged at 10000 x g for 5 min through cellulose acetate spin filters (0.2 μm, VectaSpin Micro, Whatman) to remove particulate matter and lipids.

HPLC analysis was performed using Agilent Technologies 1100 series HPLC, supplied with a binary pump and diode array detector (DAD).

Six purine reference standards (uric acid, hypoxanthine, xanthine, inosine, guanosine and adenosine) were used, all of the highest analytical grade available (Sigma-Aldrich, Milan, Italy; Across Organics, Geel, Belgium).

Chromatographic separation of purine metabolites was obtained using a reverse-phased column (Agilent Zorbax 300 SB-C18, 250×4.6 mm, 5 μm). Two eluents were used for compound separation: solvent A=KH_2PO_4 25 mM (in MilliQ water) and solvent B=a mixture of 75% solvent A and 25% methanol. Elution was performed with a flow rate of 600 μl/min: 0–6 min, 0%B; 6–19 min, linear gradient 0–80% B; 19–22 min 80% B; 22–26 min, 0% B. HPLC experiments lasted 26 min. Analytes were detected at 254 nm at six specific retention times, and peak areas were compared to those of specific calibrators that were prepared immediately prior to use.

All determinations were performed in triplicate and each sample concentration is expressed as mean.

3.4.2. Statistical analysis

Statistical analysis was performed using STAT VIEW 5.0 by SAS Institute Inc. The mean and error standard (SE) of the mean was calculated for each variable. Differences between the groups were calculated using the Student's t-test. Pearson's correlation was calculated to test the relationship between parameters examined in the FMS patients. There was no evidence of non-normality among the outcome variables according to the Kolmogorov–Smirnov test.

4. RESULTS

4.1. Clinical parameters

Demographic characteristics and clinical parameters of FMS and control subjects are reported in table 5 (for analysis purine metabolite) and 6 (for analysis proteome). No associated clinical distresses or pain were reported in the control subjects (VAS pain=0).

	FMS (n= 22)	Controls (n= 22)
Age [years, mean (DE), range]	53.5±2.3 (27-72)	49.7±2.3 (27-67)
Sex	females	females
Menopause	n=15	n=11
Body Mass Index (m^2/Kg)(mean ± SD)	25.8 ± 0.94	23.3 ±0.54
Pain duration (years, mean ± SE)	5.1 ± 0.5	-
Pain score patient (VAS mm) (mean ± SE)	56.8 ± 5.8	-
Pain score physician (VAS mm) (mean ± SE)	36.2 ± 3.9	-
FIQ total score (0-80) (mean ± SE)	63.1 ±4.2	-
Associated Clinical Distresses		
Tension type headache	n=17	
Irritable colon	n=15	-
Oto vestibule syndrome	n=14	-
Paraesthesia	n=14	-
Sleep disturbance	n=13	-
Dysmenorrhea	n=11	
Hemicrania	n=7	
Urethral syndrome	n=7	
Medication		
No therapy	n=5	n=22
Paracetamol	n=13	
Tricyclic	n=9	-
Selective Serotonin Reuptake Inhibitors (SSRIs)	n=2	-
Selective Serotonin Noradrenaline Reuptake Inhibitors (SSNRIs)	n=6	-

Table 5. Characteristics and scores of examined subjects for analysis purine metabolite (FMS = fibromyalgia syndrome; FIQ = FMS impact questionnaire; VAS = visual analogue scale.

	FMS (n= 16)	Controls (n= 12)
Age [years, mean (DE), 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
Pain duration (years, mean ± SE)	4.9 ± 0.5	-
Pain score patient (VAS mm) (mean ± SE)	57 ± 31	-
Pain score physician (VAS mm) (mean ± SE)	34 ± 18	-
FIQ total score (0-80) (mean ± SE)	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=4	n=12
Tricyclic	n=6	-
Selective Serotonin Reuptake Inhibitors (SSRIs)	n=1	-
Selective Serotonin Noradrenaline Reuptake Inhibitors (SSNRIs)	n=4	-

Table 6. Characteristics and scores of examined subjects for analysis proteome (FMS = fibromyalgia syndrome; FIQ = FMS impact questionnaire; VAS = visual analogue scale.

4.2. Proteomics data analysis

4.2.1. Proteome profile of serum

Using Image Master 2D Platinum software (GE) of University of Cagliari and Progenesis SameSpots (Nonlinear Dynamics) of University of Vigo, we compared 2D maps of sera between sixteen patients FMS and twelve healthy controls. The images of gels were found to be similar between groups. The results were satisfactory: the number of protein spots in each gels of the same group was stable and the average spot positional deviations were slight. There were approximately more than 100 spots that could be visualized manually in the 2D gel images of both FMS patients and controls. Simultaneously, 88 ± 23 spots were detected for control gels and 119 ± 26 for patient gels using Image Master 2D Platinum Software (GE).

Using Progenesis SameSpots (Nonlinear Dynamics) all two groups were mutually compared using one way ANOVA analysis. Both manual and automatic image alignment were used. Specific pre-filtering and statistical criteria were applied to all spots that were automatically detected by the Progenesis SameSpots software (in total more than 2200 spots detected by the software). Applying pre-filtering (edge and area exclusion, minimal spot area, or normalized volume) altogether more than 151 spots remained in the analysis. In Figure 7 is presented a typical control gel (A) and a typical patient gel (B).Using statistical criteria in the next step (Anova $p < 0.05$, FDR smaller than 7%; minimum fold change 1.2), we detected 17 spots that significantly differed in the normalized volumes; positions of these spots are highlighted in figure 8. In figure 9 shows the enlarge images of 106, 1065 and 2334 spot differentially expressed found in

different quantity in FMS respect to control. Principal Component Analysis showed an evident separation of samples into two aggregates, corresponding to the FMS and control groups (Figure 10).

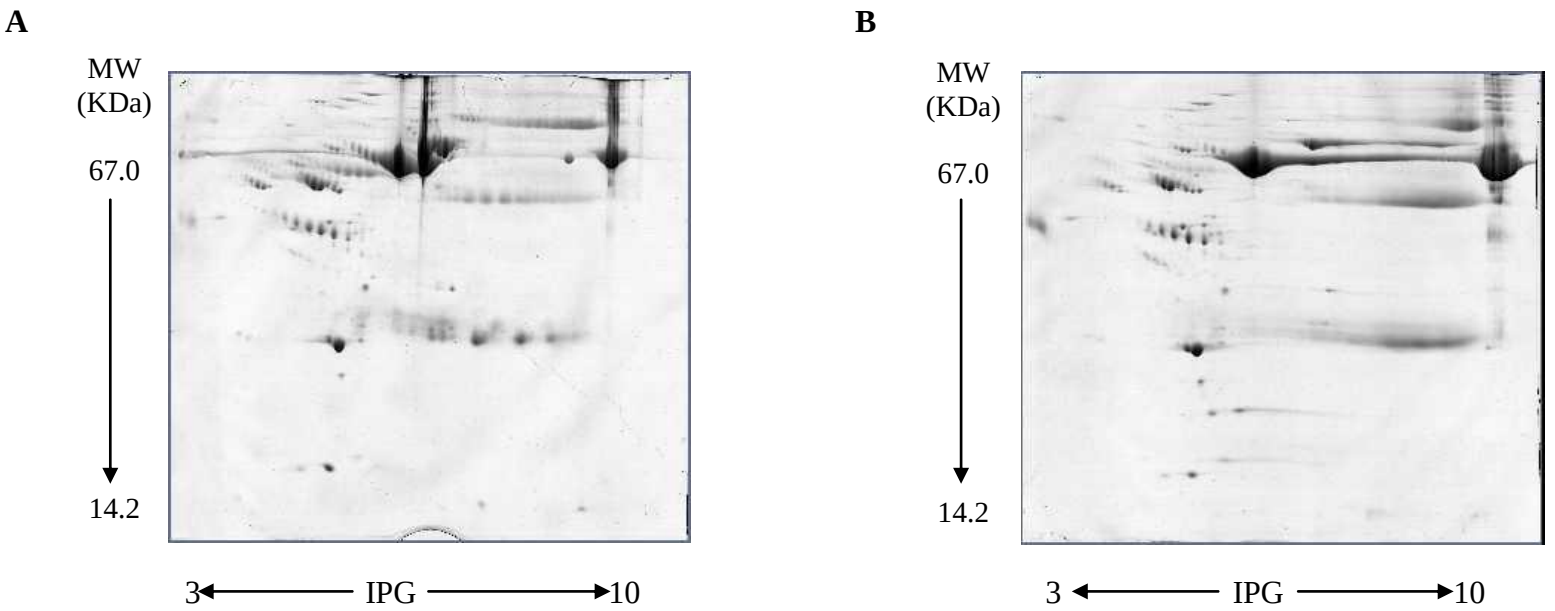
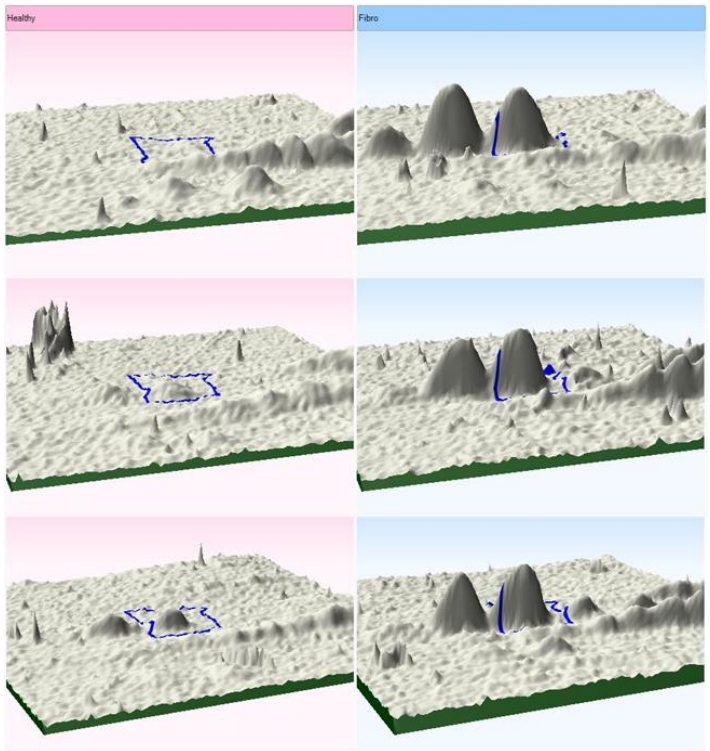
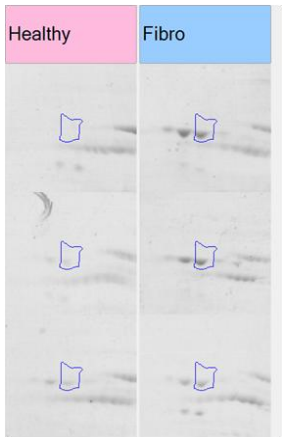


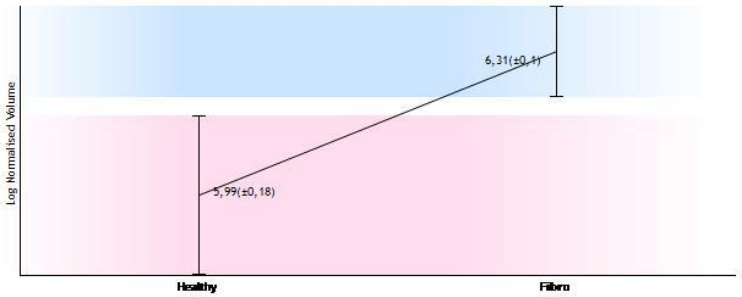
Figure 7. Representative 2-DE gel map of control (A) and fibromyalgic (B) serum proteins.
The protein samples were loaded onto linear IPG strips (pH 3-10, 18 cm) in an IEF cell and then separated by 10% SDS-PAGE. The protein spots were visualized by Colloidal Coomassie Brillant Blue G-250 stain.

Figure 8. The map was analyzed by the Progenesis SameSpots (Nonlinear Dynamics) Software. Spot numbers indicate all the proteins differentially expressed in FMS with respect to the control.

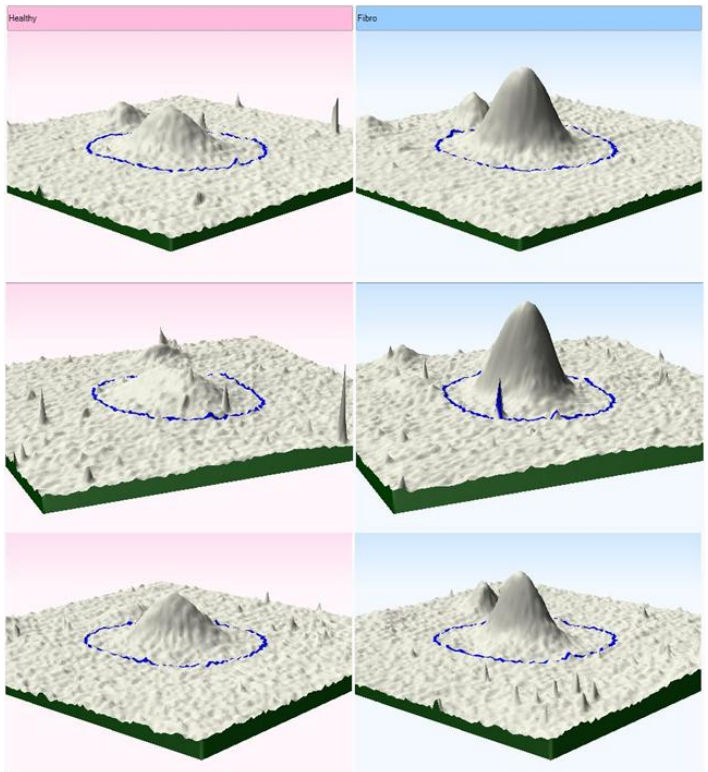
SPOT ID 106

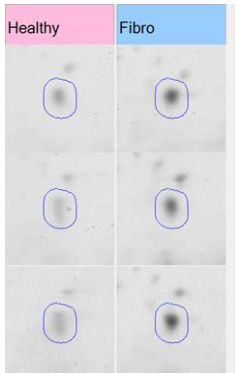

Healthy
Fibro

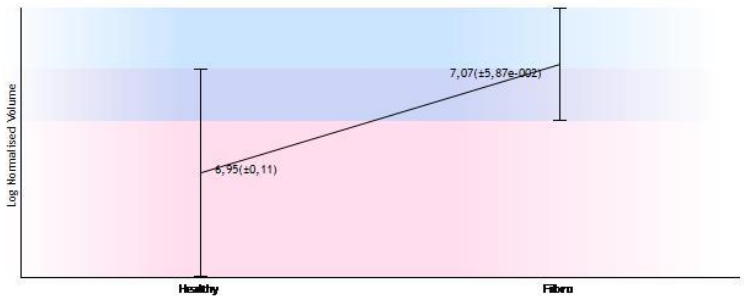

Healthy
Fibro


Log Normalised Volume
6,31(±0,11)
5,99(±0,18)
Healthy
Fibro

SPOT ID 1065


Healthy
Fibro


Healthy
Fibro


Log Normalised Volume
7,07(±5,87e-002)
6,95(±0,11)
Healthy
Fibro

SPOT ID 2334

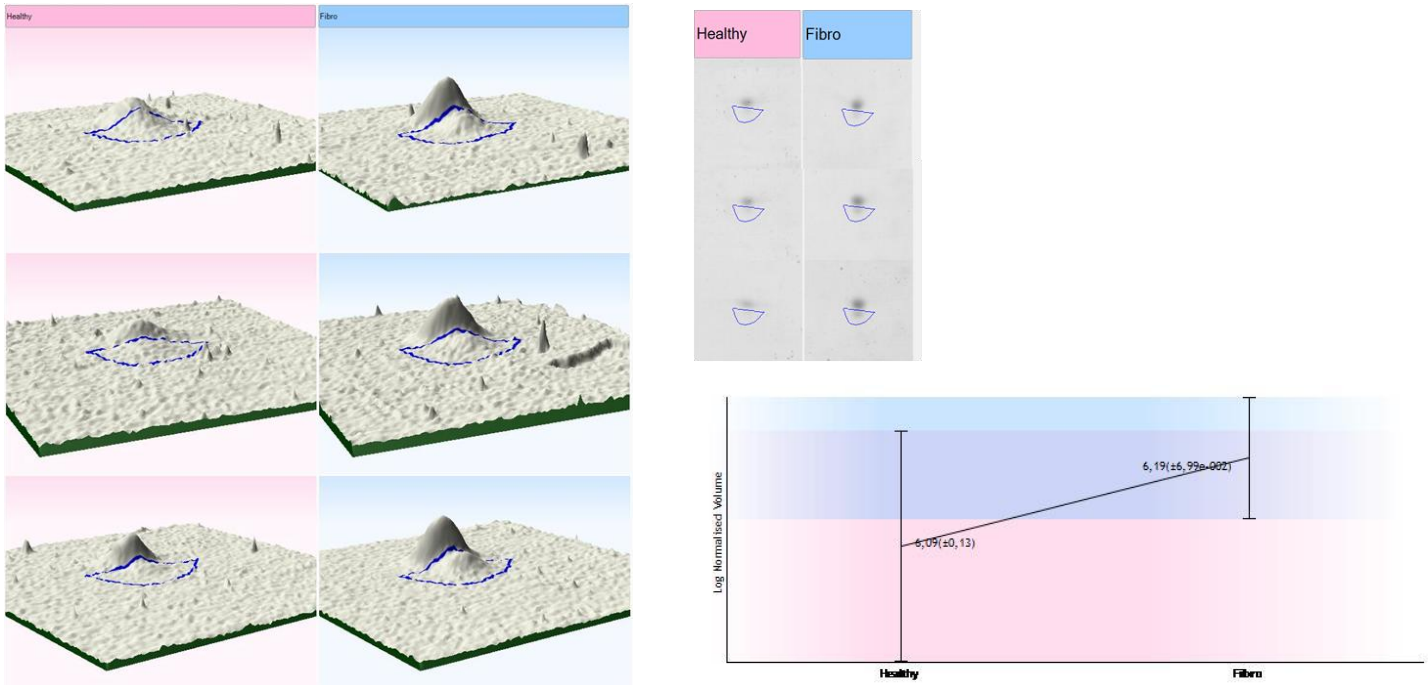


Figure 9. Enlarged images of the 2-DE gels represent proteins spots and expression profiles with the logarithms of spot normalized volumes and their standard deviations showing statistically significant changes between controls and FMS for six representative gels. Gel areas containing the same spot from each group (three exemplary gel area from each group) are presented above the logarithm values. Data were obtained from 2-DE gels using Progenesis SameSpots (Nonlinear Dynamics) Software.

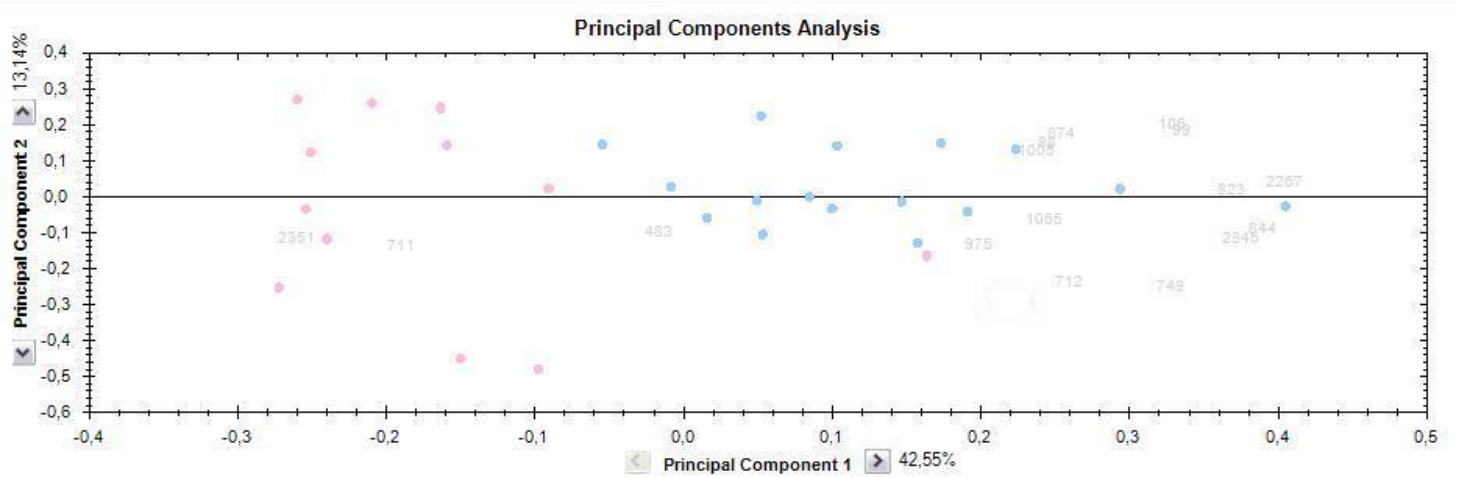


Figure 10. Principal Component Analysis. PCA was performed to assess whether grouping of patients and healthy controls based on proteomic methods reflects their stratification using classical clinical diagnosis. Analysis was based on spots that significantly differ according the mentioned statistical criteria ($p<0.05$, ANOVA and q<0,07, FDR). Principal Component Analysis (PCA) showed the separation of all samples into two aggregates that corresponded to the FMS (blue dots) and the control group (pink dots).

4.2.2. Characterization of differentially expressed proteins

After normalization, volume calculation, and statistical analysis with the Progenesis Samespots software, three spots (106, 1065 and 2334) were chosen for Mass Spectrometry/Mass Spectrometry (MS/MS) analysis and were characterized. The MALDI spectra obtained by the trypsin digestion of the 2D gel spots are reported in Figure 11. Table 6 shows the characteristics of the identified proteins, including the number of peptides matched, the percent coverage, the pI, the MW, the accession number, the score, the matched sequence and the fold change. To confirm the results has been performed MS/MS:MS/MS analysis has been performed in order to obtain the fragmentation spectra (Table 7). In practice, each m/z (mass-to-charge ratio) of interest (precursor ion) was selected and its fragments (product ions) were simultaneously detected. The peaks list has been used for peptides/proteins identification via on-line search engines such as: Mascot (http://www.matrixscience.com) and MS-Tag (http://prospector.ucsf.edu). MS/MS analysis has been indicated the same proteins confirming the results of MS. The search for the Peptide Mass Fingerprinting obtained for spot ID1065 and spot ID 106 in the Mascot database allow their identification: spot ID1065 is due to Transthyretin (TTR), spot ID 106 is due to Alpha-1 Antitrypsin (A1AT). These identifications were confirmed by MS/MS experiments on selected peptides present in the tryptic digestion mixture of each spot. In the case of spot ID 2334, only through MS/MS experiments on selected digestion products ions (m/z 2694 and m/z 2018) and by search in Mascot database it was possible to identify the protein as Retinol Binding Protein 4 (RBP4). All 3 proteins identified in this study were categorized into different cellular functional groups based on the available literature and

the protein databases Pfam http://pfam.sanger.ac.uk and/or InterPro http://www.ebi.ac.uk/interpro/ (Apweiler R et al, 2001).

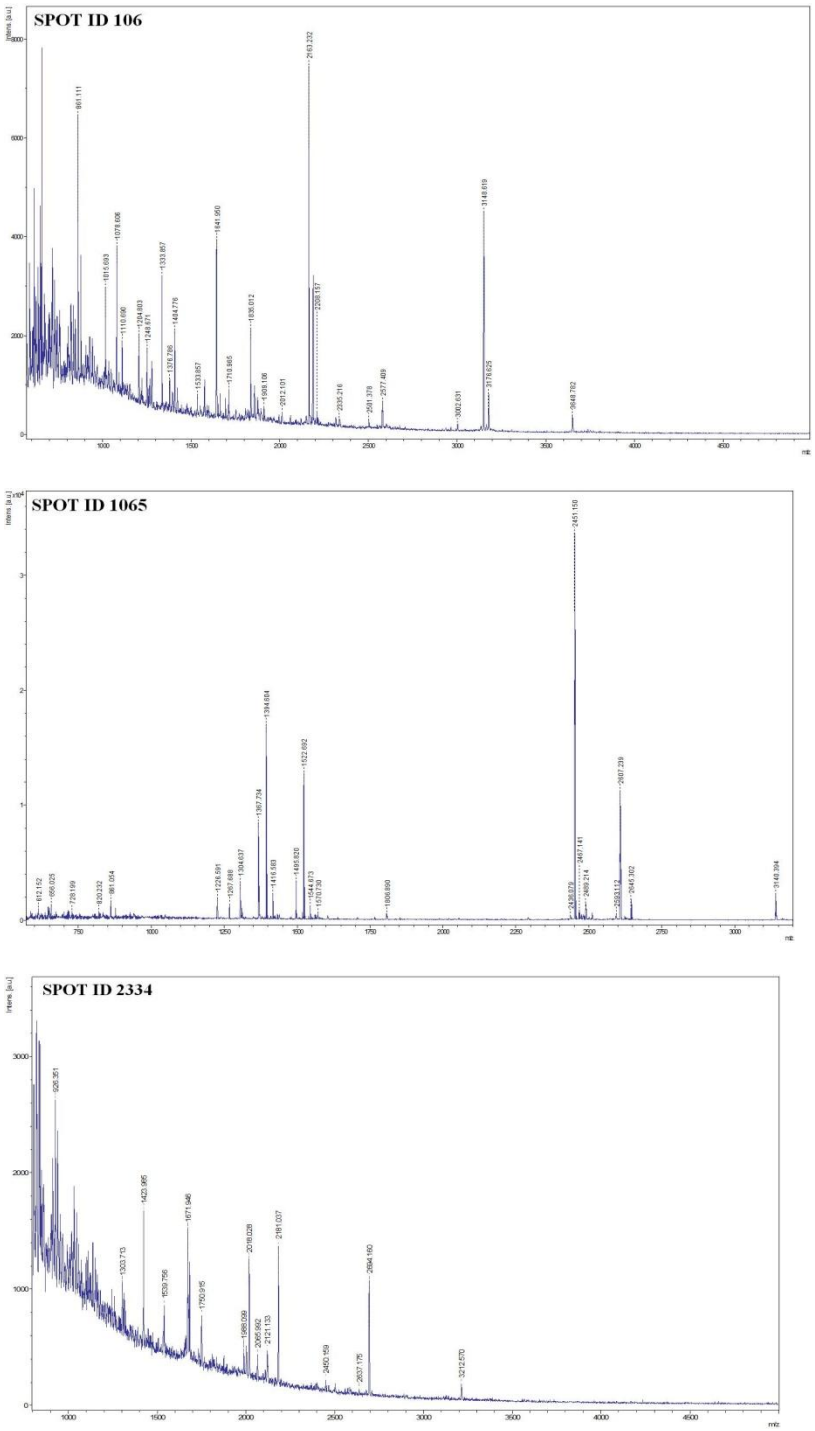


Figure 11. Representative peptide MS spectra from three proteins differentially expressed.

Spot ID	Methods	Protein name	Short name	Accession number	Mw (KDa)	pI	Score	Coverage (%)	p-value	Fold-increase
106	MS/MS	**Alpha-1-antitrypsin**	A1AT	P01009	46	5.27	91	51%	3,228e-005	2,0
1065	MS	**Transthyretin**	TTR	P02766	15	5.81	121	100%	0,007	1,3
2334	MS	**Retinol-binding protein 4**	RBP4	P02753	23	5.8	70	12%	0,039	1,2

Table 6. Protein identification of differentially expressed proteins in serum of FMS patients identified by MALDI-TOF/TOF MS analysis.

Spot ID	Protein name	Mr(expt)[a]	Mr(calc)[b]	Score	Sequence
106	**Alpha-1-antitrypsin**	1333.7927	1332.7653	40	K.LVDKFLEDVKK.L
		1833.9927	1832.9156	91	K.VFSNGADLSGVTEEAPLK.L
		2161.9927	2161.1266	127	K.VFSNGADLSGVTEEAPLKLSK.A
		3147.9927	3147.5149	57	K.GKWERPFEVKDTEEEDFHVDQVTTVK.V
1065	**Transthyretin**	1393.9927	1393.6150	85	K.AADDTWEPFASGK.T
		1521.9927	1521.7100	108	R.KAADDTWEPFASGK.T
		2449.9927	2450.1979	102	K.ALGISPFHEHAEVVFTANDSGPR.R
		2607.0000	2605.2990	92	K.ALGISPFHEHAEVVFTANDSGPRR.Y
		3138.9927	3139.5085	65	K.TSESGELHGLTTEEEFVEGIYKVEEIDTK.S
2334	**Retinol-binding protein 4**	2018.2726	2016.9920	78	R.LIVHNGYCDGRSERNLL.N
		2694.0000	2692.9927	70	K.GNDDHWIVDTDYDTYAVQYSCR.L

Table 7. Identified peptides by MSMS analysis using the Mascot software.
[a] Mr(expt), nominal mass calculated from the measured m/z;[b] Mr(calc), Mr of the matched peptide.

4.3. Serum purine metabolites

The chromatograms obtained during RP-HPLC were analyzed and the quantitative analysis was obtained. Serum purine levels in FMS subjects versus controls, magnitude of the changes and statistical significance are reported in Table 8.

	Fibromyalgia	Controls	Difference	Significance
Uric acid RT=9.34±0.37	315.46±16.80	318.40±28.03	−2.93	*p*=0.93
Hypoxanthine RT=11.55±0.32	79.57±6.86	11.99±0.87	67.58	*p*=0.0001
Xanthine RT=12.95±0.24	5.08±0.36	1.71±0.15	3.37	*p*=0.0001
Inosine RT=17.83±0.36	40.58±3.73	7.07±1.33	33.51	*p*=0.0001
Guanosine RT=18.27±0.44	1.58±0.20	1.78±0.27	−0.20	*p*=0.54
Adenosine RT=21.96±0.86	0.29±0.008	1.22±0.36	−0.93	*p*=0.013

Table 8. Serum purine metabolites (µmol/L, mean±SE) in examined subjects. RT=retention time (min).

The relationships between FIQ and serum purine metabolites in FMS are reported in Table 9; serum adenosine levels are significantly correlated with FIQ.

FIQ and	Pearson's correlation coefficient	Significance *p*
Uric acid	0.16	0.48
Ipoxanthine	0.24	0.29
Xanthine	0.40	0.06
Inosine	0.10	0.68
Guanosine	0.04	0.85
Adenosine	−0.46	0.03

Table 9. Pearson correlation among examined parameters.

5. DISCUSSION

5.1. Proteomics

Recently, a proteomic approach has been used to identify potential biomarkers of FMS in salivary fluid (Giacomelli C et al, 2011). However, to the best of our knowledge, no such study has been reported on sera of FMS patients. In this study, we attempted a proteomic approach such as 2-DE and MALDI-TOF MS to evaluate serum protein profile in FMS patients and in matched healthy controls. Our data showed three differentially expressed protein spots identified as Transthyretin (TTR), Retinol Binding protein 4 (RBP4) and Alpha-1 Antitrypsin (A1AT).

Transthyretin (TTR) (spot ID 1065) gene is located at chromosome 18q11 and consists of four exons (Tsuzuki T et al, 1985). TTR is a homotetrameric protein of 55 kDa mainly synthesized in the liver, that is responsible for more than 90% of TTR synthesis which is secreted into the blood. In addition to the liver, other sites of TTR synthesis have been identified in mammals: choroid plexus (Aleshire SL et al, 1983), retinal pigment epithelium (Martone RL et al, 1988) and pancreas (Schreiber G et al, 1990). Recent data (Li X, et al 2011) indicate that there is also a neuronal production of TTR. TTR has specific binding sites for Thyroxine (T4), RBP4 and also for Amyloid beta 1-42 ($A\beta_{1-42}$); it is also the plasma transporter of T4 and retinol (Raz A et al, 1969), in the latter case through binding to the RBP4, and it has been suggested that TTR may be one of the $A\beta_{1-42}$ sequestering proteins in CSF (Li X et al, 2011). In mammals TTR also binds T3 but with lower affinity. Thyroid hormones are essential for development, growth, metabolism, fertility, cardiovascular system and

brain function. TTR transports about 20% of the hormone T4 in the plasma (Myron JA et al, 2007) and 80% in the CSF (Robbins J, 1991).

RBP4 is transported in the bloodstream, attached to TTR only when bound to retinol as a 1:1 molar protein complex (Goodman DS, 1984; Blaner WS, 1989; Raz A et al, 1970). TTR association to RBP4 protects it from glomerular filtration and renal catabolism (Raghu P et al, 2004; D'Ambrosio DN et al, 2011) and from oxidation. Retinol or vitamin A is essential for vision, reproduction, growth, differentiation and metabolism. After retinol delivery to tissues, RBP4 shows a reduced affinity to TTR. At target cells the vitamin dissociates from RBP4 and readily moves into cells by diffusion across the plasma membrane according to concentration gradient (Noy N et al, 1990). TTR has been associated with a number of pathological conditions, including Alzheimer's disease, Parkinson's disease, schizophrenia and depression (Fleming CE et al, 2009). Other reports suggested that TTR was associated to several types of cancer, diabetes, rheumatoid arthritis, glomerular disease and hepatitis. Moreover, TTR is thought to be directly and causally involved in the establishment of all stages of the stress response to nutritional impairment and in acute stressful conditions, such as tissue injury. In relation to this, the serum TTR level (0.08-0.45 mg/ml) is used as a marker for malnutrition as well as inflammation, decreasing in both clinical settings (Myron JA et al, 2007). Its serum level is decreased in patients with some tumors (Kozak KR et al, 2005, Liu L et al, 2007), although it is not clear whether such decrease is related to an inflammatory response or is an intrinsic property of the tumors. Maetzler W and co-workers (2012) reported that CSF TTR values rise in response to oxidative stress. Significantly increased TTR serum levels were detected in high myopia patients compared to healthy controls (Shao J et al, 2011). The physiological role of TTR in the eye is

not completely known, but it may participate in retinol cycling. An elevated number of TTR mutations is known (Saraiva MJM, 2004). Most of the variants result from point mutations in the polypeptide chain, except the case of a deletion of one aminoacidic residue at position 122. The majority of the TTR variants are associated with peripheral neuropathy, often presenting cardiomyopathy, carpal tunnel syndrome (CTS) and vitreopathy. In FMS may mask that CTS can be an associated illness in fact Nacir B et al, (2012) find a higher frequency of CTS in FMS patients. Terazaki H et al, (1999) described an interesting compound heterozygote in the TTR gene with familial amyloidotic polyneuropathy (FAP). The total TTR and RBP4 concentrations in the serum samples of the proband were higher than in control subjects. The mutation may play a suppressing role in amyloid formation since none of the family members of the present case carrying the mutated gene showed clinical symptoms of FAP.

Retinol binding protein 4 (RBP4) (spot ID 2334) is the specific blood carrier for the transport of retinol (Vitamin A) to target tissues; it is a 21 kDa monomeric protein comprising 182 aa residues (Rask L et al, 1979). RBP4 is mainly synthesized in the liver (Goodman DS, 1984), but several extrahepatic organs have been found to contain high amounts of the protein (Soprano DR et al, 1986). As reported above, RBP4 circulates in the bloodstream as a complex with TTR, which increases the molecular mass of RBP4 complex and thus prevents its glomerular filtration (Raghu P et al, 2004; D'Ambrosio DN et al, 2011). Recently, Raghu P et al, (2012) found that elevated levels of plasma RPB4 in obesity and type 2 diabetes (T2D) could be due to either increased RBP4 expression in the adipose tissue or increased plasma TTR levels or both. TTR probably serves as a regulatory checkpoint for insulin function by modulating

plasma RBP4 levels. Akkus S et al (2009) found decreased Vitamin A concentration in FMS than in controls and supposed an imbalance between the reactive oxygen species (ROS) production and the antioxidant defense system in these patients.

Alpha-1 antitrypsin (spot ID 106), a member of the SERPINs (SERine Proteinase Inhibitor) superfamily, is a 52 kD glycoprotein serine proteinase inhibitor mainly produced by hepatocytes (Blank CA et al, 1994), with smaller amounts synthesized by intestinal epithelial cells, neutrophils, pulmonary alveolar cells, and macrophages (Aldonyte R et al, 2004, Dabbagh K et al, 2001). A1AT concentrations in healthy subjects are 1.5-3.5mg/ml and can increase fourfold during inflammation, indicating that A1AT is an acute-phase protein (Blank CA, 1994; Carrell RW et al, 1986; Massi G et al, 1994). Its major function is the inhibition of overexpressed serine proteases (American Thoracic Society, 2003; Janciauskiene S et al, 2007). A1AT is also known to regulate the activation and apoptosis of neutrophils, monocytes, endothelial, and pancreatic cells acting as an antioxidant, modulating cell-mediated immunity tolerance and cellular innate immune response and neutralizing endotoxin-mediated inflammation. Moreover, A1AT could play a role in host cell defense against microbial pathogens (Cordelier P et al, 2003; Forney JR et al, 1997). Adding A1AT to cultured human cells in vitro has revealed anti-inflammatory properties. A1AT inhibited LPS-stimulated (Lipopolysaccharide) synthesis and secretion of TNF-α and IL-1β in human blood monocytes (Janciauskiene SM et al, 2004). In addition, intracellular signaling studies showed that A1AT inhibited activation of NF-kB, a transcription factor involved in the expression of several proinflammatory cytochines (Shapiro L et al, 2001). A1AT gene is located

in chromosome 14 and has two alleles, named by the letters of the alphabet. Normal alleles are called M and are found in about 80% of individuals (American Thoracic Society, 2003); most common abnormal alleles are called S and Z. Blanco I et al (2007) suggest that a subset of FMS lacking functionally active A1AT may suffer from inflammatory processes, mediated by proteases, cytokines, oxidants, and other inflammation mediators normally regulated by A1AT. Therefore, the inherited deficiency of A1AT may constitute a genetic modifier of the expression of FMS syndrome. In another study Blanco I et al, (2010 Feb) showed higher A1AT plasma concentrations in the MM A1AT subject in the FMS group than those in the general population groups. No histological differences were found between samples from A1AT deficiency and normal A1AT phenotypes, but a significantly increased number of mast cells (MCs) in the papillary dermis of all FMS patients contained moderately high levels of A1AT. MCs are present in skin and mucosal surfaces throughout the human body, and are easily stimulated by a number of physical, psychological, and chemical triggers to degranulate, releasing several proinflammatory products which are able to generate nervous peripheral stimuli causing CNS hypersensitivity, local and systemic symptoms (Blanco I et al, 2010 Dec). High serum levels of A1AT have been reported in hepatocellular carcinoma (HCC) but the exact mechanism and role of these findings is still unclear. Results of the study by Sawaya R et al, (1987) supported hypotheses that the production of A1AT by tumor cells correlates with the regional proteolytic and inflammatory activity, which are probably involved in the protection of tumor cells. In addition to its antiprotease activity, there are evidence that A1AT molecule has a relevant antioxidant capacity. AAT has one cysteine and nine methionine residues; the sulfur groups of two of the methionines

and cysteine thiol group are potentially available for interaction with oxidant (Taggart C et al, 2000; Brantly M, 2002).

To the best of our knowledge, no proteomic study has been reported on sera of FMS patients; in this preliminary study, we described, for the first time, the human serum protein pattern of FMS patients in comparison with healthy controls in order to identify the potential biomarkers for the disease.

In pathogenesis of FMS oxidative stress has been proposed as a relevant event (Cordero MD et al PLOS one, 2011). The reactive oxygen species (ROS) are one the main products source of oxidative stress; mitochondria are one of the major sources of ROS (Raha S et al, 2000) and can generate ROS as byproducts of molecular oxygen consumption in the electron transport chain. Also if the role of ROS in FMS is controversial (Akkus S et al, 2009), Cordero and co-workers (2013) underline an important role of oxidative stress and mitochondrial dysfunction in FMS.

There is a complex defense system protecting from oxidative stress including enzymes and non-enzymatic species such vitamin C, E and A (Zingg JM et al, 2004, Chiu HJ et al, 2008). Vitamin A regulates multiple cellular processes and exerts many of its biological activities by giving rise to active metabolites (11-cis-retinaldehyde and retinoic acid); it is stored in various tissues mainly in the liver and it is secreted from storage into the circulation bound to retinol-binding protein 4 (RBP4); in most mammalians Vitamin A-bound RBP4 is associated to transthyretin (TTR) in a complex that, under normal circumstances, displays a 1:1 molar stoichiometry.

Vitamin A deprivation causes a rapid increase in mitochondria-derived ROS and results in cell death consequent to mitochondrial membrane

depolarization and rapid loss of plasma membrane integrity (Chiu HJ et al, 2008). Discordant data are reported on Vitamin A levels in FMS patients (Eisinger J et al, 1997); Akkus S et al, (2009) showed a reduction of Vitamin A in FMS patients as results of its role action on radical inhibition and hypothesized an imbalance between ROS production and the antioxidant defense system in patients with fibromyalgia.

5.2. Purine metabolites

A prevailing theory in the pathogenesis of FMS is the dysregulation of pain pathways leading to central sensitization and marked by neurotransmitter, neurohormone and sleep physiology irregularities (Mease P, 2005).

Consensus of opinion exists regarding a widespread physiological and pathological role played by purines acting via extracellular receptors and, a number of recent studies implicate the purinergic system as essential for pain transmission (Sawynok XJL, 2003). The role of extracellular purine on pain transmission has been investigated (Sawynok XJL, 2003), but clinical studies correlating purine levels and pain are limited. Some authors report that fibromyalgia could include dysfunction of purine nucleotide metabolism and nociception (Guieu R et al, 2012); adenosine exerts antinociceptive effects when released into the extracellular space. Extracellular inosine and adenosine access the cell through equilibrative and concentrative transporters and adenosine and inosine compete for these transporters (Pastor-Anglada M et al, 2001); high extracellular inosine can lead to an increase in extracellular adenosine preventing adenosine uptake. In this context, adenosine deaminase (ADA) activity might play an essential role. ADA is an enzyme that catalyzes deamination of either adenosine or deoxyadenosine. Adenosine is converted to inosine by ADA. This reaction is one of the rate limiting steps in adenosine degradation due to its irreversibility. ADA activity is altered in various diseases (Urunsak IF et al, 2012). Following extracellular release, all purine nucleotides and nucleosides undergo a complex extracellular metabolism by many cell surface located enzymes (Rathbone MP et al, 1999). ADA may play a focal role in this way; ADA exerts metabolic

functions inside the cells but, outside the cells, it also has extra enzymatic effects involving modulation of adenosine A1 receptors (Ciruela F et al, 1996). The presence of increased ADA activity to explain inosine concentrations higher than adenosine has been reported in isolated rat hepatocytes after hypoxia (Guinzberg R et al, 2006). Moreover, recent data (Guieu R et al, 2012) show that low extracellular adenosine concentration in FMS is modulated by a complex of CD26 (multifunctional glycoprotein with dipeptidylpeptidase IV activity) in association with MCADA (mononuclear cell adenosine deaminase activity). Adenosine is recognized as an important modulator of neurotransmission and has been implicated in many physiological functions such as sleep and anxiety regulation, typical distresses in FMS: Sawynok XJL, (2003) reported that spinal administration of adenosine produces analgesia in patients with central sensitization.

6. CONCLUSION

Our study revealed overexpression of TTR, A1AT and RBP4 in FMS patients compared to control subjects. Our data show increased serum RBP4; upregulation of RBP4 may induce hypovitaminosis A (Chuang MC et al, 2012). High serum TTR and RBP4 might be consequent to 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 accumulates also in vitamin A-deficient hepatocytes but it is not secreted into bloodstream (Ronne H et al, 1983).

In our study we found also an overexpression of serum A1AT. Also Blanco et al 2010 reported high A1AT 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 A1AT (Blanco I et al, 2010). The mastocyte-released chemicals could cause local manifestations (e.g. skin tenderness or pain) that might be related to enhanced pain sensitivity of these patient.

A1AT is one of major circulating inhibitor of proteases but it is well known that it is the target of reactive oxidants (Ueda M et al, 2002). Previous study (Marzatico F et al, 1998) reported slight increase of A1AT associated to decrease of vitamin A suggesting that genetic or environmental factors may influence the activity of A1AT and that the activity of A1AT is dependent on the antioxidant capacity of liposoluble vitamins. A1AT can be oxidized by free radicals released from oxidative stress, resulting in protein dysfunction that leads to the imbalance between A1AT and proteases, which can cause tissue damage (Jamnongkan W et al, 2012). Then there may be high levels of A1AT (as reported in our

study) but with ineffective action as protease inhibitor but might act as an antioxidant.
Considering that the TTR and RBP4 are both retinoid transporters, retinoid dysfunction is related to oxidative stress as well as A1AT.

In purine study, our data show an increase of serum inosine, hypoxanthine and xanthine and a decrease of adenosine in FMS subjects. In this context, adenosine deaminase (ADA) activity might play an essential role. In FMS, ADA activity might be increased and consequently convert almost all adenosine to inosine, unbalancing their extracellular concentrations. Moreover, significantly lower serum adenosine levels in FMS subjects with respect to controls are confirmed by recent data reported by Guieu et al (2012). Furthermore, adenosine is recognized as an important modulator of neurotransmission and has been implicated in many physiological functions such as sleep and anxiety regulation, typical distresses in FMS, and our data show a negative correlation of adenosine with the patients' clinical feature (FIQ) that is consistent with enhanced pain sensitivity in FMS patients; in fact adenosine is associated with the mechanisms underlying pain transmission, consequently, low plasma adenosine, acting at a peripheral level, supports the low nociceptive threshold in these patients where pain is a principal symptom in FMS. Moreover oxidative stress in FMS might be consistent with these data that showed unexpected normal levels of uric acid despite high levels of inosine, hypoxanthine, xanthine in FMS patients. Uric acid is a powerful free radical scavenger in humans (Waring WS, 2002) then might be not increased in FMS patients due to its consumption as ROS scavenger.
Our results are consistent with the underlying hypothesis that there is an imbalance between ROS production and the antioxidant defense system of

patients with fibromyalgia. The oxidative stress in FMS might increase serum TTR, RBP4 and A1AT expression and utilize uric acid as ROS scavenger. Moreover, adenosine is associated with the mechanisms underlying pain transmission, where the pain is a principal symptom in FMS.

Further investigation will be required to confirm this observation.

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BIBLIOGRAPHY

Aaron LA, Burke MM, Buchwald D. *Overlapping conditions among patients with chronic fatigue syndrome, fibromyalgia, and temporomandibular disorder*. Arch Intern Med. 2000 Jan 24; 160(2): 221-7.

Aaron LA, Buchwald D. *A review of the evidence for overlap among unexplained clinical conditions*. Ann Intern Med. 2001; 134(9 Pt 2): 868-81.

Adkins JN, Varnum SM, Auberry KJ, Moore RJ, Angell NH, Smith RD, Springer DL, Pounds JG. *Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry.* Mol Cell Proteomics 2002 Dec; 1(12): 947-55.

Akkuş S, Naziroğlu M, Eriş S, Yalman K, Yilmaz N, Yener M. *Levels of lipid peroxidation, nitric oxide, and antioxidant vitamins in plasma of patients with fibromyalgia*. Cell Biochem Funct., 2009 Jun; 27(4): 181-5.

Aldonyte R, Jansson L, Ljungberg O, Larsson S, Janciauskiene S. *Polymerized alpha-antitrypsin is present on lung vascular endothelium. New insights into the biological significance of alpha-antitrypsin polymerization*. Histopathology 2004 Dec; 45(6): 587-92.

Aleshire SL, Bradley CA, Richardson LD, Pari FF. *Localization of human prealbumin in choroid plexus epithelium*. J Histochem Cytochem. 1983 May; 31(5): 608-12.

Allard L, Burkhard PR, Lescuyer P, Burgess JA, Walter N, Hochstrasser DF, Sanchez JC. *PARK7 and nucleoside diphosphate kinase A as plasma markers for the early diagnosis of stroke*. Clin Chem. 2005 Nov; 51(11): 2043-51.

Ambrose KR, Gracely RH, Glass JM. *Fibromyalgia dyscognition: concepts and issues*. Reumatismo 2012 Sep 28; 64(4): 206-15.

American Thoracic Society/European Respiratory Society Statement. *Standards for the diagnosis and management of individuals with alpha 1-antitrypsin deficiency*. Am J Respir Crit Care Med. 2003; 168: 818-900.

Anderson L, Anderson NG. *High resolution two-dimensional electrophoresis of human plasma proteins*. Proc Natl Acad Sci U S A 1977 Dec; 74(12): 5421-5.

Anderson L, Seilhammer J. *A comparison of selected mRNA and protein abundances in human liver*. Electrophoresis 1997 Mar-Apr; 18(3-4): 533-7.

Anderson NL, Anderson, NG. *The human plasma proteome: history, character, and diagnostic prospects*. Mol. Cell Prot. 2002; 1, 845-867.

Anderson NL, Polanski M, Pieper R, Gatlin T, Tirumalai RS, Conrads TP, Veenstra TD, Adkins JN, Pounds JG, Fagan R, Lobley A. *The human plasma proteome: a nonredundant list developed by combination of four separate sources*. Mol Cell Proteomics 2004 Apr; 3(4): 311-26.

Apweiler R, Biswas M, Fleischmann W, Kanapin A, Karavidopoulou Y, Kersey P, Kriventseva EV, Mittard V, Mulder N, Phan I, Zdobnov E.

Proteome analysis database: online application of InterPro and CluSTr for the functional classification of proteins in whole genomes. Nucleic Acids Res 2001; 29:44-48.

Arnold LM. *The pathophysiology, diagnosis, and treatment of fibromyalgia*. Psychiatr Clin North Am 2010; 33:375-408.

Arnold LM, Fan J, Russell IJ, Yunus MB, Khan MA, Kushner I, Olson JM, Iyengar SK. *The fibromyalgia family study: A genome-scan linkage study*. Arthritis Rheum. 2012 Dec 28.

Baldwin MA, Medzihradszky KF, Lock CM, Fisher B, Settineri TA, Burlingame AL. *Matrix-assisted laser desorption/ionization coupled with quadrupole/orthogonal acceleration time-of-flight mass spectrometry for protein discovery, identification, and structural analysis*. Anal Chem. 2001 Apr 15; 73(8): 1707-20.

Barkhuizen A. *Rational and targeted pharmacologic treatment of fibromyalgia*. Rheum Dis Clin North Am. 2002 May; 28(2): 261-90.

Bazzichi L, Rossi A, Giuliano T, De Feo F, Giacomelli C, Consensi A, Ciapparelli A, Consoli G, Dell'osso L, Bombardieri S. *Association between thyroid autoimmunity and fibromyalgic disease severity*. Clin Rheumatol. 2007 Dec; 26(12): 2115-20.

Becker S, Schweinhardt P. *Dysfunctional neurotransmitter systems in fibromyalgia, their role in central stress circuitry and pharmacological actions on these systems*. Pain Research and Treatment 2012; 2012: 741746.

Bennett RM, Clark SR, Campbell SM, Burckhardt CS. *Low levels of somatomedin C in patients with the fibromyalgia syndrome: a possible link between sleep and 14 Pain Research and Treatment muscle pain*. Arthritis Rheum. 1992 Oct; 35(10): 1113-6.

Bennett R. *The Fibromyalgia Impact Questionnaire (FIQ): a review of its development, current version, operating characteristics and uses*. Clin Exp Rheumatol 2005; 23: S154-62.

Bennett RM, Friend R, Jones KD, Ward R, Han BK, Ross RL. *The Revised Fibromyalgia Impact Questionnaire (FIQR): validation and psychometric properties*. Arthritis Res Ther. 2009; 11(4): R120.

Berggren K, Chernokalskaya E, Steinberg TH, Kemper C, Lopez MF, Diwu Z, Haugland RP, Patton WF. *Background-free, high sensitivity staining of proteins in one- and two-dimensional sodium dodecyl sulfatepolyacrylamide gels using a luminescent ruthenium complex*. Electrophoresis 2000; 21, 2509-2521.

Biewer W, Conrad I, Häuser W. *Fibromyalgia*. Schmerz. 2004 Apr; 18(2): 118-24.

Blanco I, de Serres FJ, Janciauskiene S, Arbesù D, Fernandez-Bustillo E, Carcaba V, Nita I, Astudillo A. *Estimates of the prevalence and number of fibromyalgia syndrome patients and their alpha1-antitrypsin deficiency phenotypic distribution in ten countries*. J. Musculoskeletal Pain 2007; 15(4): 9-23.

Blanco I, Janciauskiene S, Nita I, Fernández-Bustillo E, Cárcaba V, Gallo C, Alvarez-Rico M, de Serres F, Béridze N. *Low plasma levels of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNFalpha), and vascular endothelial growth factor (VEGF) in patients with alpha1-antitrypsin deficiency-related fibromyalgia*. Clin Rheumatol. 2010 Feb; 29(2): 189-97.

Blanco I, Béritze N, Argüelles M, Cárcaba V, Fernández F, Janciauskiene S, Oikonomopoulou K, de Serres FJ, Fernández-Bustillo E, Hollenberg MD. *Abnormal overexpression of mastocytes in skin biopsies of fibromyalgia patients*. Clin Rheumatol. 2010 Dec; 29(12): 1403-12.

Blaner WS. *Retinol-binding protein: the serum transport protein for vitamin A*. Endocr Rev. 1989 Aug; 10(3): 308-16.

Blank CA, Brantly M. *Clinical features and molecular characteristics of alpha 1-antitrypsin deficiency*. Ann Allergy. 1994 Feb; 72(2): 105-20.

Bradley LA. *Psychiatric comorbidity in fibromyalgia*. Curr Pain Headache Rep. 2005 Apr; 9(2): 79-86.

Branco JC, Bannwarth B, Failde I, Abello Carbonell J, Blotman F, Spaeth M, Saraiva F, Nacci F, Thomas E, Caubère JP, Le Lay K, Taieb C, Matucci-Cerinic M. *Prevalence of fibromyalgia: a survey in five European countries.* Semin Arthritis Rheum. 2010; 39(6): 448-53.

Brantly M. *Alpha1-antitrypsin: not just an antiprotease: extending the half-life of a natural anti-inflammatory molecule by conjugation with polyethylene glycol.* Am J Respir Cell Mol Biol. 2002 Dec; 27(6): 652-4.

Burckhardt CS, Clark SR, Bennett RM. *The Fibromyalgia Impact Questionnaire: development and validation*. J Rheumatol 1991; 18: 728-33.

Burckhardt CS. *Multidisciplinary approaches for management of Fibromyalgia*. Curr Pharm Design 2006; 12: 59-66.

Burnstock G. *Pathophysiology and therapeutic potential of purinergic signaling*. Pharmacol Rev 2006; 58: 58-86.

Busch AJ, Barber KA, Overend TJ, Peloso PM, Schachter CL. *Exercise for treating fibromyalgia syndrome*. Cochrane Database Syst Rev. 2007 Oct 17; (4): CD003786.

Buskila D, Gladman DD, Langevitz P, Urowitz S, and Smythe HA. *Fibromyalgia in human immunodeficiency virus infection*. J Rheumatol. 1990 Sep; 17(9): 1202-6.

Buskila D. *Fibromyalgia, chronic fatigue syndrome, and myofascial painsyndrome*. Curr Opin Rheumatol. 2001; 13: 117-127.

Buskila D, Sarzi-Puttini P. *Biology and therapy of fibromyalgia. Genetic aspects of fibromyalgia syndrome*. Arthritis Res Ther. 2006; 8(5): 218-222.

Buskila D, Sarzi-Puttini P, and Ablin JN. *The genetics of fibromyalgia syndrome*. Pharmacogenomics 2007 Jan; 8(1): 67-74.

Carrell RW. *alpha 1-Antitrypsin: molecular pathology, leukocytes, and tissue damage*. J Clin Invest. 1986 Dec; 78(6): 1427-31.

Carville SF, Choy EH. *Systematic review of discriminating power of outcome measures used in clinical trials of fibromyalgia*. J Rheumatol. 2008 Nov; 35(11): 2094-105.

Cassisi G, Sarzi-Puttini P, Cazzola M. *Chronic widespread pain and fibromyalgia: could there be some relationships with infections and vaccinations?* Clin Exp Rheumatol. 2011 Nov-Dec; 29(6 Suppl 69): S118-26.

Catley D, Kaell AT, Kirschbaum C, Stone AA. *A naturalistic evaluation of cortisol secretion in persons with fibromyalgia and rheumatoid arthritis*. Arthritis Care Res. 2000; 13(1): 51-61.

Chiu HJ, Fischman DA, Hammerling U. *Vitamin A depletion causes oxidative stress, mitochondrial dysfunction, and PARP-1-dependent energy deprivation.* FASEB J. 2008 Nov; 22(11): 3878-87.

Chuang CM, Chang CH, Wang HE, Chen KC, Peng CC, Hsieh CL, Peng RY. *Valproic acid downregulates RBP4 and elicits hypervitaminosis A-teratogenesis--a kinetic analysis on retinol/retinoic acid homeostatic system.* PLoS One. 2012; 7(9): e43692.

Ciruela F, Saura C, Canela I, Mallol J, Lluis C, Franco R. *Adenosine deaminase affects ligand-induced signaling by interacting with cell surface adenosine receptors*. FEBS Lett 1996; 380: 219-23.

Clauw DJ, Crofford LJ. *Chronic widespread pain and fibromyalgia: what we know, and what we need to know*. Best Pract Res Clin Rheumatol. 2003; 17: 685-701.

Clauw DJ, Arnold LM, McCarberg BH. *The science of fibromyalgia*. MayoClin Proc 2011; 86: 907-911.

Cohen H, Neumann L, Kotler M, and Buskila D. *Autonomic nervous system derangement in fibromyalgia syndrome and related disorders*. Isr Med Assoc J. 2001 Oct; 3(10): 755-60.

Cordelier P, Strayer DS. *Mechanisms of alpha1-antitrypsin inhibition of cellular serine proteases and HIV-1 protease that are essential for HIV-1 morphogenesis*. Biochim Biophys Acta. 2003 Jul 30; 1638(3): 197-207.

Cordero MD, Alcocer-Gómez E, Cano-García FJ, De Miguel M, Carrión AM, Navas P, Sánchez Alcázar JA. *Clinical symptoms in fibromyalgia are better associated to lipid peroxidation levels in blood mononuclear cells rather than in plasma*. PLoS One. 2011; 6(10): e26915.

Cordero MD, Díaz-Parrado E, Carrión AM, Alfonsi S, Sánchez-Alcazar JA, Bullón P, Battino M, de Miguel M. *Is inflammation a mitochondrial dysfunction-dependent event in fibromyalgia?* Antioxid Redox Signal. 2013 Mar 1; 18(7): 800-7.

Corthals GL, Gygi SP, Aebersold R, Patterson SP. *Identification of proteins by mass spectrometry*, in Rabilloud T. (Ed.), Proteome Research: Two–Dimensional Gel Electrophoresis and Identification Methods, Springer Verlag, New York, NY, (1999), pp. 197-231.

Cristea IM, Gaskell SJ, Whetton AD. *Proteomics techniques and their application to hematology*. Blood. 2004 May 15; 103(10): 3624-34.

Culpepper L. *Evaluating the patient with fibromyalgia*. J Clin Psychiatry 2010 Sep; 71(9): e25.

Dabbagh K, Laurent GJ, Shock A, Leoni P, Papakrivopoulou J, Chambers RC. *Alpha-1-antitrypsin stimulates fibroblast proliferation and procollagen production and activates classical MAP kinase signalling pathways*. J Cell Physiol. 2001 Jan; 186(1): 73-81.

D'Ambrosio DN, Clugston RD, Blaner WS. *Vitamin A metabolism: an update*. Nutrients. 2011 Jan; 3(1): 63-103.

De Rezende Pena C, Grillo LP, Das Changas Medeiros MM. *Evaluation of 25- hydroxyvitamin D serum levels in patients with fibromyalgia*. J Clin Rheumatol 2010; 16: 365-9.

DeLeao JA, Yezierki RP. *The role of neuroinflammation and neuroimmune activation in persistent pain*. Pain 2001; 90: 1-6.

Demirbağ B, Erci B. *The effects of sleep and touch therapy on symptoms of fibromyalgia and depression*. Iranian journal of public health 2012; 41: 44-53.

Di Franco M, Iannuccelli C, Atzeni F, Cazzola M, Salaffi F, Valesini G, & Sarzi-Puttini P. *Pharmacological treatment of fibromyalgia*. Clin Exp Rheumatol. 2010; 28(6 Suppl 63): S110-6.

Eisinger J, Gandolfo C, Zakarian H, Ayavou T. *Reactive oxygen species, antioxidant status and fibromyalgia.* J Musculoskeletal pain 1997; 5(4): 5-15.

Eltzschig HK. *Adenosine: an old drug newly discovered*. Anesthesiology 2009; 111(4): 904-915.

Epstein SA, Kay G, Clauw D, Heaton R, Klein D, Krupp L, Kuck J, Leslie V, Masur D, Wagner M, Waid R, Zisook S. *Psychiatric disorders in patients with fibromyalgia: a multicenter investigation*. Psychosomatics. 1999 Jan-Feb; 40(1): 57-63.

Fleming CE, Nunes AF, Sousa MM. *Transthyretin: more than meets the eye*. Prog Neurobiol. 2009 Nov; 89(3): 266-76.

Forney JR, Yang S, Healey MC. *Synergistic anticryptosporidial potential of the combination alpha-1-antitrypsin and paromomycin*. Antimicrob Agents Chemother. 1997 Sep; 41(9): 2006-8.

Garrison L, Breeding PC. *A metabolic basis for fibromyalgia and its related disorders: the possible role of resistance to thyroid hormone*. Med Hypotheses 2003 Aug; 61(2): 182-9.

Geenen R, Jacobs JWG, Bijlsma JWJ. *Evaluation and management of endocrine dysfunction in fibromyalgia*. Rheum Dis Clin North Am. 2002 May; 28(2): 389-404.

Gerdle B, Björk J, Cöster L, Henriksson KG, Henriksson C, Bengtsson A. *Prevalence of widespread pain and associations with work status: a population study*. BMC Musculoskelet Disord 2008; 9: 102-112.

Giacomelli C, Bazzichi L, Giusti L, Ciregia F, Baldini C, Da Valle Y, De Feo F, Sernissi F, Rossi A, Bombardieri S, Lucacchini A. *MALDI-TOF and SELDI-TOF analysis: "tandem" techniques to identify potential biomarker in fibromyalgia.* Reumatismo 2011; 63: 165-170.

Giesecke T, Williams DA, Harris RE, Cupps TR, Tian X, Tian TX, Gracely RH, Clauw DJ. *Subgrouping of fibromyalgia patients on the basis of pressure-pain thresholds and psychological factors*. Arthritis Rheum. 2003 Oct; 48(10): 2916-22.

Godovac-Zimmermann J, Kleiner O, Brown LR, Drukier AK. *Perspectives in spicing up proteomics with splicing*. Proteomics. 2005 Feb; 5(3): 699-709.

Goodman DS. *Plasma retinol-binding protein. In: The retinoids.* 1984, Vol. 2, MB Sporn, AB Roberts and DS Goodman (eds), Academic Press, New York, pp 41-88.

Görg A, Postel W, Domscheit A, Günther S. *Two-dimensional electrophoresis with immobilized pH gradients of leaf proteins from barley (Hordeum vulgare): method, reproducibility and genetic aspects*. Electrophoresis 1988 Nov; 9(11): 681-92.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. *The current state of two-dimensional electrophoresis with immobilized pH gradients*. Electrophoresis 2000 Apr; 21(6): 1037-53.

Görg A, Weiss W, Dunn MJ. *Current two-dimensional electrophoresis technology for proteomics*. Proteomics 2004 Dec; 4(12): 3665-85.

Gowans SE, Dehueck A, Voss S, Silaj A, Abbey SE. *Six-month and one-year followup of 23 weeks of aerobic exercise for individuals with fibromyalgia*, Arthritis Rheum. 2004; 51(6): 890-8.

Gowers WR. *A lecture on lumbago: its lessons and analogues: delivered at the national hospital for the paralysed and epileptic*. Br Med J. 1904 Jan 16; 1(2246): 117-21.

Graham W. *The fibrositis syndrome*. Treat Serv Bull. 1953 Jul-Aug; 8(7): 400-4.

Graven-Nielsen T, Aspegren Kendall S, Henriksson KG, Bengtsson M, Sörensen J, Johnson A, Gerdle B, Arendt-Nielsen L. *Ketamine reduces muscle pain, temporal summation, and referred pain in fibromyalgia patients*. Pain 2000 Apr; 85(3): 483-91.

Griep EN, Boersma JW, and de Kloet ER. *Altered reactivity of the hypothalamic-pituitary-adrenal axis in the primary fibromyalgia syndrome*. J Rheumatol. 1993 Mar; 20(3): 469-74.

Guieu R, Guedj E, Giorgi R, Dousset A, Tuzzolino V, By Y, Leveque JM, Peragut JC, Régis J, Ruf J, Fenouillet E, Roussel P. *High cell surface CD26-associated activities and low plasma adenosine concentration in fibromyalgia*. Ann Rheum. Dis. 2012; 71: 1427-8.

Guinzberg R, Cortés D, Díaz-Cruz A, Riveros-Rosas H, Villalobos-Molina R, Piña E. *Inosine released after hypoxia activates hepatic glucose liberation through A3 adenosine receptors*. Am J Physiol Endocrinol Metab. 2006;290:940-51.

Han C, Lee SJ, Lee SY, Seo HJ, Wang SM, Park MH, Patkar AA, Koh J, Masand PS, Pae CU. *Available therapies and current management of fibromyalgia: focusing on pharmacological agents*. Today (Barc). 2011; 47(7): 539-57.

Haskó G, Sitkov MV, Szabó C. *Immunomodulatory and neuroprotective effects of inosine*. Trends Pharmacol Sci. 2004; 25: 152-7.

Häuser W, Schmutzer G, Brähler E, Glaesmer H. *A cluster within the continuum of biopsychosocial distress can be labeled "fibromyalgia syndrome"-evidence from a representative German population survey*. J Rheumatol. 2009; 36(12): 2806-12.

Heidari B, Shirvani JS, Firouzjahi A, Heidari P, Hajian-Tilaki KO. *Association between nonspecific skeletal pain and vitamin D deficiency*. Int J Rheum Dis. 2010; 13: 340-6.

Humphrey-Smith I, Cordwell SJ, Blackstock WP. *Proteome research: complementarity and limitations with respect to the RNA and DNA worlds*. Electrophoresis 1997 Aug; 18(8): 1217-42.

International Human Genome Sequencing Consortium. *Finishing the euchromatic sequence of the human genome*. Nature 2004 Oct 21; 431(7011): 931-45.

Jacobs JM, Adkins JN, Qian WJ, Liu T, Shen Y, Camp DG 2nd, Smith RD. *Utilizing human blood plasma for proteomic biomarker discovery*. J Proteome Res. 2005 Jul-Aug; 4(4): 1073-85.

Jamnongkan W, Techasen A, Thanan R, Duenngai K, Sithithaworn P, Mairiang E, Loilome W, Namwat N, Pairojkul C, Yongvanit P. *Oxidized alpha-1 antitrypsin as a predictive risk marker of opisthorchiasis-associated cholangiocarcinoma.* Tumour Biol. 2012 Nov 28.

Janciauskiene SM, Larsson S, Larsson P, Virtala R, Jansson L, Stevens T. *Inhibition of lipopolysaccharide-mediated human monocyte activation, in vitro, by alpha1-antitrypsin*. Biochem Biophys Res Commun. 2004 Aug 27; 321(3): 592-600.

Janciauskiene SM, Nita IM, Stevens T. *Alpha1-antitrypsin, old dog, new tricks. Alpha1-antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevating cAMP*. J Biol Chem. 2007 Mar 23; 282(12): 8573-82.

Jones KD, Deodhar P, Lorentzen A, Bennett RM, Deodhar AA. *Growth hormone perturbations in Fibromyalgia: a review*. Semin Arthritis Rheum. 2007 Jun; 36(6): 357-79.

Kadetoff D, Kosek E. *Evidence of reduced sympatho-adrenal and hypothalamic pituitary activity during static muscular work in patients with fibromyalgia*. J Rehabil Med. 2010 Sep; 42(8): 765-72.

Karakuş İ, Topaloğlu D. *Diagnosis and Treatment in Fibromyalgia and Myofacial Pain Syndrome*. Turk J Rheum. 2004; 42:367.

Kim MR, Kim CW. *Human blood plasma preparation for two-dimensional gel electrophoresis*. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 Apr 15; 849(1-2): 203-10.

Klingmann PO, Kugler I, Steffke TS, Bellingrath S, Kudielka BM, Hellhammer DH. *Sex-specific prenatal programming: a risk for fibromyalgia?* Ann. N.Y. Acad. Sci. 2008; 1148, 446-455.

Klose J, Spielmann H. *Gel isoelectric focusing of mouse lactate dehydrogenase: heterogeneity of the isoenzymes A4 and X4*. Biochem Genet. 1975 Oct; 13(9-10): 707-20.

Kooh M, Martínez-Lavín M, Meza S, Martín-del-Campo A, Hermosillo AG, Pineda C, Nava A, Amigo MC, Drucker-Colín R. *Simultaneous heart rate variability and polysomnographic analyses in fibromyalgia*. Clin Exp Rheumatol. 2003 Jul-Aug; 21(4): 529-30.

Kosek E, Hansson P. *Modulatory influence on somatosensory perception from vibration and heterotopic noxious conditioning stimulation (HNCS) in fibromyalgia patients and healthy subjects*. Pain. 1997 Mar; 70(1): 41-51.

Kötter I, Neuscheler D, Günaydin I, Wernet D, Klein R. *Is there a predisposition for the development of autoimmune diseases in patients with fibromyalgia? Retrospective analysis with long term follow-up*. Rheumatol Int. 2007 Sep; 27(11): 1031-9.

Kozak KR, Su F, Whitelegge JP, Faull K, Reddy S, Farias-Eisner R. *Characterization of serum biomarkers for detection of early stage ovarian cancer*. Proteomics 2005; 5: 4589-4596.

Laemmli UK. *Cleaveage of structural proteins during the assembly of the head of bacteriophage T4*. Nature 1970; 227, 680-685.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, et al. *Initial sequencing and analysis of the human genome*. Nature 2001; 409, 860–921.

Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, Heyse SP, Hirsch R, Hochberg MC, Hunder GG, Liang MH, Pillemer SR, Steen VD, Wolfe F. *Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States*. Arthritis Rheum. 1998 May; 41(5): 778-99.

Lee YH, Choi SJ, Ji JD, Song GG. *Candidate gene studies of fibromyalgia: a systematic review and meta-analysis*. Rheumatol Int. 2012; 32: 417–426.

Leventhal LJ, Naides SJ, Freundlich B. *Fibromyalgia and parvovirus infection*. Arthritis Rheum. 1991 Oct; 34(10): 1319-24.

Li J, Orlandi R, White CN, Rosenzweig J, Zhao J, Seregni E, Morelli D, Yu Y, Meng XY, Zhang Z, Davidson NE, Fung ET, Chan DW. *Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry*. Clin Chem. 2005; 51(12): 2229-35.

Li X, Buxbaum JN. *Transthyretin and the brain re-visited: is neuronal synthesis of transthyretin protective in Alzheimer's disease?* Mol Neurodegener. 2011; 23; 6: 79.

Li X, Masliah E, Reixach N, Buxbaum JN. *Neuronal production of transthyretin in human and murine Alzheimer's disease: is it protective?* J Neurosci. 2011 Aug 31; 31(35): 12483-90.

Liolios K, Chen IM, Mavromatis K, Tavernarakis N, Hugenholtz P, Markowitz VM, Kyrpides NC. *The Genomes On Line Database (GOLD) in 2009: status of genomic and metagenomic projects and their associated metadata.* Nucleic Acids Res. 2010 Jan; 38(Database issue): D346-54.

Liu L, Liu J, Dai S, Wang X, Wu S, Wang J, Huang L, Xiao X, He D. *Reduced transthyretin expression in sera of lung cancer*. Cancer Sci. 2007; 98: 1617-1624.

Liu Z, Welin M, Bragee B, Nyberg F. *A high-recovery extraction procedure for quantitative analysis of substance P and opioid peptides in human cerebrospinal fluid*. Peptides. 2000 Jun; 21(6): 853-60.

Lopez MF, Berggren K, Chernokalskaya E, Lazarev A, Robinson M, Patton WF. *A comparison of silver stain and SYPRO Ruby Protein Gel Stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling*. Electrophoresis 2000; 21, 3673-3683.

Maetzler W, Tian Y, Baur SM, Gauger T, Odoj B, Schmid B, Schulte C, Deuschle C, Heck S, Apel A, Melms A, Gasser T, Berg D. *Serum and cerebrospinal fluid levels of transthyretin in Lewy body disorders with and without dementia*. PLoS One 2012; 7(10): e48042.

Mannerkorpi K, Henriksson C. *Non-pharmacological treatment of chronic widespread musculoskeletal pain*. Best Pract Res Clin Rheumatol. 2007 Jun; 21(3): 513-34.

Martone RL, Herbert J, Dwork A, Schon EA. *Transthyretin is synthesized in the mammalian eye*. Biochem Biophys Res Commun. 1988; 151: 905-912.

Marzatico F, Gaetani P, Tartara F, Bertorelli L, Feletti F, Adinolfi D, Tancioni F, Rodriguez y Baena R. *Antioxidant status and alpha1-antiproteinase activity in subarachnoid hemorrhage patients.* Life Sci. 1998; 63(10): 821-6.

Massi G, Chiarelli C. *Alpha 1-antitrypsin: molecular structure and the Pi system*. Acta Paediatr Suppl. 1994 Feb; 393: 1-4.

Mease P. *Fibromyalgia syndrome: review of clinical presentation, pathogenesis, outcome measures, and treatment*. J Rheumatol 2005; 32: 6-21.

Mease P, Arnold LM, Bennett R, Boonen A, Buskila D, Carville S, Chappell A, Choy E, Clauw D, Dadabhoy D, Gendreau M, Goldenberg D, Littlejohn G, Martin S, Perera P, Russell IJ, Simon L, Spaeth M, Williams D, Crofford L. *Fibromyalgia syndrome*. J Rheumatol 2007 Jun; 34(6): 1415-1425.

Mease PJ, Dundon K, Sarzi-Puttini P. *Pharmacotherapy of fibromyalgia*. Best Pract Res Clin Rheumatol. 2011; 25(2): 285-97.

Menzies V, Lyon DE. *Integrated review of the association of cytokines with fibromyalgia and fibromyalgia core symptoms*. Biol Res Nurs. 2010; 11(4): 387-94.

Merchant ML, Klein JB. *Proteomics and diabetic nephropathy*. Curr Diab Rep. 2005 Dec; 5(6): 464-9.

Middleton GD, McFarlin JE, Lipsky PE. *The prevalence and clinical impact of fibromyalgia in systemic lupus erythematosus*. Arthritis Rheum. 1994 Aug; 37(8): 1181-8.

Moldofsky H, Inhaber NH, Guinta DR, Alvarez-Horine SB. *Effects of sodium oxybate on sleep physiology and sleep/wake-related symptoms in patients with fibromyalgia syndrome: a double-blind, randomized, placebo-controlled study.* J Rheumatol. 2010; 37(10), 2156-66.

Moyer SC, Budnik BA, Pittman JL, Costello CE, O'Connor PB. *Attomole peptide analysis by high-pressure matrix-assisted laser desorption/ionization Fourier transform mass spectrometry*. Anal Chem. 2003 Dec 1;75(23):6449-54.

Myron JA, Merlini G, Sheldon J, Ichihara K; Scientific Division Committee on Plasma Proteins (C-PP), International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). *Clinical indications for plasma protein assays: transthyretin (prealbumin) in inflammation and malnutrition*. Clin Chem Lab Med. 2007; 45(3): 419-26.

Nacir B, Genc H, Duyur Cakit B, Karagoz A, Erdem HR. *Evaluation of upper extremity nerve conduction velocities and the relationship between fibromyalgia and carpal tunnel syndrome.* Arch Med Res. 2012 Jul; 43(5): 369-74.

Nedelkov D, Kiernan UA, Niederkofler EE, Tubbs KA, Nelson RW. *Investigating diversity in human plasma proteins*. Proc Natl Acad Sci USA 2005 Aug 2; 102(31): 10852-7.

Neeck G. *Neuroendocrine and hormonal perturbations and relations to the serotonergic system in fibromyalgia patients.* Scand J Rheumatol Suppl. 2000; 113: 8-12.

Neuhoff V, Arold N, Taube D, Ehrhardt W. *Improved Staining of Proteins in Polyacrylamide Gels Including Isoelectric-Focusing Gels with Clear Background at Nanogram Sensitivity using Coomassie Brilliant Blue G-250 and R-250*. Electrophoresis 1988; *9*, 255-262.

Noy N, Xu ZJ. *Kinetic parameters of the interactions of retinol with lipid bilayers*. Biochemistry 1990 Apr 24; 29(16): 3883-8.

O'Farrell PH. *High resolution two-dimensional electrophoresis of proteins*. J Biol Chem. 1975 May 25; 250(10): 4007-21.

Okifuji A, Hare BD. *Nightly analyses of subjective and objective (actigraphy) measures of sleep in fibromyalgia syndrome: what accounts for the discrepancy?* Clin J Pain 2011; 27: 289-96.

Ozgocmen S, Ozyurt H, Sogut S, Akyol O, Ardicoglu O, Yildizhan H. *Antioxidant status, lipid peroxidation and nitric oxide in fibromyalgia: etiologic and therapeutic concerns*. Rheumatol Int. 2006 May; 26(7): 598-603.

Pastor-Anglada M, Casado FJ, Valdés R, Mata J, García-Manteiga J, Molina M. *Complex regulation of nucleoside transporter expression in epithelial and immune system cells*. Mol Membr Biol 2001; 18: 81-5.

Patton WF. *Detection technologies in proteome analysis*. J. Chromatogr. B 2002; 771, 3-31.

Payne TC, Leavitt F, Garron DC, Katz RS, Golden HE, Glickman PB, Vanderplate C. *Fibrositis and phsycologic disturbance*. Arthritis Rheum. 1982 Feb; 25(2): 213-7.

Perrot S. *Fibromyalgia syndrome: a relevant recent construction of an ancient condition?* Curr Opin Support Palliat Care, 2008 Jun; 2(2): 122-127.

Prinz PN, Moe KE, Dulberg EM, Larsen LH, Vitiello MV, Toivola B, Merriam GR. *Higher plasma IGF-1 levels are associated with increased delta sleep in healthy older men.* J Gerontol A Biol Sci Med Sci. 1995 Jul; 50(4): M222-6.

Raghu P, Sivakumar B. *Interactions amongst plasma retinol-binding protein, transthyretin and their ligands: implications in vitamin A homeostasis and transthyretin amyloidosis*. Biochim Biophys Acta. 2004 Dec 1; 1703(1): 1-9.

Raghu P, Palika R, Ghosh S, Reddy GB. *Contrasting effects of type 2 and type 1 diabetes on plasma RBP4 levels: the significance of transthyretin*. IUBMB Life. 2012 Dec; 64(12): 975-82.

Raha S, Robinson B H. *Mitochondria, oxygen free radicals, disease, and aging.* Trends Biochem Sci. 2000 Oct; 25(10): 502-8.

Rai AJ, Vitzthum F. *Effects of preanalytical variables on peptide and protein measurements in human serum and plasma: implications for clinical proteomics*. Expert Rev Proteomics 2006; 3: 409-426.

Rask L, Anundi H, Peterson PA. *The primary structure of the human retinol-binding protein*. FEBS Lett. 1979 Aug 1; 104(1): 55-8.

Rathbone MP, Middlemiss PJ, Gysbers JW, Andrew C, Herman MA, Reed JK, Ciccarelli R, Di Iorio P, Caciagli F. *Trophic effects of purines in neurons and glial cells*. Prog Neurobiol 1999; 59: 663-90.

Raz A, Goodman DS. *The interaction of thyroxine with human plasma prealbumin and with the prealbumin-retinol-binding protein complex*. J Biol Chem. 1969 Jun 25; 244(12): 3230-7.

Raz A, Shiratori T, Goodman DS. *Studies on the protein-protein and protein-ligand interactions involved in retinol transport in plasma*. J Biol Chem. 1970 Apr 25; 245(8): 1903-1912.

Rivera J, de Diego A, Trinchet M, García Monforte A. *Fibromyalgia-associated hepatitis C virus infection*. Br J Rheumatol. 1997 Sep; 36(9): 981-5.

Robbins J. *Thyroid hormone transport proteins and the physiology of hormone binding*. In: The Thyroid. Braverman LE and Utiger RD (eds). JB Lippincott Company, Philadelphia, 1991, pp 111-125.

Ronne H, Ocklind C, Wiman K, Rask L, Obrink B, Peterson PA. *Ligand-dependent regulation of intracellular protein transport: effect of vitamin a on the secretion of the retinol-binding protein.* J Cell Biol. 1983 Mar; 96(3): 907-10.

Russell IJ, Orr MD, Littman B, Vipraio GA, Alboukrek D, Michalek JE, Lopez Y, MacKillip F. *Elevated cerebrospinal fluid levels of substance P in patients with the fibromyalgia syndrome.* Arthritis Rheum. 1994 Nov; 37(11): 1593-601.

Russell IJ, Perkins AT, Michalek JE. *Sodium oxybate relieves pain and improves function in fibromyalgia syndrome: a randomized, double-blind, placebo-controlled, multicenter clinical trial*. Arthritis Rheum. 2009; 60(1), 299-309.

Salaffi F, De Angelis R, Grassi W; MArche Pain Prevalence; INvestigation Group (MAPPING) study. *Prevalence of musculoskeletal conditions in an Italian population sample: results of a regional community-based study. I. The MAPPING study*. Clin Exp Rheumatol. 2005 Nov-Dec; 23(6): 819-28.

Saraiva MJM. *Transthyretin mutations in familial amyloidotic polyneuropathy*. Hot Thyroidology (www.hotthyroidology.com), 2004 July , No 1.

Sarzi-Puttini P, Atzeni F, Salaffi F, Cazzola M, Benucci M, Mease PJ. *Multidisciplinary approach to fibromyalgia: what is the teaching?* Best Pract Res Clin Rheumatol. 2011; 25(2), 311-319.

Sawaya R, Zuccarello M, Highsmith R. *Alpha-1-antitrypsin in human brain tumors*. J Neurosurg. 1987; 67(2): 258-62.

Sawynok XJL. *Adenosine in the spinal cord and periphery: release and regulation of pain*. Prog Neurobiol 2003; 69: 313-40.

Schmidt-Wilcke T, Clauw DJ. *Fibromyalgia: from pathophysiology to therapy*. Nat Rev Rheumatol 2011 Sep; 7(9): 518-527.

Schreiber G, Aldred AR, Jaworowski A, Nilsson C, Achen MG, Segal MB. *Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus*. Am J Physiol. 1990 Feb; 258(2 Pt 2): R338-45.

Shao J, Xin Y, Li R, Fan Y. *Vitreous and serum levels of transthyretin (TTR) in high myopia patients are correlated with ocular pathologies*. Clin Biochem. 2011 Jun; 44(8-9): 681-5.

Shapiro L, Pott GB, Ralston AH. *Alpha-1-antitrypsin inhibits human immunodeficiency virus type 1*. FASEB J. 2001 Jan; 15(1): 115-122.

Shevchenko A, Wilm M, Vorm O, Mann M. *Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels*. Anal Chem. 1996 Mar 1; 68(5): 850-8.

Soprano DR, Soprano KJ, Goodman DS. *Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat.* J Lipid Res. 1986; 27: 166-171.

Sörensen J, Bengtsson A, Ahlner J, Henriksson KG, Ekselius L, Bengtsson M. *Fibromyalgia--are there different mechanisms in the processing of pain? A double blind crossover comparison of analgesic drugs*. J Rheumatol. 1997 Aug; 24(8): 1615-21.

Sörensen J, Graven-Nielsen T, Henriksson KG, Bengtsson M, Arendt-Nielsen L. *Hyperexcitability in fibromyalgia*. J Rheumatol. 1998 Jan; 25(1): 152-5.

Spicuzza L, Di Maria G, Polosa R. *Adenosine in the airways: implications and applications*. Eur J Pharmacol, 2006; 533(1-3): 77-88.

Sprott H. *What can rehabilitation interventions achieve in patients with primary fibromyalgia?* Curr Opin Rheumatol. 2003 Mar; 15(2): 145-50.

Staud R, Smitherman ML. *Peripheral and central sensitization in fibromyalgia: pathogenetic role.* Curr Pain Headache Rep. 2002 Aug; 6(4): 259-66.

Staud R, Cannon RC, Mauderli AP, Robinson ME, Price DD, Vierck CJ. *Temporal summation of pain from mechanical stimulation of muscle tissue in normal controls and subjects with fibromyalgia syndrome*. Pain. 2003 Mar; 102(1-2): 87-95.

Stisi S, Cazzola M, Buskila D, Spath M, Giamberardino MA, Sarzi-Puttini P, Arioli G, Alciati A, Leardini G, Gorla R, Marsico A, Ceccherelli F, Bazzichi L, Carignola R, Gracely RH, Salaffi F, Marinangeli F, Torta R, Di Franco M, Biasi G, Cassisi G, Casale R, Altomonte L, Atzeni F. *Italian Fibromyalgia Network. Etiopathogenetic mechanisms of fibromyalgia syndrome.* Reumatismo 2008 Jul-Sep; 60 Suppl 1: 25-35.

Stuifbergen AK, Phillips L, Carter P, Morrison J, Todd A. *Subjective and objective sleep difficulties in women with fibromyalgia syndrome.* J Am Acad Nurse Pract 2010; 22: 548-56.

Taggart C, Cervantes-Laurean D, Kim G, McElvaney NG, Wehr N, Moss J, Levine RL. *Oxidation of either methionine 351 or methionine 358 in alpha 1-antitrypsin causes loss of anti-neutrophil elastase activity.* J Biol Chem. 2000 Sep 1; 275(35): 27258-65.

Tanriverdi F, Karaca Z, Unluhizarci K, Kelestimur F. *The hypothalamo-pituitary-adrenal axis in chronic fatigue syndrome and fibromyalgia syndrome*. Stress 2007 Mar; 10(1): 13-25.

Terazaki H, Ando Y, Misumi S, Nakamura M, Ando E, Matsunaga N, Shoji S, Okuyama M, Ideta H, Nakagawa K, Ishizaki T, Ando M, Saraiva MJ. *A novel compound heterozygote (FAP ATTR Arg104His/ATTR Val30Met) with high serum transthyretin (TTR) and retinol binding protein (RBP) levels*. Biochem Biophys Res Commun. 1999 Oct 22; 264(2): 365-70.

Thadikkaran L, Siegenthaler MA, Crettaz D, Queloz PA, Schneider P, Tissot JD. *Recent advances in blood-related proteomics*. Proteomics 2005 Aug; 5(12): 3019-34.

Toda K. *Preliminary diagnostic criteria for fibromyalgia should be partially revised: comment on the article by Wolfe et al*. Arthritis Care Res (Hoboken) 2011 Feb; 63(2): 308-9.

Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M. *Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology*. Proteomics 2001 Mar; 1(3): 377-96.

Trams EG, Lauter CJ. *On the sidedness of plasma membrane enzymes*. Biochim Biophys Acta 1974; 345(2): 180-197.

Tsuzuki T, Mita S, Maeda S, Araki S, Shimada K. *Structure of the human prealbumin gene*. J Biol Chem. 1985 Oct 5; 260(22): 12224-7.

Űceyler N, Hauser W, Sommer C. *Systemic review with meta-analysis: cytokines in fibromyalgia syndrome*. BMC Musculoskeltal Disorders 2011; 12: 245.

Ueda M, Mashiba S, Uchida K. *Evaluation of oxidized alpha-1-antitrypsin in blood as an oxidative stress marker using anti-oxidative alpha1-AT monoclonal antibody.* Clin Chim Acta. 2002 Mar; 317(1-2): 125-31.

Unlu M, Morgan ME, Minden JS. *Difference gel electrophoresis: A single gel method for detecting changes in protein extracts.* Electrophoresis 1997; 18, 2071-2077.

Urunsak IF, Gulec UK, Paydas S, Seydaoglu G, Guze AB, Vardar MA. *Adenosine deaminase activity in patients with ovarian neoplasms*. Arch Gynecol Obstet 2012; 286(1): 155-9.

Vaerøy H, Helle R, Førre O, Kåss E, Terenius L. *Elevated CSF levels of substance P and high incidence of Raynaud phenomenon in patients with fibromalgia: new features for diagnosis*. Pain. 1988 Jan; 32(1): 21-6.

Van Cauter E, Plat L, Copinschi G. *Interrelations between sleep and the somatotropic axis*. Sleep. 1998 Sep 15; 21(6): 553-66.

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, et al. *The sequence of the human genome*. Science. 2001 Feb 16; 291(5507): 1304-51.

Verrills NM. *Clinical proteomics: present and future prospects*. Clin Biochem Rev. 2006 May; 27(2): 99-116.

Wallace DJ, Margolin K, Waller P. *Fibromyalgia and interleukin-2 therapy for malignancy*. Ann Intern Med. 1988 Jun; 108(6): 909.

Wallace DJ, Linker-Israeli M, Hallegua D, Silverman S, Silver D, and Weisman MH. *Cytokines play an aetiopathogenetic role in fibromyalgia: a hypothesis and pilot study*. Rheumatology (Oxford) 2001; 40: 743-749.

Waring WS. *Uric acid: an important antioxidant in acute ischaemic stroke.* QJM. 2002 Oct; 95(10): 691-3.

Watkins LR, Milligan ED, Maier SF. *Glial activation: a driving force for pathological pain*. Trends Neurosci. 2001 Aug; 24(8): 450-5.

Watkins LR, Maier SF. Immune regulation of central nervous system functions: from sickness responses to pathological pain. J Intern Med. 2005 Feb; 257(2): 139-55.

Weissbecker I, Floyd A, Dedert E, Salmon P, Sephton S. *Childhood trauma and diurnal cortisol disruption in fibromyalgia syndrome*. Psychoneuroendocrinology. 2006 Apr; 31(3): 312-24.

White KP, Speechley M, Harth M, Ostbye T. *Comparing self-reported function and work disability in 100 community cases of fibromyalgia syndrome versus controls in London, Ontario: the London Fibromyalgia Epidemiology Study*. Arthritis Rheum. 1999; 42(1): 76-83.

Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, Tugwell P, Campbell SM, Abeles M, Clark P, et al. *The American College of Rheumatology 1990. Criteria for the classification of fibromyalgia. Report of the Multicenter Criteria Committee*. Arthritis Rheum. 1990 Feb; 33(2): 160-72.

Wolfe F, Ross K, Anderson J, Russell IJ, Hebert L. *The prevalence and characteristics of fibromyalgia in the general population*. Arthritis Rheum 1995 Jan; 38(1): 19-28.

Wolfe F, Michaud K. *Severe rheumatoid arthritis (RA), worse outcomes, comorbid illness, and socio demographic disadvantage characterize RA patients with fibromyalgia*. J Rheumatol. 2004 Apr; 31(4): 695-700.

Wolfe F. *The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptoms severity*. Arthritis Care Res 2010 May; 62(5): 600-610.

Wolfe F, Clauw DJ, Fitzcharles MA, Goldenberg DL, Häuser W, Katz RS, Mease P, Russell AS, Russell IJ, Winfield JB. *Fibromyalgia criteria and severity scales for clinical and epidemiological studies: a modification of the ACR Preliminary Diagnostic Criteria for Fibromyalgia*. J Rheumatol. 2011 Jun; 38(6): 1113-22.

Woolf AD. *Te bone and joint decade 2000-2010*. Ann Rheum Dis. 2000 Feb; 59(2): 81-82.

Yates JR 3rd. *Mass spectrometry and the age of the proteome*. J Mass Spectrom. 1998 Jan; 33(1): 1-19.

Yegutkin GG, Samburski SS, Jalkanen S. *Soluble purine-converting enzymes circulate in human blood and regulate extracellular ATP level via counteracting pyrophosphatase and phosphotransfer reactions*. FASEB J 2003; 17: 1328-30.

Yunus MB. *Towards a model of pathophysiology of fibromyalgia: aberrant central pain mechanisms with peripheral modulation*. J Rheumatol. 1992 Jun; 19(6): 846-50.

Yunus MB. Arslan S, Aldag JC. *Relationship between body mass index and fibromyalgia features*. Scand J Rheumatol. 2002; 31(1): 27-31.

Zimmermann-Ivol CG, Burkhard PR, Le Floch-Rohr J, Allard L, Hochstrasser DF, Sanchez JC. *Fatty acid binding protein as a serum marker for the early diagnosis of stroke: a pilot study.* Mol Cell Proteomics. 2004 Jan; 3(1): 66-72.

Zingg JM, Azzi A. *Non-antioxidant activities of vitamin E.* Curr Med Chem. 2004 May; 11(9): 1113-33.

Zöllner N, Reiter S, Gross M, Pongratz D, Reimers CD, Gerbitz K, Paetzke I, Deufel T, Hübner G. *Myoadenylate deaminase deficiency: successful symptomatic therapy by high dose oral administration of ribose*. Klin Wochenschr. 1986 Dec 15; 64(24): 1281-90.

INTERNET RESOURCES

http://www.expasy.org/

http://www.nonlinear.com/

http://www3.gehealthcare.co.uk/

http://www.myalgia.com/

http://pfam.sanger.ac.uk

http://www.ebi.ac.uk/interpro/

http://www.mendeley.com/

https://nilde.bo.cnr.it/

http://www.genomesonline.org/cgi-bin/GOLD/index.cgi

APPENDIX: Italian FIQ

FIBROMYALGIA IMPACT QUESTIONNARIE

FIQ

ISTRUZIONI: nelle domande dal numero 1 al numero 11 del questionario che segue, verranno poste delle domande riguardo alle attività che si è stati in grado di svolgere nell'ultima settimana.

Rispondere a ciascuna domanda, mettendo una crocetta nella casella corrispondente (solo una risposta per ciascuna domanda).

Se normalmente non si svolge l'attività cui la domanda si riferisce, barrare l a casella 4

NEL CORSO DELL'ULTIMA SETTIMANA E' STATO IN GRADO DI	**SEMPRE 0**	**QUASI SEMPRE 1**	**QUALCHE VOLTA 2**	**MAI 3**	**ATTIVITA' NON SVOLTA ABITUALMENTE**
1) ANDARE A FARE LA SPESA	0	1	2	3	4
2) FARE IL BUCATO (LAVATRICE)	0	1	2	3	4
3) PREPARARE I PASTI	0	1	2	3	4
4) LAVARE I PIATTI	0	1	2	3	4
5) PASSARE L'ASPIRAPOLVERE	0	1	2	3	4
6) RIFARE I LETTI	0	1	2	3	4
7) CAMMINARE PER QUALCHE ISOLATO	0	1	2	3	4
8) ANDARE A FAR VISITA A PARENTI O AMICI	0	1	2	3	4
9) FARE LAVORI DI GIARDINAGGIO – ORTO	0	1	2	3	4
10) GUIDARE L'AUTO	0	1	2	3	4
11) SALIRE LE SCALE	0	1	2	3	4

12) QUANTI GIORNI SU 7 DELL'ULTIMA SETTIMANA SI E' STENTITA BENE?

0	1	2	3	4	5	6	7

13) QUANTI GIORNI SU 7 DELL'ULTIMA SETTIMANA NON E' ANDATA A LAVORO O NON HA POTUTO FARE LAVORI DOMESTICI A CAUSA DELLA FIBROMIALGIA?

0	1	2	3	4	5	6	7

Risponda alle seguenti domande apponendo un segno sulla riga sottostante

(estrema sinistra = nessun problema estrema destra = massima difficoltà)

14) SUL POSTO DI LAVORO O A CASA DURANTE I LAVORI DOMESTICI, QUANTA DIFFICOLTA' HA AVVERTITO A CAUSA DEL DOLORE O DEGLI ALTRI SINTOMI DELLA FIBROMIALGIA?

_____

15) QUANTO E' STATO FORTE IL SUO DOLORE?

_____

16) QUANTO SI E' SENTITA STANCA?

_____

17) COME SI E' SENTITA AL RISVEGLIO?

_____

18) QUANTO SI E' SENTITA RIGIDA?

_____

19) QUANTO SI E' SENTITA ANSIOSA O NERVOSA?

_____

20) QUANTO SI E' SENTITA DEPRESSA O TRISTE?

_____