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Fibromyalgia: a proteomic approach to study a double-face disease.

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INDEX

ABSTRACT

Fibromyalgia is a chronic non inflammatory musculoskeletal disorder characterized by a variety of symptoms related to pain. The first criterion for the diagnosis requires patient to report at least 3 months of widespread pain. The second is widespread pain in response to a tender point examination. Common unspecific symptoms include fatigue, nonrestorative sleep, morning stiffness, mood disorders, anxiety, depression, cognitive dysfunction (e.g., memory problems, concentration difficulties, diminished mental clarity), irritable bowel and bladder syndrome, sexual dysfunction and sicca symptoms. In the last few years many attempts have been carried out for the research of specific biomarkers in fibromyalgia, but, at present, there are no specific markers and the diagnosis is basically clinical.

In the present work we used two complementary proteomic approaches to obtain the whole saliva protein map of fibromyalgia patients: two-dimensional electrophoresis in combination with mass spectrometry and Surface enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF-MS). The aim of this study was the evaluation of the global changes of protein profiles which occur in the disease and the research for any eventual diagnostic or prognostic salivary biomarkers, which could be used routinely, in the future, for the management of fibromyalgia patients. 22 fibromyalgia patients and 26 healthy subjects were enrolled in the analysis with two-dimensional electrophoresis;

while for SELDI-TOF-MS technique saliva samples were collected from 63 patients and 63 controls.

With two-dimensional electrophoresis we found the significant over-expression of transaldolase and phosphoglycerate mutase I. These findings were validated by Western blot analysis and the total optical density confirmed their significant up-regulation in fibromyalgia samples with respect to healthy subjects. It was noteworthy that seven further salivary proteins resulted differentially expressed: calgranulin A, calgranulin C, cyclophilin A, profilin 1, Rho GDP-dissociation inhibitor 2, proteasome subunit-a-type-2 and haptoglobin-related protein precursor. With SELDI-TOF-MS technique we highlighted also the presence of a pattern of proteins potentially useful to discriminate fibromyalgia patients from healthy subjects.

These results demonstrated the utility of proteomic analysis in the identification of salivary biomarkers in fibromyalgia patients.

INTRODUCTION

FIBROMYALGIA

Fibromyalgia syndrome (FM) is a chronic non inflammatory musculoskeletal disorder which can occur as a primary disease or in association with other autoimmune diseases (i.e. Rheumatoid arthritis, Systemic sclerosis, Sjögren's syndrome, autoimmune thyroiditis, tetany and chronic fatigue syndrome). In 1990, The American College of Rheumatology laid out several sets of criteria for the diagnosis of FM. [1]. The first criterion required patients to report at least 3 months of widespread pain. Pain was considered widespread if it was present in four quadrants of the body, the right and left side as well as above and below the waist. Axial skeleton pain is also very commonly present in FM and is often considered a fifth "quadrant." The second diagnostic criterion was widespread pain in response to a tender point examination. In this assessment the clinician presses on 18 specific areas, the patient's report of pain in at least 11 of these tender points completes the requirements for the diagnosis of FM.

Common unspecific symptoms associated with FM include fatigue, nonrestorative sleep, morning stiffness, mood disorders, anxiety, depression, cognitive dysfunction (e.g., memory problems, concentration difficulties, diminished mental clarity), irritable bowel and bladder syndrome, sexual dysfunction and sicca symptoms. [2, 3, 4, 5, 6]. FM has a different prevalence depending on the

population studies and criteria used, ranging from 0.5% and 5.0% [7], with a female to male ratio of approximately 9:1 [4].

Symptoms usually appear between the ages of 20 and 55 although juvenile FM in patients as young as 10 years old or even less has been reported and is probably under-recognised [8]. The disorder may be dormant for years until triggered by infection, injury, physical or emotional stress or sleep disturbance [9]. Although the key alerting symptom is chronic widespread pain or tenderness, FM is not just a pain disorder. The Fibromyalgia Impact Questionnaire (FIQ; table 1) gives a good idea of the full range of symptoms that are regularly found. The patients fill out a questionnaire consisting of 10 items, from which derives a score that indicates the impact of the disease on life (FIQ total score), the total score reflects the impact of FM and ranged from 0 (no impact) to 100 (maximum impact). Although there is no official consensus of what constitutes a clinically significant score on this scale most patients diagnosed with FM have an FIQ total score of at least 50 (out of a maximum of 100-see table 1). Severely afflicted patients frequently score 70 or more [10].

Table 1. Fibromyalgia Impact Questionnaire (FIQ).

Question 1 Physical functioning
During the past week were you able to:
- Do shopping?
- Do laundry with a washer and dryer?
- Prepare meals?
- Wash dishes/cooking utensils by hand?
- Vacuum a rug?
- Make beds?
- Walk several blocks?
- Visit friends or relatives?
- Do yard work or gardening?
- Drive a car?
- Climb stairs?

Question 2
In the past week, how many days did you feel good?
(1–7)

Question 3
How many days last week did you miss work, including housework, because of fibromyalgia ?
(1–7)

Question 4
When you worked, how much did pain or other symptoms of your fibromyalgia interfere with your ability to do your work, including housework?
(No problem with work < > Great difficulty with work)

Question 5
How bad has your pain been?
(No pain < > Very severe pain)

Question 6
How tired have you been?
(No tiredness < > Very tired)

Question 7
How have you felt when you get up in the morning?
(Awoke well rested < > Awoke very tired)

Question 8
How bad has your stiffness been?
(No stiffness < > Very stiff)

Question 9
How nervous or anxious have you felt?
(Not anxious < > Very anxious)

Question 10
How depressed or blue have you felt?

Table 1. The items in question 1 are scored 0, 1, 2 or 3 for always, most of the time, occasionally or never. Because some patients may not do some of the tasks listed, they are given the option of deleting items from scoring. In order to obtain a comparable score for all patients, the mean of the scores for the rated items is used. The average score is thus 0–3. This score is multiplied by 3.33 to obtain an adjusted score (maximum 10). Question 2 is scored inversely of the number of days (0 ¼ 7, 1 ¼ 6, 2 ¼ 5, 3 ¼ 4, 4 ¼ 3, 5 ¼ 2, 6 ¼ 1 and 7 ¼ 0). It is multiplied by 1.43 to obtain an adjusted score (maximum 10). Question 3 is directly the number of days. It is multiplied by 1.43 to obtain an adjusted score (maximum 10). Questions 4–10 are visual analogue scales scored on a 100 mm line with the limits given in parentheses. The score (0–10) is the distance (in centimetre from the left hand end). These values are not adjusted. Scoring: The FIQ is scored so that a higher score indicates a greater impact of the syndrome. Each of the 10 items has a maximum possible score of 10. The maximum possible score is thus 100.

Autoantibodies.

Although FM is generally regarded as a non-inflammatory and non-autoimmune disease, there is a broad spectrum of organ and non organ specific autoantibodies which have been widely detected in the patients sera [11].

Two autoantibodies, the anti-68/48 kD and the anti-45 kD, have been reported as possible markers for certain clinical subsets of primary FM and chronic fatigue syndrome and of secondary FM/psychiatric disorders, respectively [12]. In particular, the anti-68/48 kD antibodies were considered to be closely associated with FM /chronic fatigue syndrome patients presenting with hypersomnia and/or cognitive disorders. Pamuk and Cakir [13] reported that thyroid autoimmunity in FM patients was similar in frequency to that in rheumatoid arthritis (RA) patients but higher in frequency when compared to the control group. Although the frequencies of TPO antibodies (antithyroid peroxidase) in both FM and RA patients were significantly higher than in the controls, the increase in the frequency of thyroglobulin antibodies (antithyroglobulin) was not significant. Thyroid autoimmunity, especially the presence of TPO antibodies, was found to be associated with the presence of migraine and tension headaches [13].

Compared with healthy subjects or with patients affected by other diseases, FM patients present anti-polymer antibodies (APA) with contrasting results in literature. Wison et al. found a higher prevalence of APA (67%) in FM patients in the USA population [14], while Bazzichi and collaborators found a lower percentage of APA seropositivity (23%) [15].

In 1986 Dinerman and team [16] found that 14% of FM patients had a positive antinuclear antibody (ANA) test, 30% had a history of Raynaud's phenomenon, and 18% had symptoms of Sjögren's syndrome, but none of them progressed to a classic connective tissue disease [11]. Al-Allaf and co-authors [17] suggested that patients with FM have the same rate of positive ANA as do osteoarthritis patients (8.8% and 8.9%, respectively). Results from their study did not show that ANA are a good predictor of the future development of connective tissue disease in FM patients and the majority of ANA-positive patients became ANA negative on follow-up [11]. Recently it was reported that there was no significant difference in the frequency of ANA or thyroid antibodies between FM patients and controls, and that the risk of connective tissue diseases is not increased in FM [11, 18].

Other antibodies (anti-serotonin, antiganglioside and anti-phospholipids) were identified in FM patients compared to healthy subjects [19], but according to Werle and collaborators the prevalence of autoantibodies against serotonin and thromboplastin that they found in patients with FM has no diagnostic relevance [20].

So far, nonetheless, no laboratory tests and none of the above mentioned autoantibodies have yet been appropriately validated for the disease.

FM and inflammation.

Based on the hypothesis that the origin of all pain is inflammation and the inflammatory response [21] special attention has been focused on the

inflammatory hypothesis of FM. Since 1988 it has been known that increased levels of the inflammatory transmitter substance P (SP) are found in the spinal fluid of FM patients [22].

In recent years, inflammatory cytokines have also been suggested to be involved in the FM syndrome. This hypothesis was based on the assumption that IL-6 and IL-8, release of which is stimulated by SP, may have an important role in FM symptoms, since IL-8 promotes sympathetic pain and IL-6 is associated with hypersensitivity to pain, fatigue and depression. [23, 11]. Taken together, the profile of pro- and anti- inflammatory cytokines in FM patients has recently attracted considerable attention. Several studies have focused on circulating pro-inflammatory cytokines as possible "inflammatory markers" in FM patients. Higher levels of IL-8 and tumor necrosis factor-alpha (TNFα) were found in FM patients than in controls [24, 25]. On the other hand, results for IL-10, an anti-inflammatory cytokine which is an antagonist of TNFα and IL-1β, appear to be controversial, since both increases [24] or no significant changes [25, 26] have been reported in serum IL-10 concentration in FM patients compared with healthy controls.

The sources of inflammation triggering the FM syndrome remain to be elucidated. It has been proposed that FM is due to neurogenic inflammatory response to allergens, infectious agents, chemicals or emotional stress [21]. Bearing in mind that mechanical abnormalities of the cervical or lumbar spine are included among the possible aetiology of FM, an open question may be that neck

or lumbar disorders can be a source of inflammation, and thus may be one cause of FM rather than a consequence [23].

Frequently, FM and depression present symptomatic similarities. If FM and depression coexist, the question is whether depression must be regarded as an associated affection or whether it is the actual cause of the chronic pain disorders [27]. Several studies indicate that major depression is accompanied by the activation of inflammatory response, with an increased production of pro-inflammatory cytokines [23, 28]. In fact, many FM patients are treated with antidepressant agents, which may suppress the production of pro-inflammatory cytokines, such as IFNγ, and stimulate the production of anti-inflammatory cytokine IL-10 [29]. However, it is known that FM and depression do not always coexist [27]; even different profiles of pro- inflammatory and anti-inflammatory cytokines between patients with or without diagnosis of depression have been reported. For example, Müller and co-workers [27] reported higher circulating levels of pro-inflammatory (IL-1α and TNFα) and anti-inflammarory (IL-10) cytokines in FM patients without signs of depression. However, Bazzichi and co-workers [24] found increased levels of IL-10 and IL-1 in patients with depression, but they did not find differences in the circulating level of IL-8 between FM patients with or without depression, both of them strongly higher compared with the levels found in control healthy people. This finding strongly suggested that IL-8 may be an "inflammatory marker" for FM syndrome regardless of associated depression [23].

FM and Genetic Factors.

Genetic factors may predispose individuals to FM. FM in families clusters suggests a genetic predisposition. Environmental and psychological factors, which could impact various members of the same family, may contribute to the symptoms of the disease [30, 31, 32].

Significant data suggest that FM is genetically related to a wide range of conditions subsumed under the rubric of "affective spectrum disorders" (ASD), including major depression disorder (MDD) and anxiety disorders, premenstrual dysphoric disorder, attention deficit hyperactivity disorder (ADHD), irritable bowel syndrome (IBS) as well as migraine and cataplexy conditions [33].

For example, Hudson et al. [34] concluded that patients with FM were twice as likely to have at least one of these other conditions, compared with individuals without FM.

A recent study has refined our understanding of genetic links between mood and pain/somatic disorders by suggesting intriguing patterns of genetic overlap and environmental specificity for these conditions [33, 35]. Briefly, in a large twin study on the relationship between two psychiatric disorders (MDD and generalized anxiety disorder [GAD]) and somatic syndromes (FM, chronic fatigue, IBS and recurrent headache), multivariate analyses suggested the influence of two factors: one, most likely genetic, shared between somatic disorders, MDD and GAD and a second one, more specific to somatic conditions, that was more environmentally based [35].

Genes implicated in mood disorders have been identified as risk factors for FM and related pain states. These genes include the serotonin transporter (5HTT), the serotonin 5HT2A receptor, catechol-O-methyltransferase (COMT) and the dopamine D4 receptor. [33].

Although not found by all studies [36], the association between FM and the "s" allele of serotonin transporter promoter locus (5HTTLPR) is particularly interesting given its association with a wide range of conditions that are risk factors for FM, including anxiety, neuroticism (tendency towards excessive emotional reactivity to stressful stimuli), MDD, Bipolar Disorder, Psychosis and even ADHD [33].

Results from several investigations indicate that a single nucleotide polymorphism (SNP) in the serotonin transporter (5-HTT) gene may contribute to enhanced pain sensitivity in patients with FM. Offenbaecher and colleagues [37] were the first to report that the short (S) allele of this SNP (i.e., the S/S genotype) in the regulatory region of the 5-HTT gene occurs significantly more frequently in patients with FM than in healthy controls. Cohen and colleagues [38] subsequently replicated this observation in an independent sample [39]. There also evidence that the presence of a SNP in the 5HTT gene may moderate the association between exposure to stressful life events and depression [39]. Caspi and colleagues studied young adults in a large, prospective, longitudinal study and found that, 1 or 2 copies of the short allele of the polymorphism reported more depressive symptoms and more frequently met criteria for MDD in

relation to stressful life events from age 21 to age 26 than those who were homozygous for the long allele (*L/L*) genotype [40].

Another area of investigation is the 5HT2A receptor gene; the analysis of genetic polymorphisms showed that the serotonin 5-HT2A receptor polymorphism T/T phenotype occurred more often in FM patients than in normal controls [41].

Interestingly, a separate study [42] noted that the same T/T allele, in the presence of high maternal nurturance, was associated with lower depressive symptoms than the C/C genotype, consistent with the notion that environmental factors may play a role in triggering the development of mood disorders.

COMT is also a candidate for involvement in FM. The COMT gene encodes an enzyme that metabolizes catecholamines (i.e., norepinephrine and dopamine) and thereby influences several cognitive-affective phenotypes, including pain phenotypes. COMT also has been implicated in the pathogenesis of migraine and anxiety disorders, as well as a variety of cardiovascular diseases [39]. Initial studies focused on the *val*^{158}*met* polymorphism, a SNP in codon 158 of the COMT gene that substitutes valine for methionine and results in reduced activity of the enzyme. Individuals homozygous for the *met*^{158} allele of this polymorphism showed a diminished mu-opioid receptor response to pain, and a stronger subjective experience of pain when compared to heterozygous subjects. Opposite effects in pain and negative affect have been found in *val*^{158} homozygotes [43]. Other studies have found that this COMT polymorphism may play a role in the

stress response, the trait of novelty seeking, cognition, MDD, schizophrenia, anxiety disorders and ADHD [33].

Finally, some reports have established a connection between FM and dopamine D4 receptor. For example, Buskila and colleages [44] have demonstrated an association between FM and the *DRD4* exon III 7 repeat genotype, respect to healthy controls; the frequency of the 7-repeat genotype was significantly lower in persons with FM. Treister and collaborators [45] found an association between the dopamine transporter gene (DAT-1) polymorphism and cold pain tolerance. The results underlined the association between the DAT-1 polymorphism and a decrease in pain threshold.

In conclusion, genetic studies of FM have noted alterations in genes regulating (likely in a convergent manner with depression and pain) monoamine and inflammatory signalling. It is tempting to speculate that shared genetic vulnerabilities towards depression and pain states may be reflected in deregulation of circuitry involved in modulating stress responses, pain and emotional states [33]. However, no single candidate gene has been strongly associated with FM. It is very likely that FM, depression and chronic pain have complex genetic factors, some of which may be shared [46].

FM and Environmental Triggers.

Environmental factors may play a role in triggering the development of FM and a number of "stressors," such as physical trauma (especially involving the trunk), certain infections (e.g., hepatitis C virus, Epstein-Barr virus, HIV, and Lyme

disease), emotional stress, catastrophic events, autoimmune disease, and other pain conditions have been correlated with the onset of the syndrome, [47, 48]. Of note, each of these stressors leads to chronic widespread pain or FM in approximately 5% to 10% of affected individuals. In other words, these stressors do not act as triggers in the overwhelming majority of individuals who regain their baseline state of health after experiencing infections or traumatic events [49]. Commonly reported psychosocial triggers include chronic stress, emotional trauma, and emotional, physical, or sexual abuse [4]. The effects of psychosocial stressors may be especially pervasive because, in addition to being associated with the onset of chronic widespread pain, they may also contribute to enhanced pain responses via involvement of the neuroendocrine system [39].

Owing to the fact that different "stressors" can trigger the development of this condition, the human stress response has been closely examined for a causative role. This system is primarily mediated by the activity of corticotropin releasing hormone (CRH) and norepinephrine [50].

The role of the peripheral and central nervous system in FM.

Pain pathways implicated in FM have peripheral and central components. Pain signals are detected by peripheral nociceptive nerve endings and conveyed to neurons located in dorsal root ganglia (DRG). From the DRG pain information is conducted by lightly myelinated A-delta and un-myelinated slow C-fibers to secondary sensory neurons localized in the dorsal column of the spinal cord [33].

Aside from functional alterations in nerve membranes and endocellular signaling, peripheral sensitization may occur as a result of alterations in synaptic connectivity resulting from sprouting of sympathetic axons within DRG (which may further augment pain transmission), ectopic discharges and ephaptic (direct electrical transfer of signal) communication. Central sensitization in neuropathic pain (NeP) may in part be mediated by collateral sprouting as well as damage to inhibitory GABA inter-neurons [33, 51]. On the other hand, there are fewer data to support an important role for abnormalities in peripheral or spinal cord pain signaling in FM. Nonetheless, some evidence does indicate potential peripheral contributions to the disorder [33]. For example, Salemi et al. have performed skin biopsies in 53 FM patients and found mononuclear and fibroblast-like cells adjacent to nociceptive neuronal fibres that stained positive for inflammatory cytokines, suggesting a role for neurogenic inflammation in the etiology of the FM [52].

After synaptic processing in the dorsal horn of the spinal cord, pain signals are propagated via spinothalamic (paleo-spinothalamic) and spinoparabrachial (neo-spinothalamic) tracts to higher central nervous system (CNS) pain centres. Spinothalamic signals are relayed through thalamus to somatosensory cortices I and II (SI and SII) and associated areas, including insula, anterior cingulate cortex (ACC) and posterior cingulate cortex (PCC). ACC, in turn, has close bidirectional connections with amygdala and hippocampus [33]. Spinoparabrachial fibers convey information to the parabrachial nucleus in the brainstem and then on to

amygdala, hippocampus and hypothalamus. Ascending pain signals and information from supraspinal pain circuitry are integrated in the mesencephalic periaqueductal gray area (PAG), which also has a pivotal role in regulating descending pain pathways [33]. Dorsolateral prefrontal cortex (DLPFC) and lateral-orbital prefrontal cortex (LOPFC) appear to initiate the descending pain modulatory sequence, explaining how attention and anticipation may influence the intensity of pain [33]. These prefrontal areas, richly innervated by dopamine fibers, can trigger opioid release in PAG, substantially reducing the intensity of experienced pain [33].

Imaging studies have consistently identified several brain areas as having a major role in pain processing, including primary and secondary somatosensory cortices (S1 and S2), thalamus, insula, ACC and PFC [33]. Together these brain areas are commonly referred to as the "pain matrix" [53] and many studies indicate that function is disrupted in this matrix in the context of chronic pain states, including FM.

For example, Bailiki et al. utilized functional magnetic resonance imaging (fMRI) to study chronic back pain patients. Functional neural imaging enables investigators to visualize how the brain processes the sensory experience of pain. These authors reported an association between the intensity of spontaneous pain and activation of medial prefrontal cortex (mPFC) [54], an area known to have a role in automatic emotional regulation [55]. On the other hand, in this

population, duration of pain was most strongly associated with increased activity in the insula [54].

A second fMRI study noted a greater activation in DLPFC and ACC in response to nonpainful stimuli in patients with FM relative to control subjects, a finding likely to reflect alterations in processes central to the cognitive and emotional aspects of pain, such as attention and anticipation [56]. In response to an equivalent pressure stimulus, patients with FM have been shown to demonstrate increased activity in several areas of the CNS pain matrix when compared to normal control subjects, including S2, insula, posterior cingulate cortex (PCC), ACC, superior temporal gyrus and inferior parietal lobule [57]. Moreover, mild pressure applied to subjects with FM elicited subjective pain and cerebral responses similar to the responses seen in normal subjects when twice as much pressure was applied [57]. fMRI studies like these provide objective evidence of altered cerebral processing of painful stimuli in FM patients.

In addition to functional differences, several studies have found significant structural changes in the brains of FM patients. Kuchinad and co-workers reported significantly reduced gray matter density in the cingulate cortex, insula, mPFC and the para-hippocampal lobe of FM patients when compared to a control group [58]. As with depression, the physiological changes that accompany FM may themselves damage brain structures over time, given that in this study duration of illness correlated with greater gray matter changes, such that each year of disease had an impact equivalent to 9.5 times the loss due to normal aging

[33]. Changes in these areas appear to contribute to the compromised pain regulation, emotional modulation, stress responsivity and cognitive functioning, often described in FM patients [58]. For example, Luerding et al. reported that neurocognitive deficits in FM patients correlated with reduced gray matter volume in DLPFC and ACC (areas typically associated with executive function), additionally pain scores were noted to be negatively correlated with gray matter volume in mPFC [33, 59].

Diagnosis and Therapy.

Currently, there is no therapy formally approved by the European Agency for the Evaluation of Medicinal Products or by the US Food and Drugs Administration for treatment of the pain of FM or the syndrome as a whole [23]. Treatment is largely empiric, although experience and small clinical studies have proved the efficacy of low-dose antidepressant therapy and exercise [60]. Nowadays, treatment of the FM syndrome includes both pharmacologic and non-pharmacologic therapies. Pharmacologic therapies are based on antidepressants, analgesics, muscle relaxants and antiepileptics. Non-pharmacologic therapies include exercise, massage, cognitive behavioural therapy etc [61]. Today non-pharmacological therapies, such as exercise, are recommended in the management of FM symptoms together with pharmacological treatment. In fact, it has been shown that aerobic exercise training improves physical function, psychological distress and other quality of life parameters in FM patients.

However, non-pharmacological therapies cannot replace pharmacological ones yet.

The difficulty with making a formal diagnosis of FM is that laboratory tests are normal and many of the symptoms mimic those of other conditions including many rheumatic complaints, psychiatric conditions and other somatic disorders. It is therefore important to exclude rheumatic disorders before proceeding to a diagnosis of FM. It has been estimated that it takes an average of 5 years from the time the patient's first reports symptoms to the time when FM is formally diagnosed [10, 62].

In spite of the alterations found in the different studies and although some criteria were established to standardize patients for research studies, the diagnosis of FM is basically clinical [63] and the lack of easily accessible laboratory measures makes difficult to collect under the term of FM, patients presenting with homogeneous features and prognosis [64, 65]. At present, there are no specific markers of FM, and many of them are used only to understand the pathogenetic mechanisms and to identify patient subgroups.

Therefore it is desirable to identify precise biomarkers of FM according to feasibility and reproducibility criteria, for diagnostic and therapeutic purposes [63].

PROTEOMICS

In the last few years, it has become widely recognized that the genome only represents the first layer of complexity. Biological function is carried by the dynamic population of proteins, moreover, only the characterization of the proteins themselves can reveal posttranslational modifications (e.g., phosphorylation, sulfation, glycosylation, ubiquitination, and methylation) and give insight into protein-protein interactions and subcellular localization, thus providing clues about function. For these reasons, there is increasing interest in the field of proteomics: the large-scale identification of proteins contained in cells, tissues or body fluids [66]. The proteome was originally defined as the complete protein complement expressed by a genome [67]. However, this definition does not take into account that the proteome is a highly dynamic entity that will change based on cellular state and the extracellular milieu. Therefore, the definition of a proteome should specify that it is the protein complement of a given cell at a specified time, including the set of all protein isoforms and protein modifications [68].

Proteomic analyses can be used to identify the protein content in complex biological samples such as biological fluids and tissue extracts, and to determine the quantitative or qualitative differences for each polypeptide contained in different samples. It is expected that the proteomic profiling patterns resulting from such analyses define comprehensive molecular signatures in health and disease [69]. The exploitation of a proteomic approach for the study of different

diseases has led to the hypothesis that multiple biomarkers or a panel of biomarkers shown by proteomic profiling may correlate more reliably with a specific disease than a single biomarker or protein. Expression pattern of a known biomarker or correlation of expression of several known biomarkers can be a valuable research and clinical tool for monitoring disease or treatment progression [70].

For our study we used two complementary proteomic technique: two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and surface enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF/MS).

Two-dimensional electrophoresis.

The identification of proteins from complex biological sample has traditionally been performed using 2-D PAGE coupled with mass spectrometry (MS). Two-dimensional electrophoresis (2-DE) separates proteins by both their isoelectric point (pI) and molecular weight. With this technique proteins are resolved into discrete spots, each of which represents a single protein that can be selectively excised and identified by MS. The high resolution of 2-DE allows the researcher to pick only the proteins of interest while bypassing the more abundant or less interesting proteins [71].

Sample preparation.

Preparation of samples for 2-D PAGE involves solubilization, denaturation and reduction to completely break up the interactions between the proteins [72]. Although desirable, there is no single method of sample preparation that can be universally applied due to the diverse samples which are analyzed by 2-DE gel electrophoresis [73]. The ideal sample solubilization procedure for 2-D PAGE would result in the disruption of all non-covalently bound protein complexes and aggregates into a solution of individual polypeptides [74]. However, whatever method of sample preparation is chosen, it is most important to minimize protein modifications which might result in arte-factual spots on the 2-DE maps [73]. Samples containing urea must not be heated as this may introduce considerable charge heterogeneity due to carbamylation of the proteins by isocyanate formed from the decomposition of urea. Generally speaking, samples should be subjected to as minimum handling as possible and kept cold at all times [75].

Protein extracts should not be too diluted to avoid loss of protein due to adsorption to the wall of the vessel (glass or plastic). If samples are rather diluted and contain relatively high concentrations of salts which can interfere with IEF, samples may be desalted [73].

Alternatively, proteins can be precipitated with ice-cold TCA / acetone to remove salts. Diluted samples with a low salt concentration may also be applied directly without further treatment, if the dried IPG strips are reswollen in sample

solution. In this case, solid urea, CHAPS and dithiothreitol (DTT) are added to the sample until the desired concentration is obtained [73, 76].

First dimension.

Iso-electro focusing (IEF) represents the first dimension of 2-DE and it is performed in individual immobilized pH gradients (IPG) strips. Each sample protein applied to an IPG strip will migrate to its isoelectric point (pI), the point at which its net charge is zero. There are strips with broad or narrow pH gradient (e. g., 3-10; 4-7; 4-9; 6-10; 5-6; 9-12; 10-12).

Dried gel strips containing immobilized pH gradient were commercially introduced in 1991 (Pharmacia Biotech, Immobiline® DryStrip Gel), their adoption for the first dimension of 2-DE has produced significant improvement over the classical O' Farrell carrier ampholyte-based 2-DE separation [77]. In the original 2-DE the required pH gradient is established by the migration of individual species of carrier ampholytes to their respective pI. Variations of the complex carrier ampholyte mixtures result in variations in the shape of the pH gradient [77]. The use of commercially prepared IPG DryStrip, introduced by Bjellqvist et al. [78]. and Gorg et al. [79] eliminates these variations.

The pH gradient is immobilized by covalent incorporating Immobiline® acrylamido buffers into the acrylamide matrix during polymerization. Since Immobiline consists of discrete, relatively simple molecules, they can be manufactured very reproducibly pure, eliminating batch effect as demonstrated

by interlaboratory comparison [77, 80]. Further, pI resolution to 0.01 pH unit can be achieved [78]. The acrylamide matrix with the Immobiline, acrylamido buffers is cast onto a backing sheet, polymerized, washed and dried. The backing gives the strips size stability and simplify handling. The dried strips can be rehydrated in various buffers and additives that would inhibit polymerization if included at the time of casting [77].

Second dimension.

Prior to the second dimension (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis-SDS-PAGE), the IPG strips are equilibrated twice with gentle shaking in a solution containing urea and glycerol in order to diminish electroendosmotic effects [79] which are held responsible for reduced protein transfer from the first to the second dimension. DTT, a reducing agent, is added to the first and iodoacetamide (IAA) to the second equilibration step [81]. IAA is added to the equilibration buffer in order to remove excess DTT (responsible for the "point streaking" in silver stained patterns) [82]. After equilibration the strips are applied to vertical SDS gels in order to perform electrophoresis and to separate proteins according to their molecular weight. Polypeptides separated can be visualized by Coomassie Blue, silver staining, fluorescence or autoradiography, or by "specific" stains such as glycoprotein staining or immunochemical detection methods [73]. Whereas the "general" protein stains are carried out in the electrophoresis gel directly, immunochemical detection methods are usually

performed after electrophoretic transfer ("blotting") of the separated polypeptides from the electrophoresis gel onto an immobilizing membrane [83, 84]. Silver staining methods are about 10-100 times more sensitive than various Coomassie Blue staining techniques. Consequently, they are the method of choice when very low amounts of protein have to be detected on electrophoresis gels. A huge number of silver staining protocols have been published, based on the silver nitrate staining technique of Merril et al. [85] and modifications.

Stained spots excised from the electrophoresis gel can be identified by mass spectrometry.

Mass Spectrometry, MALDI-TOF-MS.

In traditional protein chemistry, proteins were identified by de novo sequencing using automated Edman degradation. Today, this technique is replaced by mass spectrometry, which is becoming one of the most powerful techniques in protein chemistry. The reason for this is a 100 fold increase in sensitivity and 10 fold increase in speed. Until today, the matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been the most widespread technique used for protein identification [86, 87].

Spots of interest are excised from the gel and treated with tripsin, an enzyme that cleaves C-terminal to arginine (R) and lysine (K). The mixture of protein fragments (peptides) obtained after digestion is purified and subjected to mass analysis.

Basically, a mass spectrometer consists of three parts. In the first part (ion source) the molecules to be analysed are ionised. The energy required for ionization of the ions is provided by a laser (wavelength 337 nm). Once ionised the molecules are accelerated and fly into the next part of the mass spectrometer, the analyser. Here the individual ions are separated based on the size and charge of the molecule. The last part of the instrument is a detector, which records the signals from all ion [86].

Before transferred into the ionisation source the peptide or protein samples are mixed with a matrix and the mixtures are placed to crystallize in small droplets on a target. The matrix is an organic component like sinapinic acid (SPA), dihydroxy benzoic acid (DHB), or α-cyano hydroxy cinnamic acid (HCCA). The choice of matrix material and the method used depends on the sample and on the sizes of the expected peptides.

The MALDI source is conveniently interfaced to a time-of-flight (TOF) analyzer. In the TOF-analyzer the m/z ratio of the individual ions is measured based on the flight time in a field-free drift tube. The m/z ratio of a peptide is calculated based on the energy equation $E=\frac{1}{2}mv^2$ (E=kinetic energy of the ion, m=mass, v=velocity). At a constant energy, peptides with a high mass will have a longer flight time in the tube before it reaches the detector at the end of the flight tube. Therefore, the peptides are sorted due to the size of the individual peptides. Flight times of ions are inversely proportional to their molecular mass. However, constant energy is an assumption. There is a small spread of time and kinetic

energy during ionization. An ion mirror (a reflectron) and a time-lag focusing (delayed extraction) are two approaches built into the MALDI-TOF instruments today to overcome this problem [86].

This technique offers a fingerprint unique for the particular protein or protein mixtures. The spectrum obtained is a graph of ion abundance versus the mass-to-charge ratio. The experimental obtained mass-to-charge (m/z) values can be matched against theoretical obtained mass data from already identified protein sequences and a score depending on the correlation can be given.

Correct identification of course requires that the database contain the specific protein sequences, therefore, the approach is best suited for generically well characterized organisms where the entire genome is known, but can also be used for organisms were only part of the genome is known or for which very homologous sequences are available.

MALDI is ideal for biological samples because it is compatible with buffers such as phosphate and Tris and low concentrations of urea, non-ionic detergents, and to a small degree alkali metal salts, however contaminants lower the sensibility of the spectra. When analyzing small amounts of biological analytes using MALDI-MS, optimizing sample preparation and removing contaminants from the sample can improves the spectra a lot [86, 87].

SELDI-TOF-MS.

Surface enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF-MS; by Bio-Rad Laboratories) is a high throughput technique, particularly appropriate for the investigation of low-molecular weight proteins (<of 25 KDa) with femtomole sensitivity and the ability to examine native proteins that provides a complementary visualization technique of the 2-DE. Furthermore SELDI-TOF-MS allows multiple samples to be analysed in a relatively short time while 2-DE permits the analysis of a little number of samples.

The principle of this technique is very simple [88]; a few microliters of a sample of interest are deposited on the spots of Protein Chip Arrays that present chromatographic surfaces with different physiochemical characteristics (hydrophobic, cationic, anionic and presenting metal ion) to obtain a broad range of proteins bound for analysis. The Protein Chip Arrays are incubated and then washed with the appropriated buffer. The proteins are captured on the chromatographic surface by depending on their properties and analyzed by TOF mass spectrometry. The result is a spectrum comprised of the mass to charge (m/z) values and intensities of the bound proteins/peptides. One of the unique strengths of SELDI-TOF is its ability to analyze proteins from a variety of crude sample types, with minimal sample consumption and processing [89].

ProteinChip® Arrays.

ProteinChip arrays are available with a variety of chromatographic surfaces. Typically, chromatographic surfaces are used for profiling of proteins and peptides in differential expression analyses.

The ProteinChip Q10 array: it is a strong anion exchange that can be used to analyze molecules with a negative charge on the surface. The active spots contain cationic, quaternary ammonium groups that interact with the negative charges on the surface of target proteins, e.g., aspartic acid or glutamic acid. By maintaining the pH of the binding/wash buffer at alkaline conditions (e.g., pH 8.0), an overall net negative charge is imparted on a greater number of proteins within the sample, and the result is more binding. By decreasing the pH of the binding/wash buffer, an overall net positive charge is imparted on the proteins, resulting in less binding (i.e., more specificity).

ProteinChip CM10 array: it is a weak cation exchange that can be used to analyze molecules with a positive charge on the surface. The active spots contain weak anionic carboxylate groups that interact with the positive charges on the surface of target proteins, e.g., lysine, arginine or histidine residues. By decreasing the pH of the binding/wash buffer, an overall net positive charge is imparted on a greater number of proteins within the sample and the result is more binding. By increasing the pH of the binding/wash buffer, an overall net negative charge is imparted on the proteins, resulting in less binding (i.e., more specificity). Binding of proteins to ProteinChip CM10 arrays can also be affected by changing the ionic

strength of the buffer. By increasing the ionic strength, competition is generated between the charged protein on the surface and the buffer ions, causing weakly bound proteins to elute from the array surface (i.e., more specificity).

ProteinChip IMAC30 array: it is used to capture molecules that bind polyvalent cationic metals such as nickel, gallium, copper, iron, and zinc. The active spots contain nitrilotriacetic acid (NTA) groups on the surface that chelate metal ions. Proteins applied to the array surface may bind to the chelated metal ion through histidine, tryptophan, cysteine, and phosphorylated amino acids. To generate selectivity, the binding and/or wash buffers may contain increasing concentrations of competitors (e.g., imidazole), which compete with the metal on the NTA group for binding to the protein or peptide.

ProteinChip H50 array: it is an hydrophobic surface that is used for capturing proteins and peptides through reversed-phase or hydrophobic interactions. Active spots contain methylene chains that closely mimic the characteristics of C6 to C12 alkyl chromatographic sorbent.

Proteins less hydrophobic relative to the binding buffer will not bind to the array surface, while proteins more hydrophobic will bind to the array surface. By increasing the organic content of the wash buffer, the hydrophobic nature of the buffer increases. Proteins that had previously bound to the array will divide into the wash buffer and be washed away if their hydrophobicity is less than that of the wash buffer. Only the most hydrophobic proteins will be retained with wash buffers containing a high concentration of organic solvent [Bio-Rad Laboratories].

Desorption, Ionization, and Analysis in the ProteinChip SELDI Reader.

The technique relies on time-of-flight mass spectrometry for the accurate measurement of the mass-to-charge ratio (m/z) of peptides and proteins.

The ProteinChip SELDI reader utilizes a nitrogen laser that induces both protein ionization and a change of state from the solid, crystalline phase into the gas phase. Therefore the analyte can move very rapidly, or fly, upon application of a voltage differential. The voltage differential applies the same kinetic energy to all of the analytes in the sample, thus resulting in flight times that depend upon the mass (see the mass spectrometry chapter above). The ProteinChip SELDI reader records the TOF of the analyte; from this measurement, a highly accurate and precise mass is derived [Bio-Rad Laboratories].

SALIVA

In terms of disease diagnosis and prognosis, a human body fluid (e.g., blood, urine, or saliva) appears to be more attractive than tissue because body fluid testing provides several key advantages including low invasiveness, minimum cost, and easy sample collection and processing [69, 90]. Serum or plasma have been the fluids most often used in disease diagnosis but an issue with these samples is sample preparation and handling. Another critical point is the complexity of the proteome [90]. Most importantly, when searching for biomarkers in blood, there are two serious consideration. First, the concentration of substance can vary over 9 orders of magnitude, which severely diminishes the likelihood of detecting those at the lower end of the scale; besides, blood is composed of peptides, proteins and cells that have half-lives ranging from seconds to weeks, or even a month or more. As a consequence, the presence of a given substance might not accurately reflect the current state of the organism [91].

By contrast, human saliva is becoming a more attractive source for proteomic profiling because it can provide clues to local and systemic diseases and conditions. The physiology of the oral cavity is such that the flow of secreted fluid is continually flushing and refreshing the fluid content of the mouth. Therefore, the composition of the saliva temporally reflects the metabolic activity of the secretory elements generating that fluid at any moment [91]. The logistical advantages of salivary diagnostic are obvious; saliva is relatively easy to collect in sufficient quantities for analysis, and the costs of storage and shipping tend to be

lower than those for serum and urine. Non-invasiveness, and ease of sample processing are advantageous as well [69, 90, 92]. In addition, for health care professionals and scientists, saliva tests are safer than blood tests, which are more likely to result in exposure to HIV or hepatitis [90]. On the other hand, a variety of factors may influence the rate of salivary flow and its physiologic characteristics, including circadian rhythms and activities such as exercise, and these factors should be taken into account when saliva is used as a diagnostic fluid [92].

AIM

In the present work, for the first time, we used 2-DE in combination with MS and SELDI-TOF-MS to obtain the whole saliva protein map of FM patients. The aim of this study was the evaluation of the global changes of the protein profiles, which occur in the disease and the research for any eventual diagnostic or prognostic salivary biomarkers, which could be used routinely, in the future, for the management of FM patients.

MATERIAL AND METHODS

Chemicals.

Iodoacetamide (IAA), CHAPS, urea, thiourea, glycerol, SDS, TEMED, ammonium persulfate, glycine, 30% acrylamide-N,N,N-bisacrylamide, trifluoroacetic acid (TFA), HEPES and copper sulphate were acquired from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl), acetonitrile (ACN) from J.T. Baker. Sodium acetate, Trizma base, SDS, DTT and trichloroacetic acid (TCA) from AppliChem. IPGs pH 3–10 L, pharmalyte 3–10 and dry strip cover fluid were purchased from GE Health Care, Europe (Uppsala, Sweden). Coomassie Brilliant Blue G 250 was from Merck (Darmstadt, Germany). Transaldolase (T-20) and phosphoglycerate mutase I (PGAM1) specific primary antibodies were from Santa Cruz Biotechnology (CA, USA). Secondary antibodies (horseradish peroxidase (HRP)-conjugated), donkey anti-goat and goat anti-mouse, were from Santa Cruz Biotechnology and from PerkinElmer, respectively. DC and RC/DC protein assay kit, chips CM10 and IMAC30, sinapinic acid (SPA) were provided by Bio-Rad. All other reagents were supplied by standard commercial sources and were of the highest grade available.

Human subjects.

A total of 85 patients were consecutively recruited from Department of Internal Medicine, Rheumatology Unit, University of Pisa; (Dr Laura Bazzichi).

Twenty-two women with a diagnosis of FM (mean age 43.38±13.23 years, M±SD), made according to the ACR criteria for the disease [1], were enrolled in the study of two-dimensional electrophoresis. Twenty-six healthy women, with similar mean age (48.57±8.22; M±SD) and demographic characteristics, were included as controls.

For Western blot and SELDI-TOF-MS analysis saliva samples were collected from sixty-tree patients (mean age 45.39±11.09 years, M±SD; 55 females, 8 males), with a diagnosis of FM made according to the ACR criteria and sixty-tree healthy subjects (mean age 40.92±10.03 years, M±SD; 47 females, 16 males).

This study was approved by the local Ethics Committee. An informed consensus was obtained for diagnostic or clinical purposes. Medical history and physical examination of each patient was carefully recorded focusing in particular on the number of tender points at the time of sample collections. Patients were also asked to fulfil the FIQ and a visual analogue score scale (VAS) in order to assess their overall pain levels. Moreover, as far as serological data is concerned, complete blood tests, nonorgan-specific autoantibodies (Antinuclear antibodies. ANA; Extractable Nuclear Antigen, ENA; Rheuma-test, Ra-test; Anti-cardiolipin antibodies, ACLA) and thyroid hormones and thyroid specific autoantibodies were detected in all the cases. Hepatitis B and hepatitis C infections were excluded in all the participants. Patients affected by secondary FM who were affected by FM and any other concomitant defined systemic connective disorders

were excluded from the study. Patients with serious hepatic, cardiac, renal or lung comorbidities were also not included in the study.

Finally, every FM patient had a psychiatric specialist evaluation to assess the presence of psychiatric concomitant disorders. This psychiatric evaluation was based on the administration of the Structured Clinical Interview for DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th. Edition) axis-I disorders (SCID-I/P) [93].

The clinical and serological data of the patients have been summarized in tables 2-4.

Table 2: Clinical and serological features of FM patients enrolled in the study of 2-DE.

CLINICAL/SEROLOGICAL FEATURES		
Patients number/sex	22/F	
Age (years)	43.38±13.23	(mean value ± SD)
Xerostomia	9/22	(41%)
ANA	7/22	(32%)
Autoimmune thyroiditis	10/22	(45%)
Drugs potentially inducing xerostomia	19/22	(86%)
Fibromyalgia Impact Questionnaire	66.8±14.4	(mean value ± SD)
Pain VAS (visual analogic scale)	7.9±1.9	(mean value ± SD)
Tenders points	14±3	(mean value ± SD)

Table 3: Clinical and serological features of FM patients enrolled in the WB and SELDI-TOF-MS analysis.

CLINICAL/SEROLOGICAL FEATURES		
Patients number/sex	55/F 8/M	
Age (years)	45.39±11.09	(mean value ± SD)
Xerostomia	28/63	(44%)
ANA	16/63	(25%)
Autoimmune thyroiditis	29/63	(46%)
Drugs potentially inducing xerostomia	52/63	(83%)
Fibromyalgia Impact Questionnaire	68.5±194	(mean value ± SD)
Pain VAS (visual analogic scale)	7.8±2.1	(mean value ± SD)
Tenders points	15±3	(mean value ± SD)

Table 4: Results of psychiatric evaluation		
FM patients enrolled in the study of 2-DE		
PSYCHIATRIC COMORBIDITIES	8/22	(36%)
Depression	4/22	(18%)
Anxiety Disorder	3/22	(14%)
Panic Disorder	1/22	(4.5%)
FM patients enrolled in the WB and SELDI-TOF-MS analysis		
PSYCHIATRIC COMORBIDITIES	26/63	(41%)
Panic Disorder	10/63	(16%)
	2/10	Eating Disorder
	1/10	Depression
Anxiety Disorder	6/63	(9.5%)
	2/6	Depression
Depression	5/63	(7.9%)
Bipolar Disorder	4/63	(6.3%)
	1/4	Panic Disorder
Eating Disorder	1/63	(1.6%)

Sample collection and preparation.

Unstimulated WS samples were collected early in the morning in standard conditions, i.e. all the subjects were asked no to eat (including gum or candies), smoke or drink since the night before. About 2 ml of saliva were obtained from each subject, processed immediately and kept on ice in order to minimize proteins degradation. Immediately after collection, saliva samples were centrifuged at 17000 *g* for 30 min at 4°C to discard bacteria, exfoliated epithelial cells and debris. The resulting supernatants were stored at -80°C until use. The protein amount was determined using Bio-Rad DC-protein assay. Bovine serum albumin (BSA) was used as a standard.

2-DE analysis.

For analytical gels, 150 μg of proteins, for each sample, were filled up to 350 μl in 7M urea, 2M thiourea, 4% CHAPS, 60mM DTT, 0.5% 3-10 ampholytes and 0.002% bromophenol blue (rehydration solution). Isoelectrofocusing (IEF) was carried out by using 18 cm Immobiline Dry-Strips (GE Healthcare) with a linear, pH 3-10, gradient. IEF was performed at 16°C on an Ettan IPGphor II apparatus (Amersham Biosciences), according to the following schedule: the samples were applied by in-gel rehydration for 10 h using low voltage (30 V), then the voltage was linearly increased from 200 to 5000 during the first 4 h, and then the proteins were focused for up to 70 000Vh at a maximum voltage of 8000 V. To prepare the IPG strips for the second dimension, the strips were first equilibrated 15 min at room temperature in a buffer containing 50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT, followed by a second equilibration for 10 min in the same buffer except that DTT was replaced by 2.5% IAA. Subsequently, the IPG strips were applied horizontally on top of 12.5% SDS-polyacrylamide gels (20x18x0.15 cm) and electrophoresis was performed using the PROTEAN-II Multi Cell system (Bio-Rad) with constant amperage (40mA/gel) at 10 °C until the dye front reached the bottom of the gel (about 5 h) applying a continuous buffer system.

Staining and image analysis.

The analytical gels were stained with ammoniacal silver nitrate. The procedure of silver staining consists of five sequential phases including protein fixation, sensitization, silver impregnation, image development and stopping. To ensure that the spot staining was within the values of the linearity range, the silver stain was performed in standard conditions of time and temperature. All solutions were kept at 4 °C, except for silver solution, while room temperature was controlled at 18 °C. All steps were performed on an orbital shaker.

Briefly, at the end of the second dimension run, the gels were removed from the glass plates, washed in deionized water for 5 min, soaked in ethanol: acetic acid: water (40: 10: 50) for 1 hour and then soaked in ethanol: acetic acid: water (5: 5: 90) overnight. After protein fixation, the gels were washed in deionized water for 5 min at 4°C and soaked in a solution containing glutaraldehyde (1%) and sodium acetate (0.5 M) for 30 min. After washing 3 times in deionized water for 10 min at 4 °C, the gels were soaked twice in a 2.7 naphtalene-disulfonic acid solution (0.05% w/v) for 30 min at 4 °C in order to obtain homogeneous dark brown staining of the proteins. Then the gels were rinsed 4 times in deionized water for 15 min at 4 °C. Staining was carried out in a freshly made ammoniacal (30%) silver nitrate (2.5%) solution for 30 minutes at 18 °C. After staining, the gels were washed 4 times in deionized water for 4 min at 4 °C. The images were developed in a solution containing citric acid (0.01% w/v) and formaldehyde (0.1% v/v) for 5

minutes. Development was stopped with a solution containing Tris (0.4M) and acetic acid (2% v/v).

The stained gels were scanned using an Epson Expression 1680 Pro scanner and the images were analyzed using Image-Master 2D Platinum 6.01 (GE Health Care Europe, Uppsala).

Spots were automatically detected, manually edited and then counted. After spot detection in gels, a match set and a synthetic image for each class was generated. A synthetic gel was obtained by averaging the positions, shapes, and optical densities of the matched spots in the set of gels class. This produces an intersection of all the gels, showing only the spots found in almost 75% of the images of each class.

Statistical analysis.

The optical density of the proteins was expressed as a percentage of the volume (mean±SD) of the spots representing a certain protein that was determined in comparison with the total number of proteins present in the 2-DE gel. The significance of the differences (p-value<0.05) was calculated using Mann-Whitney test and Spearman's Correlation for non-parametric variables. The sensibility and specificity of the potential disease biomarkers was assessed by receiver operating characteristic (ROC) curves. (MedCalc 9.6.4.0 software).

Exclusively the proteins whose expression showed over two-fold spot quantity change in FM samples compared with control samples were selected and

identified by matrix-assisted laser desorption ionization time-of-flight/time-of-flight (MALDI-TOF/TOF).

Preparative gels.

In order to identify these proteins of interest, preparative gels are performed and stained with Coomassie Brilliant Blue G-colloidal. This detection method is compatible with mass spectrometry but less sensitive than silver staining therefore, for preparative gels, we had to load 1500µg of proteins. Protein precipitation is required to prepare a concentrated protein sample from a dilute sample such as WS.

WS, previously centrifuged, was precipitated using 10 % (w/v) TCA and 0.05% DTT. After incubation at 0 °C for one hour, insoluble material was pelleted at 17000 g. Pellets were washed three times with pure acetone, air dried and solubilized in rehydration solution. The protein amount was estimated using an RC/DC protein assay from BioRad. This colorimetric assay allows the determination of protein concentration in the presence of reducing agents and detergents. BSA was used as a standard.

2-DE analysis of preparative gels.

For first dimension, a preliminary step at 200 V for 12 h was introduced, while second dimension is the same as analytical gels. The preparative gels for mass

spectrometric analysis were stained with Coomassie Brilliant Blue G-colloidal (0,12%Coomassie G-250, 10% ammonium sulfate, 2% phosphoric acid) according to Candiano et al. [94]. Briefly, after protein fixation with acetic acid (7%) and methanol (40%) for 1 h, the gels were stained overnight with Coomassie Brilliant Blue G-colloidal diluted with methanol (4:1 v/v). The gels were then rinsed 60 seconds with a solution of acetic acid (10%) and methanol (25%) and finally washed twice for few seconds with a solution of methanol (25%).

Both analytical and preparative gels showed the same protein pattern. Protein spots of interest were cut from gel and sent to Core Facility Proteomic (Université de Genève) for analysis by MALDI-TOF/TOF.

Western Blot.

Proteins of interest detected by MS/MS were also identified by Western Blotting (WB). WS samples were mixed with SDS sample buffer (Laemmli solution) and heated at 100°C for 5 min. Amounts of the samples, corresponding to 50 µg of proteins for transaldolase and 2,5 µg for PGAM1, were run on 12 % SDS-PAGE gels and transferred onto nitrocellulose membranes (0.2 µm) using a voltage of 100 V for 30 min (Criterion Blotter, Biorad). Non-specific binding was prevented by blocking the membranes with 3% low fat dried milk, 0.2% (v/v) Tween 20 in PBS (10 mM NaH_2PO_4, pH 7.4, 0.9% NaCl) (PBS/milk) overnight at 4°C. After blocking, the membranes were incubated for 2 h at RT in PBS/milk containing primary antibody transaldolase (goat polyclonal, 1:100 dilution) and

phosphoglycerate mutase (mouse monoclonal, 1:200 dilution). After 4 washes with PBS/milk, we incubated the membranes with peroxidase-labeled secondary antibody (donkey anti-goat, 1:5000 dilution; goat anti-mouse, 1:5000 dilution). Proteins were revealed with an enhanced chemiluminescence detection method according to the manufacturer's instructions (PerkinElmer).

SELDI-TOF-MS.

The experiments with SELDI-TOF-MS were carried out with an approach revised on the basis of the advices obtained from the Plate-forme de Protéomique Clinique (Montpellier; Prof. Silvain Lehmann) and the GIGA center (Liège; Dr. Dominique de Seny). We used two different Protein Chip Arrays (Bio-Rad): CM10 (that captures proteins with positive surface charges) and IMAC30 (Immobilized Metal Affinity Capture, for proteins that bind polyvalent metal ions). Aliquots of WS (corresponding to 20μg) were mixed (2:3 v/v) with denaturing buffer solution (9M urea, 2% CHAPS) and incubated for 30 min before loading onto Protein Chip arrays. Each chip was prepared according to Bio-Rad instructions. We first washed the chips two times with the binding buffers specific (100 mM sodium acetate pH 4 for CM10; PBS, NaCl 0.5M, Triton 0.1% for IMAC30) then we applied on the spots the sample. The chips IMAC30 need also an activation with copper sulphate 100 mM before the wash with the binding buffer. After an incubation of 1h, under agitation, the chips were washed three times with the specific binding buffer, twice with 150 μL of HEPES (10mM,

pH7.0) and then air-dried for 5 min. Finally 1μL of a 50% saturated solution of SPA in 50% ACN, 1%TFA was applied twice to each spot to facilitate desorption and ionization. The chips were read on a ProteinChip SELDI reader (Personal Edition, Bio-Rad). using an automated protocol (laser energy 3500 nJ; matrix attenuation 1000; focus mass 10 kDa; acquired mass range from 0 to 100 kDa). Analysis of the spectra was carried out using Protein Chip data manager software 3.5. Spectra were visually examined and poor quality spectra were excluded from further analysis.

Pre-processing of data is required before analysis. These processing steps include: calibration, baseline subtraction, normalization and peak detection. Calibration, carried out according to the manufacture's instructions, is necessary for mass accuracy. The software was externally calibrated using All-in-One Protein Standard and All-in-one Peptide Standard (BioRad). Baseline subtraction was achieved by using an algorithm that eliminates any baseline signal caused by matrix distortions. Peak intensities were normalized between samples in each study group to the total ion current (TIC) for avoiding the signal interference from SPA. Auto-detection of peaks was performed with "expression difference mapping" (EDM) under the following conditions: signal/noise ratio of 3 or higher for the first pass, 2 for the second pass, presentation in at least 10% of spectra for identification, 0.1% mass window and mass range 2,000-100,000 Da. Peaks having a m/z ratio <2 KDa were not used for analysis because they overlap with SPA signal.

Statistical Analysis.

The data of SELDI-TOF-MS were analyzed by an univariate and 2 multivariate analysis: extremely randomized trees machine learning algorithm (extra-trees) and Tree Boosting algorithm.

The univariate analysis determines if the intensity of a peak is significantly different in the experimental group spectrum as compared to controls; p-values associated with every peak were calculated using the Mann–Whitney test (significant when <0.05).

The multivariate analysis were performed by the GIGA Bioinformatics Platform & Bioinformatics and Modeling Unit (Université de Liège-Belgium) as described by Geurts et al. [95].

We used multivariate analysis to build classification models ("predictive models") and extract potentially relevant peaks with variable importance < 1% for tree-based methods. Biomarkers were thus ranked according to their importance (%) provided by the machine learning method. To assess accuracy, it was used the leave-one-out protocol. It consists in building a model using all the individuals except one, and classifying the remaining individual with the model. The procedure is repeated for all individuals to count the overall frequency of different types of error and finally it's provide the sample misclassification error rate.

RESULTS and DISCUSSION

Results of 2-DE analysis.

Figure 1 shows the typical WS 2-DE pattern obtained from a healthy control subject (fig.1 A) and from a FM patient (fig.1 B), respectively.

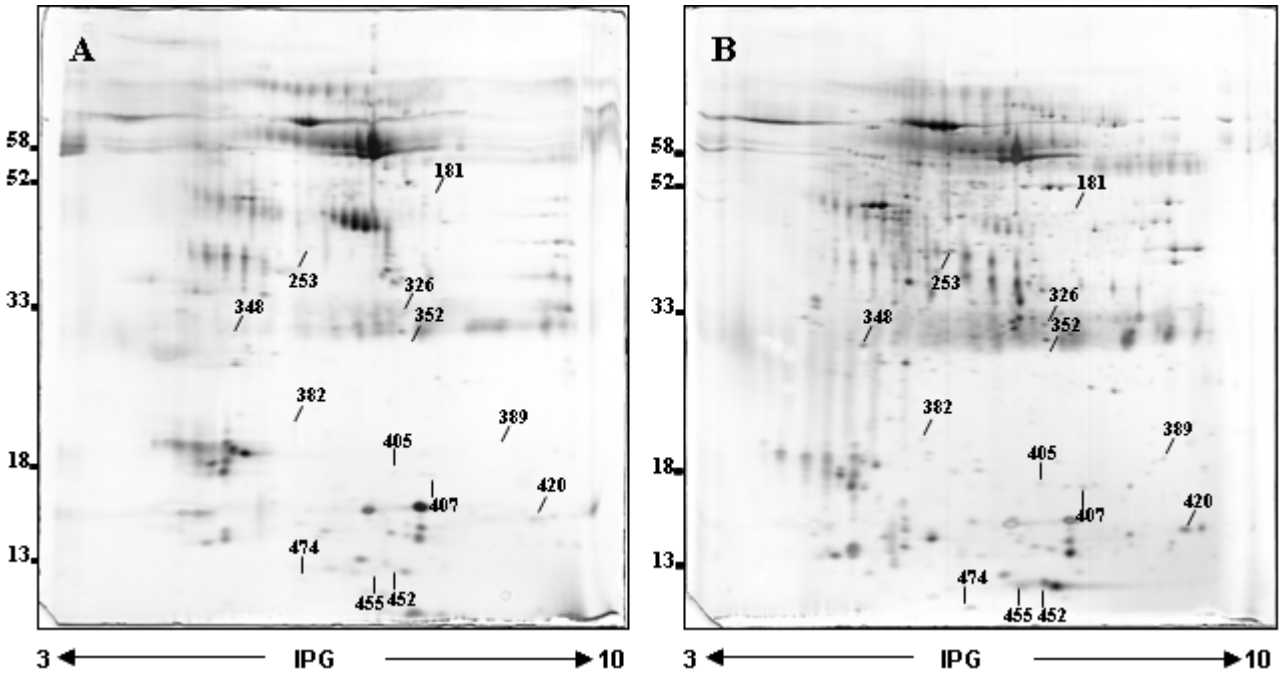

Figure 1: Representative 2-DE gel map of salivary proteins Control (A) and a FM patient (B). A total of 150 μg proteins were separated by 2-DE using 18 cm pH 3–10L strip and 12.5% SDS-PAGE. Proteins were detected by silver staining. The map was analyzed by the Image Master 2-D Platinum Software. Spot numbers indicate all the proteins differentially expressed in FM with respect to the control, identified by MS/MS and refer to the number reported in Table 5.

To assess intra-class variability the correlation coefficients were measured (FM patients' correlation coefficients 0.75–0.86; healthy subjects' correlation coefficients 0.77–0.90). The analysis of the obtained protein profiles allowed us to

focus many quantitative and qualitative differences in WS pattern between the two groups. A list of identified proteins, MW, pI, score and coverage values of MS/MS, fold of change in expression level and p-value calculated using the Mann-Whitney test, are shown in table 5.

Table 5. Protein identification of differentially expressed proteins in WS of FM patients by MS/MS. (n.s. not significant).

Spot n°	Protein name	Accession number	Mw KDa	pI	score	coverage (%)	fold-increase	p-value
181	gelsolin	A2A418	81	5.6	113	2	1.2	n.s.
253	transaldolase	P37837	38	6.3	181	42	3.02	<0.0001
326	phosphoglycerate mutase 1	P18669	29	6.7	501	44	2.5	0.0011
352	proteasome subunit alpha type-2	P25787	26	6.9	38	2	3.4	0.0258
348	Rho GDP-dissociation inhibitor 2	P52566	23	5.1	126	13	2.0	0.024
382	haptoglobin-related protein precursor	P00739	39	6.4	96	9	5.2	0.0029
389	cofilin-1	P23528	19	8.2	98	22	2.7	0.069
405	cyclophilin A	P62937	18	7.7	242	40	2.1	0.05
407	cyclophilin A	P62937	18	7.7	450	58	2.1	0.0157
420	profilin-1	P07737	15	8.4	400	43	2.0	0.0176
452	calgranulin A	P05109	11	6.5	313	47	2.6	0.0036
455	calgranulin A	P05109	11	6.5	187	37	4.7	0.0002
474	calgranulin C	P80511	11	5.8	68	7	12.9	0.001

Figure 2 shows the enlarged images of spots differentially expressed and the histogram of the percentage volumes of the proteins found in different quantity in FM respect to controls. The representative peptide spectra from three proteins among these differentially expressed are shown in Fig. 3.

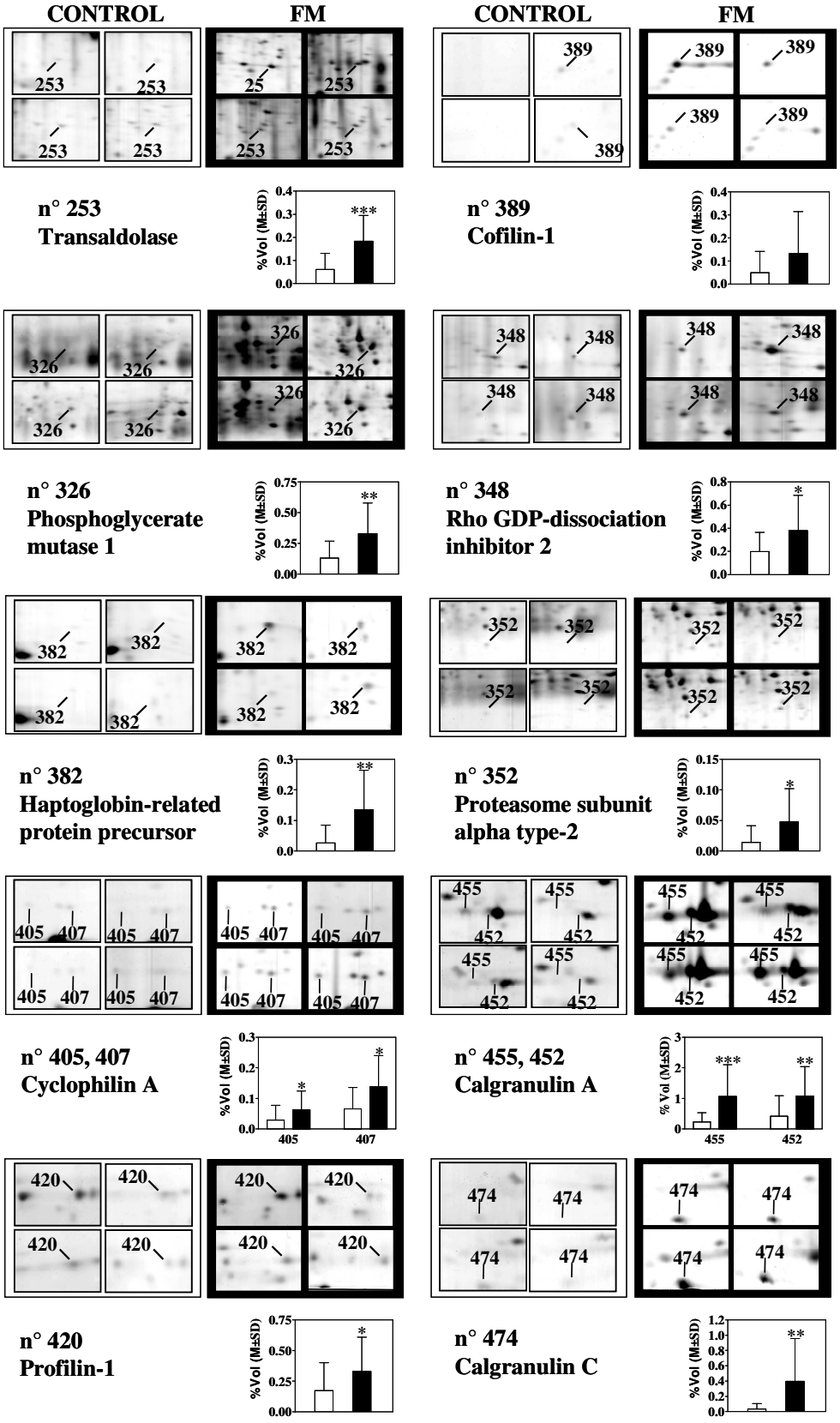

Figure 2: Enlarged images of the 2-DE gels highlighting the differentially expressed proteins for four representative gels. The arrows indicate the position of the differentially expressed proteins. Histograms show the percentage volumes of the proteins found in significantly different quantities in WS of FM patients with respect to the healthy subjects. Each bar represents the mean±SD of the mean of each spot. Significant differences from control WS are based on Mann-Whitney test; (*p<0.05, **p<0.01, ***p<0.001).

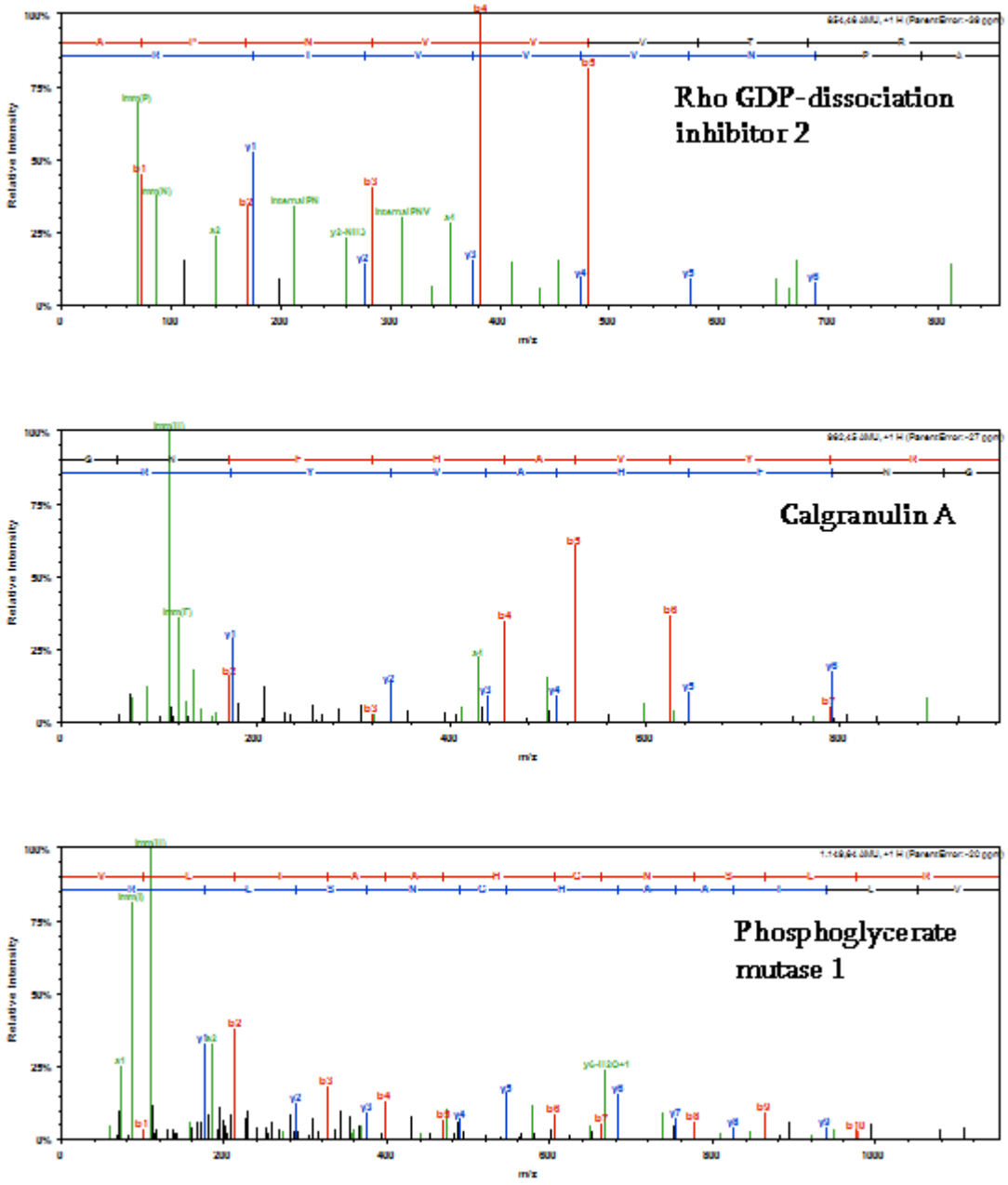

Figure 3: Representative peptide MS/MS spectra from three proteins differentially expressed as obtained from Scaffold software (Proteome Software, Portland, OR, USA).

Transaldolase and PGAM1 ROC curves.

ROC curve was calculated to evaluate the ability of transaldolase and PGAM1 to separate patients and control groups (Fig. 4). The sensitivity and the specificity of the transaldolase and PGAM1 were respectively 77.3 and 84.6% and 95.5 and 50%. No statistically significant correlation was detected between transaldolase and PGAM1 expression and any of the following FM clinical parameters: FIQ (p-value=0.98 for transaldolase and p-value=0.71 for PGAM1, respectively), visual analogic scale (VAS) (p-value=0.16 for transaldolase and p-value=0.47 for PGAM1, respectively) and the number of tender points (p-value=0.89 for transaldolase and p-value=0.94 for PGAM1, respectively).

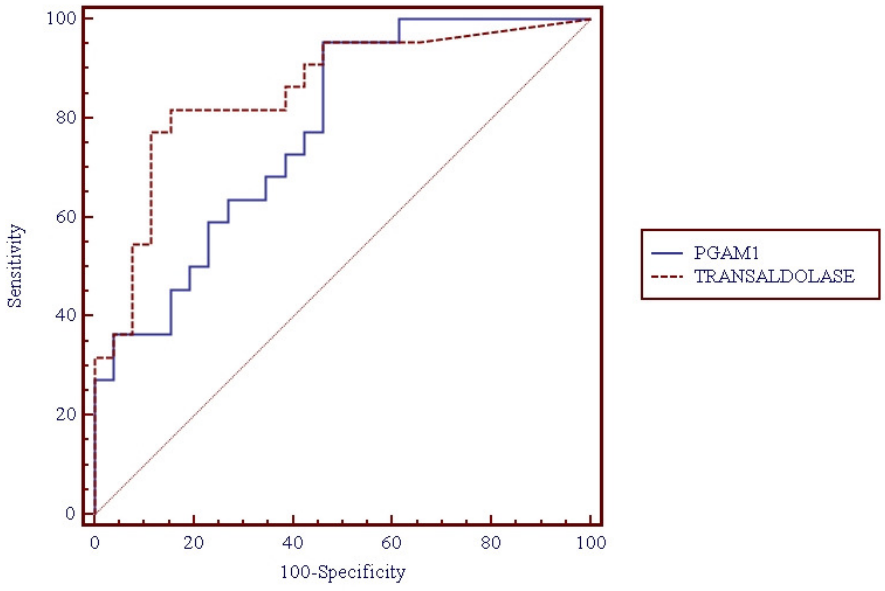

Figure 4: ROC curve of transaldolase and PGAM1.

The obtained results were validated by WB analysis of the WS proteins in 63 FM patients with respect to 63 control subjects. As depicted in Fig. 5, each antibody

recognized a specific band, corresponding to the molecular weight of the relative protein: 28KDa for PGAM1 and 38KDa for transaldolase. The optical density of specific band was detected by the software ImageJ and the mean ±SD values were compared between the two groups. Data are showed in the bar graphs (Fig. 5). The analysis, performed with Mann-Whitney test, of total optical density confirmed the significant up-regulation of transaldolase and PGAM1 in FM samples with respect to healthy subjects: p=0.02 and p=0.006, respectively (fig. 5, bar graph).

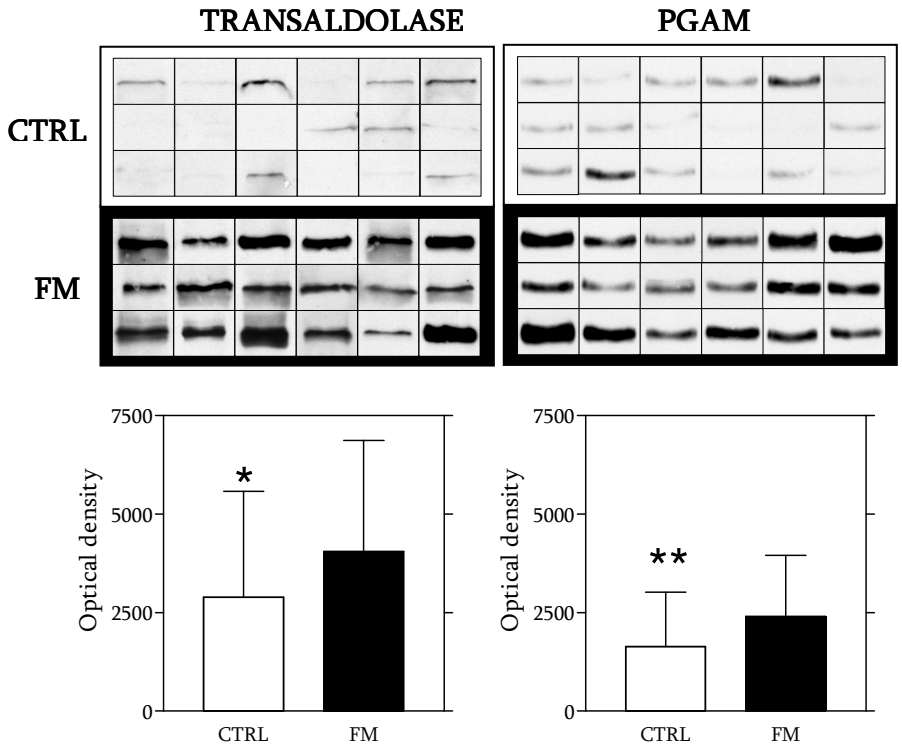

Figure 5: Western Blot analysis of transaldolase and PGAM1 in WS samples from healthy subjects (CTRL), and FM patients. Densitometry of the blots is shown. Values that are significantly different from healthy subjects ($p<0.05$) as determined by Mann-Whitney test, are indicated.

We have also investigated the correlation of the expression of transaldolase and PGAM1 between FM patients without psychiatric disorders and FM patients with concomitant psychiatric disorders. Figure 6 shows the histograms of the optical density (mean ±SD) of these proteins detected with WB analysis. We have evaluated the differences between five classes of FM patients: FM without psychiatric comorbidity (FM no psy), anxiety disorder, panic disorder, depression and bipolar disorder. We have excluded FM patients with more than one concomitant psychiatric disorder. The analysis, performed with Mann-Whitney test, of total optical density reveals an up-regulation of PGAM1 in FM patients with anxiety disorder respect to "FM no psy" patients: p-value=0.03. But also transaldolase shows a tendency to increase in FM patients with anxiety disorder respect to "FM no psy": p=0.06 (fig.6)

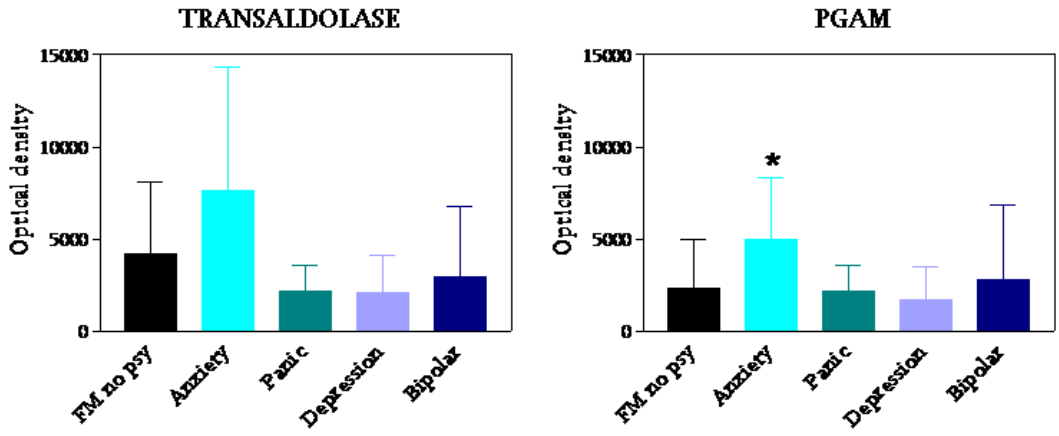

Figure 6: Histograms of the optical density (mean±SD) of transaldolase and PGAM1 detected with WB analysis in WS samples. We reported the different classes of patients: FM without psychiatric comorbidity (FM no psy), anxiety disorder, panic disorder, depression and bipolar disorder. Values that are significantly different ($p<0.05$) as determined by Mann-Whitney test, are indicated.

Discussion of 2-DE analysis.

With this study we described the human WS protein pattern of FM patients in comparison with healthy controls in order to identify the potential salivary biomarkers for the disease. The most relevant observation, which emerged from the data analysis, is the significant over-expression of transaldolase (spot n°253) in FM patients with respect to control. Transaldolase is an enzyme of the non-oxidative phase of the pentose phosphate pathway, which is involved in the generation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) [96]. Many evidences have shown that oxidative stress and nitric oxide may play an important role in FM pathophysiology [97]. However, it is still not clear whether oxidative stress abnormalities documented in FM are the cause or the effect [98, 99, 100].

The overexpression of transaldolase might be justified by the nicotinamide adenine dinucleotide phosphate reduced production, which could be involved in limiting oxidative damage to tissues. Transaldolase with a sensibility of 77.3% and a specificity of 84.6% may then be considered a reliable biomarker for FM (Fig. 4). Moreover, transaldolase links the pentose phosphate pathway to glycolysis. From this point of view, it is intriguing that another enzyme involved in the glycolysis, the PGAM1 (spot n°326), was differently expressed in FM patients [101].

We investigated the expression of these two proteins also in the 4 main psychiatric subsets of FM patients: anxiety disorder, panic disorder, depression

and bipolar disorder. Optical density of transaldolase and PGAM1 detected by WB in these patients was compared with that of FM patients without psychiatric comorbidity (fig.6).

With regard to transaldolase we can observe a tendency to up-regulation in FM patients with anxiety disorders respect to "FM no psy" patients, while the increase of PGAM1 in patients with anxiety disorders respect to "FM no psy" patients is significant.

These data are interesting considering a recent work of Gormanns and collaborators [102]. In order to capture the complex pathophysiology of anxiety and depression, they conducted a comprehensive pathway analysis taking into account all relevant transcriptome data currently deposited in the public domain. In case of anxiety disorder, besides a dysregulation of carbohydrate metabolism, tight junction, phosphatidylinositol signaling system, vascular endothelial growth factor (VEGF) signaling and long term potentiation they found a dysregulation of glycolysis pathway. Even if we had few samples to speculate any conclusion, our preliminary results can give us interesting indications for future studies.

Besides these proteins also cyclophilin A (CyPA) (spots n°405, 407), calgranulin A (spots n°452, 455) and calgranulin C (spot n°474) resulted all over-expressed in FM patients in comparison with healthy subjects. CyPA is a ubiquitously distributed protein belonging to the immunophilin family and it is recognized as the host cell receptor for the potent immunosuppressive drug cyclosporine A [103]. CyPA has also been shown to possess peptidylprolyl cis-trans-isomerase

activity and it is thought to play an important role in protein folding [103]. Although CyPA was initially believed to exist solely as an intracellular protein, later studies have revealed that it can be secreted by cells in response to inflammatory stimuli [104]. Secreted CyPA is a potent chemo attractant for monocytes, neutrophils, eosinophils and T cells in vitro. Recently, Satoh K and co-workers [105], showed that CyPA is secreted from smooth muscle cells and macrophages also in response to oxidative stress [104, 105, 106]. Calgranulins are highly homologous low-molecular weight calcium-binding proteins belonging to the S100 multigene family of proteins implicated in regulating calcium-dependent intracellular processes [107].

S100 proteins are involved in a variety of intracellular activities, cell proliferation and differentiation, cytoskeletal interactions, rearrangement and structural organization of membranes, intracellular $Ca2^{+}$ homeostasis, cell migration, inflammation and protection from oxidative cell damage [108, 109]. Considering that FM is defined as a non-inflammatory disease, we suggest that the over-expression of calgranulins in association with the other above mentioned biomarkers might be related more to the increased oxidative stress in FM rather than to an inflammatory process. According to the non-inflammatory nature of the disease another relevant observation is related to the gelsolin (GSN) expression (spot n°181). GSN was identified in 16 out of 22 salivary samples of patients affected by FM and in 10 out of 26 healthy controls. Nonethelesss, from a quantitative point of view, the protein was expressed in FM at the same levels as

in the healthy volunteers (p-value=0.18). GSN is a ubiquitous cytoplasmic actin-binding protein that mediates cell shape changes and motility [110, 111]. Recently it has been demonstrated that in every acute tissue injury (lung injury, adult respiratory distress syndrome, acute liver injury, myonecrosis, synovitis, pancreatitis, trauma, burns and bacterial sepsis) plasmatic GSN levels decrease [111, 112, 113, 114, 115, 116]. The unifying explanation for low plasmatic GSN concentrations in acute inflammatory conditions is the exposure by injury to plasma of the GSN-binding ligand, actin, a major cellular constituent ordinarily separated from the extracellular environment by intact plasma membranes [115]. The fact that in this study FM patients salivary levels of GSN were comparable with those of healthy controls is in line with the non-inflammatory nature of FM. In addition to GSN, other proteins involved in the cytoskeletal arrangement appeared to be differently expressed. In particular, profilin 1 (spot n°420) was overexpressed (p-value=0.017), while cofilin (spot n°389) showed the tendency to increase (p-value=0.06). Both of this actin binding proteins, even if in a different way, are responsible for the dynamics of filament turnover and remodeling of the actin cytoskeleton in response to appropriate signals [117]. Finally, three other proteins, Rho GDP-dissociation inhibitor 2 (spot n°348), proteasome subunit-a-type-2 (spot n°352) and haptoglobin-related protein precursor (spot n°382) resulted over-expressed with, respectively, two-, three and five-fold of increase in WS of FM patients with respect to the control subjects. Rho GDP-dissociation inhibitor 2 is a pivotal regulator of Rho GTPases, which are involved in the

control of cell morphology and motility in untrasformed cells [118, 119, 120]. Proteasome is a cytoplasmatic as well as nuclear-localized proteinase complex that is involved in the ubiquitin-dependent selective degradation of short-lived and abnormal proteins [121, 122]. Haptoglobin-related protein is a specific plasma protein associated with apolipoprotein L–I containing high-density lipoprotein particles shown to be a part of the innate immune defense [123]. The role of these proteins in FM patients need to be clarified. In conclusion, this first, preliminary study investigating salivary biomarkers in FM adds to the growing body of evidence that WS analysis performed by combining 2-DE and MALDI-TOF/TOF may be a new useful tool in the search for a potential panel of biomarkers in systemic diseases. In this study, patients' human saliva allows us to focus on some of the peculiar pathogenic aspects of FM, and namely, the non-inflammatory nature of the disease, the oxidative stress which contradistinguishes this condition and the involvement of proteins related to the cytoskeletal arrangements. None of the candidate proteins showed a statistical correlation with the patients' clinical features (i.e. FIQ, VAS, tender points). Further studies are desirable to assess any potential clinical correlation between the candidate biomarkers and the different subsets of FM patients, and to evaluate the potential therapeutic implication of our results.

Results of SELDI-TOF-MS analysis.

Protein expression profiles for each sample were generated also by SELDI-TOF-MS that provides a complementary visualization technique of the 2-DE.

Figure 7 reports a representative protein profile of salivary samples by using CM10 ProteinChips in the molecular range of 0–50 kDa.

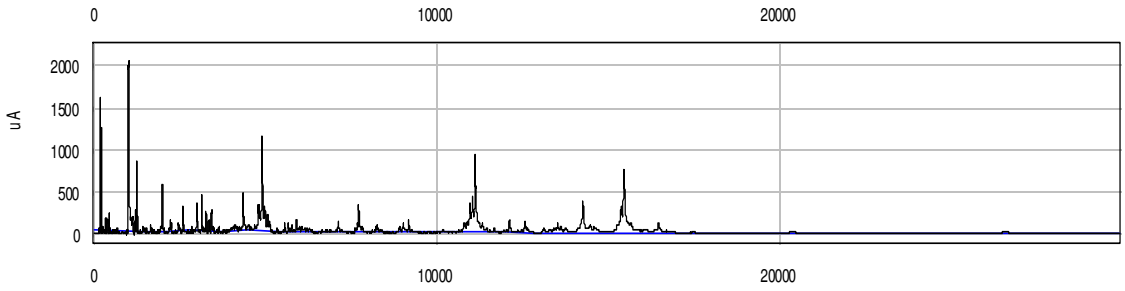

Figure 7: Representative protein profile of salivary samples by SELDI-TOF-MS using CM10 ProteinChips in the molecular range of 0–50 kDa.

SELDI-TOF-MS is often criticize for its poor reproducibility, so it's mandatory to perform at the same time all the experiments with the same chip for the different classes, furthermore the use of quality controls (QC) is highly recommended. QC is a well-characterized pool of samples processed alongside the experimental samples in order to calculate coefficient of variation (CV) for peak intensities and mass accuracy as a measure of reproducibility of the SELDI-TOF-MS analyses. The QC samples were applied randomly on different chips in order to avoid any artefact due to experimental handling. The CV (the standard deviation of the series divided by the mean of the series) was calculated using multiple protein peaks selected over the experiments. In this study the CV was 24.5% for peak

intensity, 0.008% for mass accuracy with the CM10 chips and 18.9% for peak intensity, 0.003% for mass accuracy with the IMAC30 chips. Our CVs indicated acceptable reproducibility of the spectra.

Peak detection with the ProteinChip data manager software 3.5 resolved a total of 112 peaks on CM10 and 98 peaks on IMAC30 arrays in the m/z ratio between 2000 and 100,000. Each spectra was thus described by 112 (CM10) and 98 (IMAC30) input variables where each variable correspond to the peak intensity for the given m/z. Extra-trees and tree boosting analysis provided information about peaks which presented high potential of discrimination between FM and control.

Discussion of SELDI-TOF-MS analysis.

With decision trees it is possible to compute from a tree the contribution of each variable to the classification. For each variable, this measure gives the percentage of information provided by the tree about the classification that can be attributed to this variable. Like the p-value, this measure allows m/z values to be ranked according to their relevance for differentiating FM from control groups. However, unlike the p-value approach, which takes into account each variable individually, this approach considers all variables simultaneously, and hence it can take into account interactions among variables.

Both approaches may thus provide substantially different results [95]. Table 6 provides the peaks found differentially expressed in FM respect to controls using the Mann–Whitney test (significant when p-value<0.05). 46 peaks were found

significantly differentially expressed in WS of FM patient respect to controls with CM10 arrays and 48 with IMAC30 arrays.

Table 6: Peaks found differentially expressed (p-value<0.05) in FM respect to ctrl using the Mann–Whitney test.

CM10		IMAC30	
m/z	**p-value**	**m/z**	**p-value**
11569	$1.14x10^{-5}$	10946	$6.14x10^{-7}$
9919	$1.19x10^{-5}$	22968	$1.87x10^{-6}$
7112	$2.46x10^{-5}$	11615	$2.29x10^{-6}$
13178	$3.47x10^{-5}$	10595	$3.43x10^{-6}$
6841	$6.12x10^{-5}$	22436	$3.20x10^{-5}$
8867	$6.12x10^{-5}$	5111	$4.92x10^{-5}$
7482	$7.51x10^{-5}$	24915	$7.40x10^{-5}$
7857	$8.66x10^{-5}$	9808	$8.66x10^{-5}$
7548	0.0002	14724	$8.79x10^{-5}$
5512	0.0003	8956	0.0001
10830	0.0003	11025	0.0002
11035	0.0004	8985	0.0002
22922	0.0006	24089	0.0003
13459	0.0007	5403	0.0003
9799	0.0008	7511	0.0004
12689	0.0008	9106	0.0004
24008	0.0008	10864	0.0005
12770	0.0009	6249	0.0006
7528	0.0012	14331	0.0006
5424	0.0017	11047	0.0006
9429	0.0018	28603	0.0007
7241	0.0020	7163	0.0008
5019	0.0028	9969	0.0008
9963	0.0028	8578	0.0016
5454	0.0029	29737	0.0016
14694	0.0032	3291	0.0027
5225	0.0035	8854	0.0039
13354	0.0040	4717	0.0048
8256	0.0041	15895	0.0050
8571	0.0044	7861	0.0052
51947	0.0046	4140	0.0067
4141	0.0063	4246	0.0080
6351	0.0069	12179	0.0087
11004	0.0074	4822	0.0103
5110	0.0103	9071	0.0107
13275	0.0103	3162	0.0146
1695	0.0163	23522	0.0149
4936	0.0163	4932	0.0194
5141	0.0174	6647	0.0203
16437	0.0246	6405	0.0212
12160	0.0320	11535	0.0266
9865	0.0340	27558	0.0306
18044	0.0397	66928	0.0353
11143	0.0416	5705	0.0411
16522	0.0416	15566	0.0411
2235	0.0421	28130	0.0421
		31152	0.0480
		10457	0.0496

Tables 7 and 8 illustrate the first 15 most discriminating peaks obtained with the two methods on CM10 and IMAC30 arrays. The quantity of information that a peak brings in the ensemble decision trees was referred as the percentage of importance (imp%) and was used to rank the potential biomarkers. We also provided in these tables the p-values calculated by univariate analysis with the Mann–Whitney test for these peaks. We then made the assumption that highly informative peaks were good biomarker candidates.

Table 7: The 15 most discriminating biomarkers, on **CM10** arrays, ranked according to imp (%) determined by Extra-trees and Tree-boosting and associated p-value calculated by Mann–Whitney test.

FM vs CTRL					
Extra-trees			**Tree-boosting**		
m/z	*imp%*	*p-value*	*m/z*	*Imp%*	*p-value*
11569	3.8	$1.14x10^{-5}$	11569	11.49	$1.14x10^{-5}$
4934	3.34	0.0162	7112	4.71	$2.46x10^{-5}$
9799	2.94	0.000754	7548	4.28	0.00019
7859	2.54	$8.66x10^{-5}$	3447	4.11	0.3526
5138	2.41	0.01737	7859	3.81	$8.66x10^{-5}$
7112	2.37	$2.46x10^{-5}$	8852	3.53	$6.12x10^{-5}$
3447	2.27	0.3526	9799	2.9	0.000754
5851	2.06	0.13226	13178	2.56	$3.47x10^{-5}$
13178	2.00	$3.47x10^{-5}$	6840	2.08	$6.12x10^{-5}$
8852	2.00	$6.12x10^{-5}$	5851	2.05	0.13226
7548	1.74	0.00019	22968	1.82	0.000609
8309	1,68	0.579723	7528	1.6	0.001166
3375	1,66	0.190174	5454	1.56	0.002894
3491	1.54	0.775312	4716	1.55	0.397251
5021	1.46	0.002803	52077	1.44	0.00462

Table 8: The 15 most discriminating biomarkers, on **IMAC30** arrays, ranked according to imp (%) determined by Extra-trees and Tree-boosting and associated p-value calculated by Mann–Whitney test.

FM vs CTRL					
Extra-trees			**Tree-boosting**		
m/z	*imp%*	*p-value*	*m/z*	*imp%*	*p-value*
11615	5.17	$2.293x10^{-6}$	11615	11.82	$2.293x10^{-6}$
10595	3.10	$3.429x10^{-6}$	5769	4.39	0.0609
10945	2.83	$6.137x10^{-7}$	3446	3.85	0.7042
3446	2.7	0.7042	5110	3.76	$4.294x10^{-5}$
5769	2.31	0.0609	9808	3.30	$8.66x10^{-5}$
5110	2.03	$4.294x10^{-5}$	4716	2.87	0.00481
8955	2.03	0.000125	22967	2.77	$1.87x10^{-6}$
10864	2.02	0.00049	14724	2.75	$8.79x10^{-5}$
9106	1.99	0.00045	10945	2.73	$6.137x10^{-7}$
4932	1.99	0.01939	7861	2.3	0.00525
2115	1.85	0.33347	5402	2.26	0.0003
9808	1.74	$8.66x10^{-5}$	9969	2.25	0.0008
4821	1.68	0.0103	10595	2.01	$3.429x10^{-6}$
3490	1.54	0.51279	8955	2.00	0.0001
46918	1.46	0.61715	5222	1.97	0.1009

Table 9 reports the predictions of these statistical analysis with the error rate, the error rate is the percentage of samples that are misclassified by the model.

Table 9: Predictions made with two different multivariate analysis.

CM10							
EXTRA-TREE				**TREE BOOSTING**			
Error rate: 35.714%				**Error rate: 27.778%**			
GROUP	*FM*	*CTRL*	*TOTAL*	**GROUP**	*FM*	*CTRL*	*TOTAL*
FM	42	21	63	*FM*	48	15	63
CTRL	24	39	63	*CTRL*	20	43	63
TOTAL	66	60	**126**	*TOTAL*	68	58	**126**
IMAC30							
Error rate: 32.54%				**Error rate: 29.365%**			
GROUP	*FM*	*CTRL*	*TOTAL*	**GROUP**	*FM*	*CTRL*	*TOTAL*
FM	43	20	63	*FM*	46	17	63
CTRL	21	42	63	*CTRL*	20	43	63
TOTAL	64	62	**126**	*TOTAL*	66	60	**126**

Therefore, with CM10 arrays, using the Extra-tree methods, 81 objects were correctly classified and 45 misclassified while, with the Tree-Boosting, 91 samples were correctly classified and 35 misclassified.

With IMAC30 arrays, 85 objects were correctly classified and 41 misclassified using Extra-tree analysis; 89 and 37 samples were, respectively, correctly classified and misclassified using the Tree Boosting analysis.

We can observe that for a given statistical approach, the most discriminating m/z values provided by the multivariate analysis were not necessarily identical to those provided by the p-value (see tables 7, 8). For example the peak with m/z 3446 Da was 3rd and 4th according to two different multivariate analysis (Tree-boosting and Extra-tree respectively) but its variation is not significant between FM and control according to Mann–Whitney test. Therefore a careful analysis of the generated data allowed us to focus our attention on some peaks: those for which there is a concordance between the different statistical analysis performed. Table 10 underlines the potential biomarkers detected with CM10 and IMAC30 arrays; p-value and fold variation of intensities of peaks are reported. Some of these peaks are illustrated in figure 8.

Table 10: Potential biomarkers detected on CM10 and IMAC30 arrays.

FM vs CTRL				
m/z	**imp% extra-trees**	**imp% Tree-Boosting**		**FM vs ctrl p-value**
CM10				
7112	2.37	4.71	↑	2.46×10^{-5}
7548	1.74	4.28	↑	0.00019
7859	2.54	3.81	↑	8.66×10^{-5}
8852	2.00	3.53	↑	6.12×10^{-5}
9799	2.94	2.9	↑	0.000754
11569	3.8	11.49	↑	1.14×10^{-5}
13178	2.00	2.56	↑	3.47×10^{-5}
IMAC30				
5110	2.03	3.76	↓	4.294×10^{-5}
5769	2.31	4.39	↓	0.0609
8955	2.03	2.00	↑	0.000125
9808	1.74	3.30	↑	8.66×10^{-5}
10595	3.10	2.01	↑	3.429×10^{-6}
10945	2.83	2.73	↑	6.137×10^{-7}
11615	5.17	11.82	↑	2.293×10^{-6}

In conclusion, SELDI-TOF-MS is a high-throughput method that compares expression levels of many individual proteins from multiple samples in parallel. Its strengths are the simple, rapid sample preparation and the use of small amount of proteins, so it can play an important role in discovering biomarkers but it does not allow identifying proteins. Further study are necessary to purify and identify potential biomarkers.

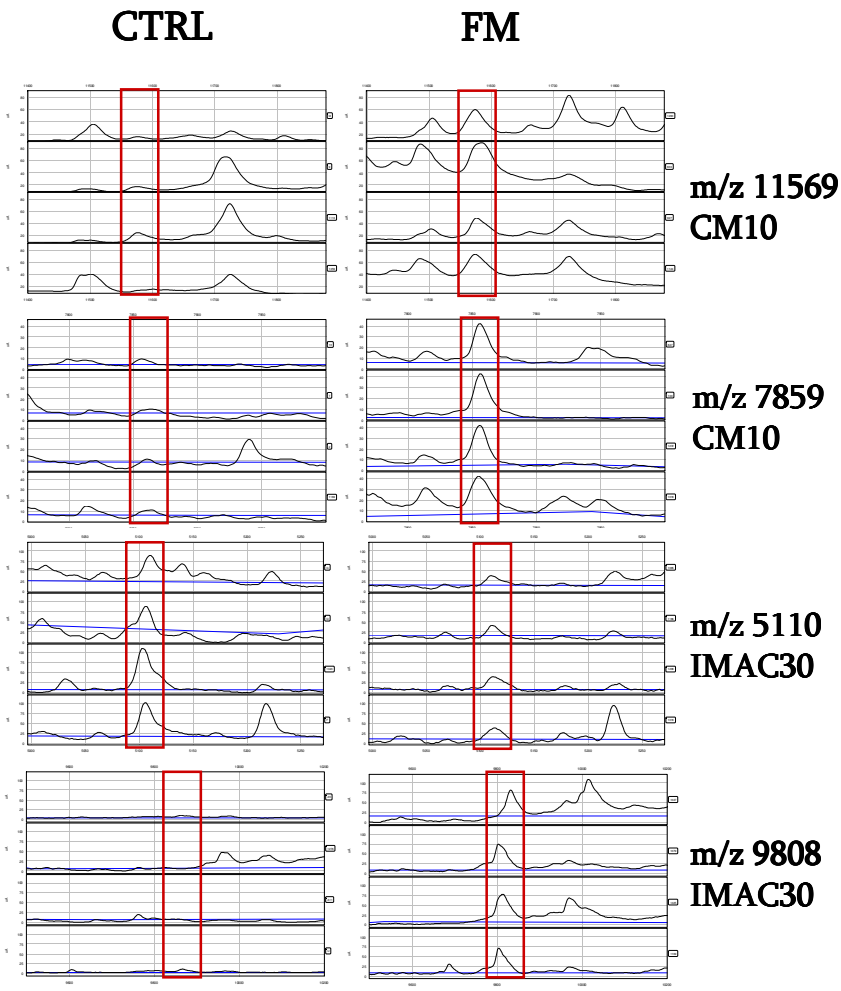

Figure 8: Enlarged image of 4 peaks with significant changes in the FM patients respect to the controls.

Future perspectives.

Our study has attested the potential usefulness of the proteomic characterization of human saliva in distinguishing FM from healthy subjects but the panel of candidate biomarkers found need to be validated in different cohort of pathological controls. Our interest is to compare FM patients with subjects affected by other disease in order to identify biomarkers really specific of FM and

to exclude any interference of concomitant disorder (e.g. psychiatric comorbidities).

With this aim we have begun to recruit patients affected by inflammatory diseases, psychiatric diseases and migraine.

REFERENCES

1. Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, Tugwell P, Campbell SM, Abeles M, Clark P, Fam AG, Farber SJ, Fiechtner JJ, Franklin M, Gatter RA, Hamaty D, Lessard J, Lichtbroun AS, Masi AT, Mccain GA, Reynolds WJ, Romano TJ, Russell J, Sheon RP. The American College of Rheumatology 1990 Criteria for the Classification of Fibromyalgia. Report of the Multicenter Criteria Committee. *Arthritis Rheum*. **1990**; 33: 160-172.
2. Moldofsky H. The significance of the sleeping-waking brain for the understanding of widespread musculoskeletal pain and fatigue in fibromyalgia syndrome and allied syndromes. *Joint Bone Spine*. **2008**; 75: 397-402.
3. Lesley M, Arnold LM. Management of fibromyalgia and comorbid psychiatric disorders. *J Clin Psychiatry*. **2008**; 69: 14–19.
4. Bennett RM, Jones J, Turk DC, Russell IJ, Matallana L. An internet survey of 2,596 people with fibromyalgia. *BMC.Musculoskele Disord*. **2007**; 8: 27–38.
5. Goebel A, Buhner S, Schedel R, Lochs H, Sprotte G. Altered intestinal permeability in patients with primary fibromyalgia and in patients with complex regional pain syndrome. *Rheumatology (Oxford).* **2008**; 47: 1223–1227.
6. Orellana C, Gratacós J, Galisteo C, Larrosa M. Sexual dysfunction in patients with fibromyalgia. *Curr Rheumatol Re.* **2009**; 11: 437-442)
7. White KP, Harth M. Classification, epidemiology, and natural history of fibromyalgia. *Curr Pain Headache Rep.* **2001**; 5: 320-329
8. Eraso RM, Bradford NJ, Fontenot CN, Espinoza LR, Gedalia A. Fibromyalgia syndrome in young children: onset at age 10 years and younger. *Clin Exp Rheumatol*. **2007**; 25: 639–644.
9. McLean SA, Clauw DJ. Biomedical models of fibromyalgia. *Disabil Rehabil*. **2005**; 27: 659–665.

10. Kasper S. The psychiatrist confronted with a fibromyalgia patient. *Hum Psychopharmacol.* **2009**; 24: S25-S30.

11. Buskila D, Sarzi-Puttini P. Fibromyalgia and autoimmune diseases: the pain behind autoimmunity. *Isr Med Assoc J.* **2008**; 10: 77-78.

12. Nishikai M, Tomomatsu S, Hankins RW, Takagi S, Miyachi K, Kosaka S, Akiya K. Autoantibodies to a 68/48 kDa protein in chronic fatigue syndrome and primary fibromyalgia: a possible marker for hypersomnia and cognitive disorders. *Rheumatology*. **2001**; 40: 806–810.

13. Pamuk ON, Cakir N. The frequency of thyroid antibodies in fibromyalgia patients and their relationship with symptoms. *Clin Rheumatol*. **2007**; 26: 55–59.

14. Wilson RB, Gluck OS, Tesser JR, Rice JC, Meyer A, Bridges AJ. Antipolymer antibody reactivity in a subset of patients with fibromyalgia correlates with severity. *J Rheumatol* **1999**; 26: 402-407.

15. Bazzichi L, Giacomelli C, De Feo F, Giuliano T, Rossi A, Doveri M, Tani C, Wilson RB, Bombardieri S. Antipolymer antibody in Italian fibromyalgic patients. *Arthritis Res Ther*. **2007**; 9: R86.

16. Dinerman H, Goldenberg DL, Felson DT. A prospective evaluation of 118 patients with fibromyalgia syndrome: prevalence of Raynaud's phenomenon, ANA, low complement, and IgG deposition at the dermal-epidermal junction. *J Rheumatol*. **1986**; 13: 368–373.

17. Al-Allaf AW, Ottewell L, Pullar T. The prevalence and significance of positive antinuclear antibodies in patients with fibromyalgia syndrome: 2-4 years follow up. *Clin Rheumatol*. **2002**; 21: 472–477.

18. Kotter I, Neuscheler D, Gunaydin I, Werent 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**; 27: 1031–1039.

19. Klein R, Berg PA. Diagnostic relevance of antibodies to serotonin and phospholipids in fibromyalgia syndrome. *J Rheumatol*. **2002**; 29: 395-396.

20. Werle E, Fischer HP, Müller A, Fiehn W, Eich W. Antibodies against serotonin have no diagnostic relevance in patients with fibromyalgia syndrome. *J Rheumatol.* **2001**; 28: 595-600.

21. Omoigui S. The biochemical origin of pain: The origin of all pain is inflammation and the inflammatory response. Part 1of 3–Inflammatory profile of pain syndrome. *Med Hypotheses*. **2007**; 69: 1169-1178.

22. Vaerøy H, Helle R, Førre O, Kåss E, Terenius L. Elevated CSF levels of substance P and high incidence of Raunaud phenomenon in patients with fibromyalgia: new features for diagnosis. *Pain*. **1988**; 32: 21-26.

23. Ortega E, García JJ, Bote ME, Martín-Cordero L, Escalante Y, Saavedra JM, Northoff H, Giraldo E. Exercise in fibromyalgia and related inflammatory disorders: known effects and unknown chances. *Exerc Immunol Rev*. **2009**; 15: 42-65.

24. Bazzichi L, Rossi A, Massimetti G, Giannaccini G, Giuliano T, De Feo F, Ciapparelli A, Dell'Osso L, Bombardieri S. Cytokine patterns in fibromyalgia and their correlation with clinical manifestations. *Clin Exp Rheumatol*. **2007**; 25:225–230.

25. Wang H, Moser M, Schiltenwolf M, Buchner M. Circulating cytokine levels compared to pain in patients with fibromyalgia—a prospective longitudinal study over 6 months. *J Rheumatol*. **2008**; 35: 1366-1370.

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

27. Müller W, Schneider EM, Stratz T. The classification of fibromyalgia syndrome. *Rheumatol Int.* **2007**; 27: 1005-1010.

28. Van West D, Maes M. Neuroendocrine and immune aspects of Fibromyalgia. *BioDrugs.* **2001**; 15: 521-531.

29. Kubera M, Lin AH, Kenis G, Bossmans E, van Bockstaele D, Maes M. Anti-Inflammatory effects of antidepressants through suppression of the interferongamma/interleukin-10 production ratio. *J Clin Psychopharmacol*. **2001**; 21: 199-206.

30. Buskila D, Neumann L, Hazanov I, Carmi R. Familial aggregation in the fibromyalgia syndrome. *Semin Arthritis Rheum.* **1996**; 26: 605–611.

31. Buskila D, Neumann L. Fibromyalgia syndrome (FM) and nonarticular tenderness in relatives of patients with FM. *J Rheumatol*. **1997**; 24: 941–944.

32. Arnold LM, J Hudson J, Hess EV, Ware AE, Fritz DA, Auchenbach MB, Starck LO, Keck PE Jr. Family study of fibromyalgia. *Arthritis Rheum.* **2004**; 50: 944–952.

33. Maletic V, Raison CL. Neurobiology of depression, fibromyalgia and neuropathic pain. *Front Biosci*. **2009**; 14:5291-5338.

34. Hudson JI, Arnold LM, Keck PE Jr, Auchenbach MB, Pope HG Jr. Family study of fibromyalgia and affective spectrum disorder. *Biol Psychiatry*. **2004**; 56: 884-891.

35. Kato K, Sullivan PF, Evengard B, Pedersen NL. A population-based twin study of functional somatic syndromes. *Psychol Med*. **2008**: 1-9.

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

37. Offenbaecher M, Bondy B, de Jonge S, Glatzeder K, Krüger M, Schoeps P, Ackenheil M. Possible association of fibromyalgia with a polymorphism in the serotonin transporter gene regulatory region. *Arthritis Rheum.* **1999**; 42: 2482-2488.

38. Cohen H, Buskila D, Neumann L, Ebstein RP. Confirmation of an association between fibromyalgia and serotonin transporter promoter region (5-HTTLPR) polymorphism, and relationship to anxiety-related personality traits. *Arthritis Rheum*. **2002**; 46: 845-847.

39. Bradley LA. Pathophysiology of fibromyalgia. *Am J Med*. **2009**; 122: S22-S30.

40. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A, Poulton R. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science*. **2003**; 301: 386-389.

41. Buskila D, Sarzi-Puttini P, Ablin JN. The genetics of fibromyalgia syndrome. *Pharmacogenomics*, **2007**; 8: 67-74.

42. Jokela M, Keltikangas-Jarvinen L, Kivimaki M, Puttonen S, Elovainio M, Rontu R, Lehtimaki T. Serotonin receptor 2A gene and the influence of childhood maternal nurturance on adulthood depressive symptoms. *Arch Gen Psychiatry*. **2007**; 64: 356-360.

43. Zubieta J K, Heitzeg MM, Smith YR, Bueller JA, Xu K, Xu Y, Koeppe RA, Stohler CS, Goldman D. COMT val158met genotype affects mu-opioid neurotransmitter responses to a pain stressor. *Science*. **2003**; 299: 1240-1243.

44. Buskila D, Cohen H, Neumann L, Ebstein RP. An association between fibromyalgia and the dopamine D4 receptor exon III repeat polymorphism and relationship to novelty seeking personality traits. *Mol Psychiatry.* **2004**; 9: 730-731.

45. Treister R, Pud D, Ebstein RP, Laiba E, Gershon E, Haddad M, Eisenberg E. Associations between polymorphisms in dopamine neurotransmitter pathway genes and pain response in healthy humans. *Pain*. **2009**; 147: 187-193.

46. Goldenberg DL. The interface of pain and mood disturbances in the rheumatic diseases. *Semin Arthritis Rheum*. **2010**; 40: 15-31.

47. 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.

48. Daoud KF, Barkhuizen A. Rheumatic mimics and selected triggers of fibromyalgia. *Curr Pain Headache Rep.* **2002**; 6: 284–288.

49. Clauw DJ. Fibromyalgia: An Overview. *Am J Med.* **2009**; 122: S3-S13.

50. Di Franco M, Iannuccelli C, Valesini G. Neuroendocrine immunology of fibromyalgia. *Ann N Y Acad Sci*. 2010; 1193: 84-90.

51. Freeman R. The treatment of neuropathic pain. *CNS Spectr*. **2005**; 10: 698-706.

52. Salemi SJ, Rethage U, Wollina BA, Michel RE, Gay S, Sprott H. Detection of interleukin 1beta (IL-1beta), IL-6, and tumor necrosis factor-alpha in skin of patients with fibromyalgia. *J Rheumatol*. **2003**: 30: 146-150.

53. Tracey I. Imaging pain. *Br J Anaesth*. **2008**; 101: 32-39.

54. Baliki MN, Chialvo DR, Geha PY, Levy RM, Harden RN, Parrish TB, Apkarian AV. Chronic pain and the emotional brain: specific brain activity associated with spontaneous fluctuations of intensity of chronic back pain. *J Neurosci*. **2006**; 26: 12165-1273.

55. Phillips ML, Ladouceur CD, Drevets WC. A neural model of voluntary and automatic emotion regulation: implications for understanding the pathophysiology and neurodevelopment of bipolar disorder. *Mol Psychiatry*. **2008**; 13: 829, 833-857.

56. Cook DB, Lange G, Ciccone DS, Liu WC, Steffener J, Natelson BH. Functional imaging of pain in patients with primary fibromyalgia. *J Rheumatol*, **2004**; 31: 364-378.

57. Gracely RH, Petzke F, Wolf JM, Clauw DJ. Functional magnetic resonance imaging evidence of augmented pain processing in fibromyalgia. *Arthritis Rheum*. **2002**; 46: 1333-1343.

58. Kuchinad A, Schweinhardt P, Seminowicz DA, Wood PB, Chizh BA, Bushnell MC. Accelerated brain gray matter loss in fibromyalgia patients: premature aging of the brain? *J Neurosci*. **2007**; 27: 4004-4007.

59. Luerding R, Weigand T, Bogdahn U, Schmidt-Wilcke T. Working memory performance is correlated with local brain morphology in the medial frontal and anterior cingulate cortex in fibromyalgia patients: structural correlates of pain-cognition interaction. *Brain*. **2008**; 131: 3222-3231.

60. Millea PJ, Holloway RL. Treating fibromyalgia. *Am Fam Physician.* **2000**; 62: 1575-1582, 1587.

61. 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**; 34: 1415-1425.

62. Millea PJ, Holloway RL. Treating fibromyalgia. *Am Fam Physician*. **2000**; 62: 1575–1582.

63. Bazzichi L, Rossi A, Giacomelli C, Bombardieri S. Exploring the abyss of fibromyalgia biomarkers. *Clin Exp Rheumatol*. **2010**; 28: S125-S130.

64. Natelson BH. Fibromyalgia syndrome: diagnosis and comorbidities. *CNS Spectr*. **2009**; 14: 8-11; discussion 17-18.

65. Clauw DJ. Assessing and diagnosing fibromyalgia in the clinical setting. *J Clin Psychiatry.* **2008**; 69: e33.

66. Van Oostrum J, Voshol H. The human genome: proteomics. *Am J Psychiatry.* **2002**; 159: 208.

67. Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez JC, Yan JX, Gooley AA, Hughes G, Humphery-Smith I, Williams KL, Hochstrasser DF. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnology (NY).* **1996**; 14: 61–65.

68. de Hoog CL, Mann M. Proteomics. *Annu Rev Genomics Hum Genet.* **2004**; 5: 267–293.

69. Al-Tarawneh SK, Bencharit S. Applications of Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight (SELDI-TOF) Mass Spectrometry in Defining Salivary Proteomic Profiles. *Open Dent J.* **2009**; 3:74-79.

70. Matthiesen R, Mutenda KE. Introduction to proteomics. *Methods Mol Biol.* **2007**; 367: 1-35.

71. Zhang X, Fang A, Riley CP, Wang M, Regnier FE, Buck C. Multi-dimensional liquid chromatography in proteomics--a review. *Anal Chim Acta.* **2010**; 664: 101-113.

72. Rabilloud T. Solubilization of proteins for electrophoretic analysis. *Electropho-resis.* **1996**; 17: 813-829.

73. Görg A, Boguth G, Harder A, Obermaier C, Scheibe B, Wildgruber R, Weiss W. Two-Dimensional Electrophoresis of Proteins using Immobilized pH Gradients. A laboratory manual. *http://www.weihenstephan.de/blm/deg* **1998.**

74. Herbert BR, Sanchez, JC, Bini L. Two-dimensional electrophoresis: The state of the art and future directions. *In:* Wilkins MR, Williams KL, Appel RD, Hochstrasser DF. (eds) *Proteome Research: New Frontiers in Functional Genomics*. *Springer Berlin Heidelberg New York.* **1997**; 13-33.

75. Dunn MJ. Gel electrophoresis of proteins. *BIOS Scientific Publishers Ltd. Alden Press, Oxford.* **1993**.

76. Rabilloud T, Valette C, Lawrence JJ. Sample application by in-gel rehydration improves the resolution of two-dimensional electrophoresis with immobilized pH-gradients in the first dimension. *Electrophoresis.* **1994**; 15: 1552-1558.

77. Fichmann J. 2-D proteome analysis protocols-Advantages of Immobilized pH Gradients. *Methods in Molecular Biology™.* **1999**; 112: 173-174.

78. Bjellqvist B, Ek K, Righetti PG, Gianazza E, Görg A, Westermeier R, Postel W. Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. *J Biochem Biophys Methods*. **1982**; 6:317-339.

79. Görg A, Postel W, Günther S. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis.* **1988**; 9: 531-546.

80. Blomberg A, Blomberg L, Norbeck J, Fey SJ, Larsen PM, Larsen M, Roepstorff P, Degand H, Boutry M, Posch A, et al. Interlaboratory reproducibility of yeast protein patterns analyzed by immobilized pH gradient two-dimensional gel electrophoresis. *Electrophoresis*. **1995**; 6:1935-1945.

81. 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**; 21:1037-1053.

82. Görg A, Postel W, Weser J, Günther S, Strahler SR, Hanash SM, Somerlot L. Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. *Electrophoresis.* **1987**; 8: 122-124.

83. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from poly-acrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc Natl Acad Sci USA.* **1979**; 76: 4350-4354.

84. Kyhse-Andersen J. Electroblotting from multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins to nitrocellulose membrans. *J Biochem Biophys Methods.* **1984**; 10: 203-209.

85. Merril CR, Goldman D, Sedman S, Ebert H. Ultrasensitive stain for proteins in poly-acrylamide gels show regional variation in cerebrospinal fluid proteins. *Science.* **1981**; 211, 1437-1438.

86. Hjernø K, Højrup P. Peptide Mass Mapping by MALDI-TOF MS. Protein identification by MALDI-TOF MS and database searching. *http://www.protein.sdu.dk/bm131/download/ProteinIdentification.pdf* . 1-24.

87. Ashcroft AE. An Introduction to Mass Spectrometry. *http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm*.

88. Al-Tarawneh SK, Bencharit S. Applications of Surface-Enhanced Laser Desorption /Ionization Time-Of-Flight (SELDI-TOF) Mass Spectrometry in Defining Salivary Proteomic Profiles. *The Open Dentistry Journal*. **2009**; 3: 74-79.

89. Xiao Z, Prieto D, Conrads TP, Veenstra TD, Issaq HJ. Proteomic patterns: their potential for disease diagnosis. *Mol Cell Endocrinol*. **2005**; 230: 95-106.

90. Hu S, Loo JA, Wong DT. Human body fluid proteome analysis. *Proteomics.* **2006**; 6: 6326-6353.

91. Streckfus CF, Dubinsky WP. Proteomic analysis of saliva for cancer diagnosis. *Expert Rev Proteomics*. **2007;** 4:329-332.

92. Papale M, Pedicillo MC, Di Paolo S, Thatcher BJ, Lo Muzio L, Bufo P, Rocchetti MT, Centra M, Ranieri E, Gesualdo L. Saliva analysis by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF/MS): from sample collection to data analysis. *Clin Chem Lab Med*. **2008**; 46: 89-99.

93. First MB, Spitzer RL, Gibbon M, Williams JBW. Structured Clinical Interview for DSM-IV Axis I Disorders, Research Version, Patient Edition. (SCID-I/P). *New York: Biometrics Research, New York State Psychiatric Institute*. **2002**.

94. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis*. **2004**; 25 (9): 1327-33.

95. Geurts P, Fillet M, de Seny D, Meuwis MA, Malaise M, Merville MP, Wehenkel L. Proteomic mass spectra classification using decision tree based ensemble methods. *Bioinformatics* **2005**; 21: 3138–3145.

96. Samland AK, Sprenger GA. Transaldolase: from biochemistry to human disease. *Int J Biochem Cell Biol* **2009**; *41*, 1482–1494.

97. Ozgocmen S, Ozyurt H, Sogut S, Akyol O, Current concepts in the pathophysiology of fibromyalgia: the potential role of oxidative stress and nitric oxide. *Rheumatol. Int.* **2006**; 26: 585–597.

98. Cordero MD, Moreno-Fernàndez AM, Demiguel M, Bonal P, Campa F, Jiménez-Jiménez LM, Ruiz-Losada A, Sánchez-Domínguez B, Sánchez Alcázar JA, Salviati L, Navas P. Coenzyme Q10 distribution in blood is altered in patients with fibromyalgia. *Clin Biochem*. **2008**; 42: 732–735.

99. Chung CP, Titova D, Oeser A, Randels M, Avalos I, Milne GL, Morrow JD, Stein CM. Oxidative stress in fibromyalgia and its relationship to symptoms. *Clin Rheumatol.* **2009**; 28: 435–438.

100. Altindag O, Celik H. Total antioxidant capacity and the severity of the pain in patients with fibromyalgia. *Redox Rep*. **2006**; 11: 131–135.

101. Kondoh H, Lleonart ME, Bernard D, Gil J. Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization. *Histol Histopathol.* **2007**; 22: 85-90.

102. Gormanns P, Mueller NS, Ditzen C, Wolf S, Holsboer F, Turck CW. Phenome-transcriptome correlation unravels anxiety and depression related pathways. *Journal of Psychiatric Research*. **2011**; doi: 10.1016/ j. jpsychires. 2010.12.010.

103. Galat A. Peptidylproline cis-trans-isomerases: immunophilins. *Eur J Biochem.* **1993**; 216: 689–707.

104. Arora K, Gwinn WM, Bower MA, Watson A, Okwumabua I, MacDonald HR, Bukrinsky MI, Constant SL. Extracellular cyclophilins contribute to the regulation of inflammatory responses. *J Immunol*. **2005**; 175: 517–522.

105. Satoh K, Matoba T, Suzuki J, O'Dell MR, Nigro P, Cui Z, Mohan A, Pan S, Li L, Jin ZG, Yan C, Abe J, Berk BC. Cyclophilin A mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation*. **2008**; 117: 3088-3098.

106. Suzuki J, Jin ZG, Meoli DF, Matoba T, Berk BC. Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells. *Circ Res*. **2006**; 98: 811–817.

107. Donato R. Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochim Biophys Acta.* **1999**; 1450: 191–231.

108. Yang Z, Tao T, Raftery MJ, Youssef P. Proinflammatory properties of the human S100 protein S100A12. *J Leukoc Biol*. **2001**; 69: 986–994.

109. Passey RJ, Xu K, Hume DA, Geczy CL. S100A8: emerging functions and regulation. *J Leukoc Biol.* **1999**; 66: 549–556.

110. Silacci P, Mazzolai L, Gauci C, Stergiopulos N. Gelsolin superfamily proteins: key regulators of cellular functions. *Cell Mol Life Sci.* **2004**; 61: 2614–2623.

111. Spinardi L, Witke W. Gelsolin and diseases. *Subcell Biochem.* **2007**; 45: 55–69.

112. Ito H, Kambe H, Kimura Y, Nakamura H. Depression of plasma gelsolin level during acute liver injury. *Gastroenterology* . **1992**; 102: 1686–1692.

113. Lind SE, Smith DB, Janmey PA, Stossel TP. Depression of gelsolin levels and detection of gelsolin-actin complexes in plasma of patients with acute lung injury. *Am Rev Respir Dis.* **1988**; 138: 429–434.

114. Smith DB, Janmey PA, Sherwood JA, Howard RJ, Lind SE. Decreased plasma gelsolin levels in patients with Plasmodium falciparum malaria: a consequence of hemolysis? *Blood*. **1988**; 72: 214–218.

115. Osborn TM, Verdrengh M, Stossel TP, Tarkowski A, Bokarewa M. Decreased levels of the gelsolin plasma isoform in patients with rheumatoid arthritis. *Arthritis Res Ther*. **2008**; 10: R117.

116. Suhler E, Lin W, Yin HL, Lee WM. Decreased plasma gelsolin concentrations in acute liver failure, myocardial infarction, septic shock, and myonecrosis. *Crit Care Med.* **1997**; 25: 594–598.

117. Bubb MR, Yarmola EG, Gibson BG, Southwick FS. Depolymerization of actin filaments by profiling, effects of profilin on capping protein function. *J Biol Chem*. **2003**; 278: 24629–24635.

118. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature.* **2002**; 420: 629–635.

119. Bishop AL, Hall A. Rho GTPases and their effector proteins. *Biochem J.* **2000**; 348: 241–255.

120. Hall A. Rho GTPases and the Actin cytoskeleton. *Science* **1998**; 279: 509–514.

121. Seufert W, Jentsch S. In vivo function of the proteasome in the ubiquitin pathway. *EMBO J.* **1992**; 11: 3077–3080.

122. Richter RB, Heinemeyer W, Wolf DH. The proteasome/multicatalytic-multifunctional proteinase: in vivo function in the ubiquitin-dependent N-end rule pathway of protein degradation in eukaryotes. *FEBS Lett.* **1992**; 11: 192–196.

123. Nielsen MJ, Petersen SV, Jacobsen C, Oxvig C. Haptoglobin-related protein is a high-affinity hemoglobinbinding plasma protein. *Blood* . **2006**; 108: 2846–2849.