UNIVERSITÀ DI PISA

PhD School in
ENDOCRINE, METABOLIC and PSYCHIATRIC SCIENCES

Course in
NEUROBIOLOGY and CLINICAL MEDICINE of AFFECTIVE DISORDERS

SSD: BIO 10

Thesis

A multidisciplinary approach to study the effects of balneotherapy and mud-bath therapy treatments on Fibromyalgia.

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THESIS FOR THE DEGREE OF PHILOSOPHIAE DOCTOR IN NEUROBIOLOGY AND CLINICAL MEDICINE OF AFFECTIVE DISORDERS
2013

INDICE

ABSTRACT

Fibromyalgia (FM) is a chronic non-inflammatory musculoskeletal disorder characterized by a variety of symptoms related to pain. A wide range of other unspecific symptoms may characterize the disease including fatigue, sleep disturbances, mood disorders, morning stiffness, 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 decade, many attempts have been carried out for the research of specific biomarkers in FM, but, at present, there are no specific markers, the diagnosis is basically clinical. The complexity of the disease means that there isn't a therapeutic treatment standards and this is demonstrated by the fact that pharmacological treatments are often applied in combination with non-pharmacological treatments. Among non-pharmacological interventions the most used there is the spa therapy, which includes hydrotherapy, balneotherapy, physiotherapy, mud-pack therapy and exercise.

In the present work, to study the effects of both balneotherapy and mud-bath therapy treatments in patients affected by FM, we used rheumatological, psychiatric, biochemical and proteomic approach.

Forty-one FM patients (39 F, 2 M), who fulfilled the American College of Rheumatology criteria received 2-week thermal therapy program comprising therapy once daily for 6 days/week. Twenty-one patients received mud-bath treatment, while the other twenty balneotherapy. Pain, symptoms, and quality of life were assessed. Oxytocin, brain-derived neurotrophic factor (BDNF), ATP and serotonin transporter levels during therapy were assayed by commercial kits. Comparative whole saliva proteomic analysis was performed using a combination of 2-D and nano-LC-ESI-MS/MS spectrometry techniques. The Elisa kit was performed to validate different expression of transaldolase, phosphoglicerate mutase1 and zinc alpha-2-glycoprotein1

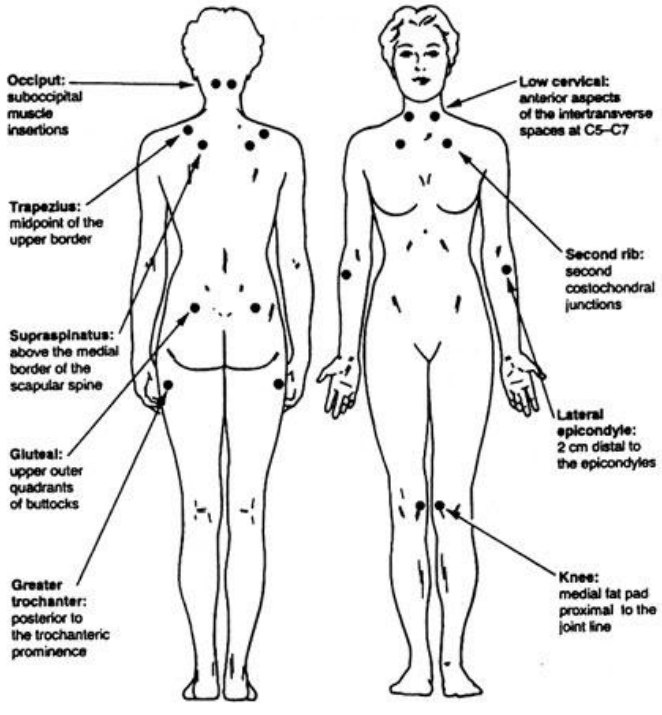
proteins. We observed reduction in pain, FIQ values and improvement of SF36 in both groups of patients treated with mud-bath or balneotherapy. The improvement of the outcome measures occurred with different timing and duration in the two spa treatments. A significant decrease of neuropeptide concentrations was observed either after balneo-theraphy or mud-bath therapy when assayed after twelve weeks, while no significant change of oxytocin levels was detected. Significant differences were observed for PGAM1 and zinc-alpha 2 glycoprotein protein expression.

Our results showed that the thermal treatment might have a beneficial effect on the specific symptoms of the disease. In particular, while balneotherapy gives results that in most patients occur after the end of the treatment but which are no longer noticeable after 3 months, the mud-bath treatment gives more lasting results.

INTRODUCTION

Fibromyalgia

Fibromyalgia (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, Sjogren'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 (Fig.1), the patient's report of pain in at least 11 of these tender points completes the requirements for the diagnosis of FM.



Fibromyalgia Tender Points
General locations of the 18 tender points that make up the criteria for identifying fibromyalgia.

A wide range of other unspecific symptoms may characterize the disease including fatigue, sleep disturbances, mood disorders, morning stiffness, anxiety, depression, cognitive dysfunction (e.g., memory problems, concentration difficulties, diminished mental clarity), sicca symptoms, sexual dysfunction and autonomic complaints, such as irritable bowel and bladder syndromes [2], [3], [5]. FM syndrome exists in all ethnic groups and is not limited to affluent or industrialized nations. Current estimates suggest that between 2 and 4% of the general population suffer from the syndrome and the condition is more common amongst women than men, while representing 30% of rheumatic diseases [6],[7]. The most recent estimates from the United States suggest that FM affects about 5% of all women, and is the third most common rheumatic disorder after low back pain and osteoarthritis [8]. In a more recent study from Europe, the estimated overall prevalence of FM was 4.7% for chronic widespread pain, and was 2.9% when stronger pain and fatigue criteria were simultaneously used. 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 [9]. The disorder may be dormant for years until triggered by infection, injury, physical or emotional stress or sleep disturbance [10]. The development of FM often leads to a premature retirement, to limitation of physical activity and waste of years with an acceptable quality of life, as well as the highest rate of medical consultations. For such reasons, FM represents a major socio-economic problem [11], and therefore, efforts should be directed towards the identification of specific diagnostic tests and specific treatment to reduce pain and disability.

Etiopathogenesis

In the last decade, significant improvements have been made in the knowledge of the mechanisms involved in the altered pain threshold of FM patients [12], [13]. FM clearly differs in pathogenesis from most other rheumatological disorders in which autoimmunity plays a central role. Nevertheless, many attempts have been made to identify immunological markers in FM, leading on the whole to inconclusive results. Several studies have demonstrated increased levels of antiserotonin antibodies in FM, but these antibodies do not appear to have diagnostic relevance. Other nonspecific autoantibodies that have been studied in FM include antiganglioside antithromboplastin antipolymer antibody, anti-68/84, and anti-45kDa. These antibodies may represent nonspecific immune activation, and hitherto none has been useful either clinically or as a research end point. Since FM is characterized by widespread pain, and the origin of pain is inflammation, many studies have focused on the inflammatory hypothesis for FM, although FM is generally regarded as a non-inflammatory disease. Special attention has been paid on circulating pro-inflammatory cytokines as possible markers in FM patients [17]. The interleukin (IL)-8 has been consistently demonstrated to be increased in FM patients in several studies and was correlated with severity of pain [14], the same is true for IL-6, which is associated with hypersensitivity to pain, fatigue and depression. The environmental factors may play a prominent role in triggering the development of FM and related conditions. Environmental 'stressors' temporally associated with the development of FM or chronic fatigue syndrome (CFS) include physical (and particularly skeletal) trauma, certain infections (e.g. hepatitis C, Epstein–Barr virus, parvovirus, Lyme disease), emotional stress and other regional pain conditions or autoimmune disorders [15]. The environmental factors may be relevant, but often are not enough to trigger the disease. In fact, extensive studies have shown that genetic factors may predispose individuals to FM. Recent familial studies have

suggested an underlying genetic susceptibility on which environmental factors trigger the expression of symptoms. Thus, first-degree relatives of individuals with FM display an eightfold greater risk of developing FM compared with the general population [14]. The principal gene polymorphisms supposed to be a risk factor for FM are those implicated in mood disorders but results are often controversial. Polymorphisms in the serotonergic 5-hydroxy tryptamine 2A receptor (T/T phenotype), the dopamine 4 receptor and the catecholamine o-methyl transferase enzyme (COMT), the serotonin transporter (5-HTT) have therefore been detected at higher frequencies in patients with FM [12]. In particular, the 5-HTT, which cleaves the synapse from the neurotransmitter, plays a crucial role in the termination and fine-tuning of serotonergic transmission. Alterations in 5-HTT function or expression may be involved in the pathogenesis of different disorders, especially those in which there are depressive symptoms. Offenbaecher and colleagues were the first to analyze the genotypes of the 5-HTT promoter locus (5HTTLPR) in patients with FM. They found that the S/S genotype of 5-HTT occurred more frequently in FM patients than in healthy controls. This association seems to be interesting considering that 5-HTT is involved in many conditions that are either risk factors for —or frequent concomitants to— FM, such as anxiety, Bipolar Disorder, Psychosis, attention deficit hyperactivity disorder, and Major Depressive Disorder [17].

FM is considered to be a prototypical example of a central sensitization syndrome. The term “central sensitization”, introduced by Woolf in 1983, implies a condition in which the central nervous system over-reacts to a variety of stimuli reaching it, thus causing what would normally be perceived as innocuous to end up being interpreted as painful and unpleasant. This basic neuro-physiological condition has been demonstrated, among other modalities, by the increased response of the central nervous system (CNS) to experimental stimulation, documented through sophisticated neuro-imaging techniques,

as well as by the decreased capacity of the brain to inhibit and modulate incoming pain signals [18].

FM is often associated with increased prevalence of depressive symptoms, major depression and anxiety. The cause and pathophysiology of FMs is unclear; pathophysiological hypotheses include impairment in the functioning of the hypothalamic-pituitary axis and alterations in specific neurotransmitters such as substance P, N-methyl-D-aspartate (NMDA), noradrenaline (norepinephrine) and serotonin (5-HT) [19]. Stressful experiences lead to depression in some people who are already genetically predisposed, and increase the probability of FM exordium.

Various areas in the CNS are responsible for inhibiting ascending pain transmission within the spinal cord through the activity of inhibitory neurotransmitters, which include 5-HT, norepinephrine, enkephalins, γ-amino butyric acid and adenosine. A decrease in this pain inhibitory loop is an important component of central sensitization syndrome [16].

The Other Aspects of FM

- ***Oxytocin and FM***

The neuropeptide oxytocin is a nonapeptide produced in the paraventricular (PVN) and supraoptic (SO) nuclei of the hypothalamus. It is well known that oxytocin is widely in many areas of the central nervous system and the spinal cord. Its effects include both hormonal and neuronal processes. Among the latest, oxytocin may modify behavior of learning and memory. Under physiological conditions oxytocin from the magnocellular neurons is mainly transported from the PVN to the posterior lobe of the pituitary and there released into the vein system and the circulation. In contrast, oxytocin acting as a neuropeptide/neuromodulator is released from parvocellular neurons projecting

elsewhere to various other brain regions. These neurons also project to the spinal cord, where they terminate on the presynaptic neurons of the sympathetic system in the area where pain modulation takes place. This latter function of oxytocin is not likely to contribute to changes in peripheral plasma oxytocin concentrations. Oxytocin may also act as a powerful stimulant for the secretion of adreno-corticotropin hormone (ACTH) at the anterior lobe of the pituitary. This is of interest, as perturbations in the hypothalamic-pituitary-adrenal (HPA) axis have been seen in FM patients. It has therefore been hypothesized that long-standing stress might be one important factor behind the pathophysiological mechanisms in the development to the FM. Oxytocin is known to have anti-nociceptive effects: it has been shown that a continuous infusion of oxytocin could increase thresholds of visceral sensory perception in Irritable Bowel Syndrome (IBS) patients through a modulation of the activity of afferent nerve pathways. Moreover, in a patient with opiate resistant cancer pain, the injection of oxytocin into the third ventricle was reported to result in effective analgesia [21]. The other studies also has revealed anxiolytic and sedative effects as well as anti-depressive and anti-stress effects of oxytocin. Differences of serum oxytocin expression in FM patients has been reported by Anderberg and colleagues [22] which suggested that depressed FM patients have a reduced level of neuropeptide with respect to non-depressed FM patients. This is of interest, if we consider that often the onset of FM coincides with the advent of some kind of physical stress and/or emotional. Moreover, the steroid hormones, and estrogen in particular, stimulate the synthesis of oxytocin and the affinity to its receptors. The effect pattern of oxytocin as well as the dependence of oxytocin on estrogen make oxytocin of special interest in FM research considering that 90% of the patients struck by the disorder are women and many of the patients have their onset of the disorder in their fifth to sixth decade when estrogen levels decline [22].

❖ ***BDNF and FM***

Neurotrophic factors and neurotransmitters are two major classes of intercellular signals that play a key role in regulating synaptic plasticity and in cell survival throughout life. Brain-derived neurotrophic factor (BDNF) is a member of the nerve-growth factor (neurotrophin) family. By promoting neurogenesis, synaptic plasticity and cell survival, BDNF plays a pivotal role in the development and plasticity of the brain. Recent research has identified BDNF and 5-HT as two prominent signals that act in concert to regulate aspects of neural plasticity in multiple brain regions. In the sensory system BDNF is produced by a subset of primary sensory neurons (nociceptors), which are located in the dorsal root ganglia (DRG), that respond to tissue injury. BDNF is then transported in the dorsal horn where it can be released and activate trkB receptors that is coupled to activation of phosphatidylinositol-3-kinase and protein kinase B (Akt). Activation of trkB receptors by BDNF modulates two types of synapse: the first pain synapse in the spinal cord and the synapses in the CA1 area of the hippocampus. Furthermore, exogenous BDNF facilitates the release of glutamate in the hippocampus and GABA in the spinal cord [23]. During development of the cerebral cortex and hippocampus, BDNF induces the differentiation of neural stem cells into neurons and promotes the survival of newly generated neurons. BDNF signaling at synapses enhances long-term potentiation (LTP), a process of synaptic strengthening associated with learning and memory. BDNF also plays an important role in preventing death of neurons during development, and promotes cell survival during stressful conditions [24]. Recent findings have suggested an involvement of BDNF as a neuromediator of hyperalgesia and spinal central sensitization. BDNF seems to have a role in neurodegenerative disorders [24], in epileptic and psychogenic non-epileptic seizures [25], and in Major Depression Disorder, but about this, there are conflicting results. Some studies have suggested an involvement of BDNF in the pathogenesis of major

depression which is often associated with pain syndrome, that at the level of Peripheral Nerve System (PNS) is mediated by BDNF [26]. Moreover, in animal and human studies, antidepressant treatments could increase central as well as peripheral BDNF levels. Recent studies have shown the significant increase of BDNF concentrations in serum [26] and in cerebrospinal fluid (CSF) [27] of FM patients when compared to healthy subjects and to chronic migraine, respectively. In a recent study [26], comparisons of FM patients with and without recurrent major depression as well as with or without antidepressive medication in low analgesic doses revealed no statistical significant differences, indicating that the found increase of BDNF serum concentrations in FM patients is independent of pre-existing major depression or antidepressive low dose medication [26]. However, BDNF levels were significantly correlated with the duration of chronic pain. This data are confirmed by Haas et al. [28] suggesting that BDNF is involved in the pathophysiology of abnormal pain syndromes.

There are different and controversial reports which discuss about the role of BDNF in the pathophysiological mechanisms responsible for the symptoms in the FM, particularly anxiety and depression. In fact, Nugraha et *al*. [29], [30] have demonstrated that BDNF serum concentration among FM patients were significantly different in depression subgroup instead that were not observed in the subgroup anxiety.

- ***SERT and FM***

Serotonin (5-hydroxytryptamine, 5-HT) is a modulating neurotransmitter produced by neurons located in the brainstem raphe nucleus; the axons of these 5-HT neurons innervate multiple cortical and subcortical brain regions to regulate an array of behaviors and physiologic variables, including sensorimotor control, cognition, mood, anxiety, sleep, food intake, and aggressive and impulsive behavior. As already said, 5-

HT and BDNF co-regulate one another and play an important role in neuronal survival and synaptic plasticity in the CNS and PNS [24], [26]. 5-HT, in particular, is theorized to have a function in stage 4 sleep and in the pain threshold. In this regard, Russell and colleagues found low levels of 5-HT and tryptophan (5-HT precursor) in serum [31] and in CSF [32] of group of FM patients respect to healthy controls, explaining, so, the use in the treatment of FM and related symptoms of all those drugs inhibiting reuptake of neurotransmitters. 5-HT exerts its actions by means of interaction with distinct receptors, which are differentiated on the basis of structures, molecular mechanisms and pharmacological profiles. In the past few decades, much attention has been focused on the specific protein which promotes the 5-HT reuptake into the pre-synaptic terminals, once released in the synaptic cleft, the so-called 5-HT transporter (SERT) which control the endogenous activity of 5-HT. For this activity, the SERT is the main target of antidepressants (SSRIs) such as paroxetine and fluoxetine. Moreover, it has been postulated that serotonergic neurotransmission has a significant function in nociception; alterations in 5-HT metabolism and transmission might therefore be important in the pathogenesis of FM. These findings support the proposal that aberrant pain perception in FM also results from an instability of the 5-HT system in FM. The SERT is widely expressed in intestinal epithelial cells, in central or peripheral serotonergic neurons and in platelets [19]. Since blood platelets and neurons share a similar reuptake system of 5-HT, platelet SERT has become a useful peripheral model of presynaptic serotonergic activity, particularly after the demonstration of its identity with the same structure expressed in the CNS [33]. Studies concerning the expression and function of SERT by estimating the number of binding sites for [^{3}H]-paroxetine and the uptake of serotonin, have shown an involvement of the serotonergic system in psychosis [34], eating disorders, such as bulimia and anorexia [35], [36] and in FM [19] with a reduction in the density of binding sites for platelet paroxetine coupled to a reduced rate of re-uptake

of 5-HT. In particular, recent clinical study have demonstrated that FM patients have fewer SERTs expressed on the cellular membrane than healthy subjects and a deficit in functionality demonstrated by a decrease in transport rate [19]. A reduced density and rate of SERT are consistent with previous observations indicating that levels of 5-HT are altered in patients with FM.

- ***ATP and FM***

All living things, require a continual supply of energy to keep the organism alive. Some of these processes occur continually, such as the metabolism of foods, the synthesis of large biologically important molecules (e.g. proteins and DNA), and the transport of molecules and ions throughout the organism. Other processes occur only at certain times, such as muscle contraction.

The Adenosine 5'-triphosphate (ATP) performs this function acting as a special carrier of energy given by its chain of phosphate groups. The ATP is produced by mitochondria, through the ATP synthase localized on their inner membrane and forming part of the oxidative phosphorylation system. It is one of the end products of photophosphorylation, cellular respiration, and fermentation and used by enzymes and structural proteins in many cellular processes, cellular respiration (as a coenzyme) biosynthetic reactions (DNA, RNA and proteins), motility and cell division (maintenance of cell structure and for muscle contraction) and cell signaling. In a last decade, in parallel with neuroendocrine studies also tissue ultrastructural analyses have been carried out in affected subjects: electron microscopy has revealed disorganized fibers and actin filaments, altered mitochondria, defects in capillary microcirculation, glycogen and lipid accumulation together with DNA fragmentation in muscle biopsies of FM patients [37]. Moreover, the use of ^{31}P magnetic resonance spectroscopy has

allowed the analysis of biochemical substrates in the FM muscle: higher concentrations of phosphodiesterase products and inorganic phosphate, have been observed in patients in comparison to controls, together with a decrease in phosphocreatine, ATP and phosphocreatine/inositol phosphate ratio in the quadriceps, confirming previous results in trapezius biopsies of FM patients. A reduction of ATP levels in FM subjects than healthy subjects is confirmed on platelets [37] suggesting a bioenergetics alteration in FM.

Diagnosis of FM

The mechanisms underlying the FM are still unclear and largely unknown, and as a direct consequence, the diagnosis is very difficult. The difficulty 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 such as depression, anxiety but also intense pain, chronic fatigue and sleep disturbances. 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 [38]. The Fibromyalgia Impact Questionnaire (FIQ) (Table 1) gives a good idea of the full range of symptoms that are regularly found. 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 [38]. 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 [13] and the lack of easily

accessible laboratory measures makes difficult to collect under the term of FM, patients presenting with homogeneous features and prognosis [39], [40].

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?

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.

To date, the diagnosis of FM is performed according to the criteria established by the American College of Rheumatology (ACR) in 1990 and by new diagnostic criteria have been proposed by Wolfe and colleagues [41] in 2010. The interpretation of these criteria is subject to criticism: a limit on net eleven "tender points" could give rise to contradictions as each and every muscle tendon insertion is sore subject FM and tenderness of the various tender points may vary spontaneously, day after day. 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 [13].

Pharmacological treatment

The complexity of the disease means that there isn't a therapeutic treatment standards and this is demonstrated by the fact that pharmacological treatments are often applied in combination with non-pharmacological treatments. Current evidence suggests a multifaceted treatment program based on patient education, medications to improve symptoms, and the aggressive use of exercise and cognitive-behavioral approaches to retain or restore function. Therefore, the treatment of FM symptoms requires a multimodal approach that has to consider somatic aspects (i.e., pain onset, location, quality, quantity, duration), emotional aspects (i.e., mood and anxiety), cognitive aspects (i.e., coping styles, beliefs about pain), and environmental aspects (i.e., social context and patient relationships). From the pharmacological point of view, categories of drugs most commonly used are: analgesics, antidepressants, muscle relaxants and anti-epileptics. duloxetine and milnacipran, two selective serotonin and norepinephrin

inhibitors, pregabalin (antiepileptic drug), and alpha2-delta agonist, have been approved by Food and Drug Administration for the treatment of FM symptoms. On the contrary, The European Medicines Agency did not approve any specific treatment for the symptoms of FM up to now. These drugs and a variety of other compounds are used for the management of FM based on their clinically meaningful and durable effect on pain in monotherapy trials and their beneficial effect on other symptom domains, such as fatigue, sleep alterations, cognition, and function [58]. The results of clinical trials of anti-inflammatory medications have been generally disappointing, but it has been found that the Tramadol (with or without acetaminophen) is effective in FM subjects [59]. Tramadol has multiple analgesic effects: it inhibits norepinephrine and serotonin reuptake, and its major metabolite binds weakly to opioid receptors. Opioids may be helpful in treating FM pain but may induce tolerance and become habit forming and are also associated with adverse effects such as constipation, sedation, and nausea. Their use should be considered only after all other medicinal and non-medicinal therapies have been tried [59]. Antidepressants may restore neurotransmitter levels and modulate receptor expression in the hypothalamus, which normalizes hyperactivity of the HPA axis. Antidepressants that increase 5-HT- and NE-mediated neurotransmission are frequently used to treat FM and other chronic pain, particularly neuropathic pain. Inhibiting both 5-HT and NE reuptake transporters with tricyclic antidepressants (TCAs) or serotonergic and noradrenergic reuptake inhibitors (SNRIs) seems to be more effective in treating pain (and FM in general) than inhibiting either transporter alone with selective serotonergic (SSRIs) or noradrenergic re-uptake inhibitors. However, the efficacy of TCAs is counterbalanced by their side effects. Among the many muscle relaxants, those who have demonstrated greater effects are cyclobenzaprine and tizanidine. Finally, among the antiepileptics, are used the Alpha2-delta ligands (i.e., gabapentin and pregabalin) that act at a number of sites that may be relevant to pain,

and particularly limit neuronal excitation and enhance inhibition [58]. Finally, in pharmacological treatment, are sometimes used antagonists of N-Methyl-D-Aspartate (NMDA) receptors, which may play a key role in the nervous system reorganization thought to be involved in maintaining chronic pain, and its blockade can relieve pain in patients with FM [59].

Non-pharmacological treatment

In recent years, at least three sets of guidelines have been developed by different medical organizations in an attempt to standardize the treatment of this condition (American Pain Society, European League Against Rheumatism, Association of the Medical Society of Germany). The current recommendations suggest that the optimal treatment of FM requires a multidisciplinary approach with a combination of non-pharmacological and pharmacological treatment modalities tailored according to pain intensity, function, associated features, such as depression, fatigue and sleep disturbances, decided through discussion with the patient, so the main strategy is symptom management [59]-[62]. Current therapeutic strategies are aimed primarily at reducing pain and other symptoms that the disease involves (sleep disorders, anxiety, depression, etc..). In recent years there has been in-depth study regarding the complementary and alternative medicine as a possible solution curative or at least, possible relief for all patients because of debilitating symptoms have seen their daily lives strongly affected. Among non-pharmacological interventions the most used are physical therapy, psychotherapy, acupuncture, massage, and balneotherapy [63]-[66]. In the context of complementary medicine, the spa therapy, which includes hydrotherapy, balneotherapy, physiotherapy, mud-pack therapy and exercise [67]-[69], is known to be one of the most recognized therapeutic strategies for the treatment of rheumatic (as well as

dermatological) disorder to pain alleviation [70]. The efficacy of this treatment for these indications underscores the applicability of spa therapy for the treatment of FM [71] but despite the long history and popularity, there are not many randomized controlled trials that demonstrate their effect on FM patients [71], and only a few were paralleled by a biological evaluation. Despite there are several articles on the effects of thermal therapies on FM management, its role in modern medicine is still not clear. About the spa treatments, balneotherapy and mud-bath therapy are the most popular methods used, and several studies are aimed at understanding the physiological mechanisms that determine these treatments in patients with FM. In Table 2 are represented the most important randomized controlled trials performed in FM in the last years[69]. Despite patients who were enrolled in these studies relate the beneficial effects of spa treatments, although in short term, in the reduction of pain, it is difficult to distinguish the effects of thermal applications from the benefits that could be derived from a stay in a spa environment [72]. The net benefit is probably the result of a combination of factors, among which, mechanical, thermal, and chemical effects are most prominent. A distinction can be made between the non-specific (hydrotherapeutic in a broad sense) mechanisms of simple bathing in hot tap water, and the specific (hydromineral and crenotherapeutic) mechanisms, which depend on the chemical and physical properties of the water used [69]. In recent years, several hypotheses have been formulated on the beneficial effect of the baths and , in particular, they have focused attention on the inflammatory process and the release of all those mediators (cytokines) involved in the inflammatory response. Recent studies have posited a connection between cytokines and certain symptoms of FM (e.g., sleep disorders, hyperalgesia, cognitive dysfunctions, fatigue, stress and anxiety), and it also appears that these molecules may play a role in the communication between the immune and nervous systems [73]. Particularly, IL-6 and IL-8, whose release is caused by substance P, seem

to have an important role in generating the symptoms of FM, since IL-8 promotes the pain by sympathetic while the IL-6 is associated with hypersensitivity to pain , fatigue and depression. It has been shown, in fact, that the condition of major depression, for example, is followed by the activation of the inflammatory response with a consequent release of pro-inflammatory cytokines mentioned above, and therefore, the FM patients are treated with antidepressants who own the aim to suppress the production of IL-8 and IL-6 as promoters of inflammation, and thus promote the release of anti-inflammatory cytokines such as IL-10 [74]. The profile of pro- and anti-inflammatory properties has attracted considerable attention in the scientific field, and not by chance are trying to identify possible markers of inflammatory cytokines such as FM. In addition to high levels of IL-8, in diseased subjects were also found high levels of TNFα that would seem to be another very important factor in the inflammatory process underlying the FM [73], [75]. The anti-inflammatory effect of balneotherapy has been assessed on the basis of serum levels of some biochemical factors in patients; it is shown that in arthrosis treatments such as mud baths can reduce the serum levels of IL-1 [76]. The mud-balneotherapy therapy appears to have a positive effect on pro-inflammatory factors particularly involved in the genesis of pain, once again reducing the serum levels, we speak in this regard of prostaglandins PGE2 and leukotriene LTB4 [76].

Table 2. Main characteristics of studies with spa therapy.

Authors	Sample size	Intervention	Outcome measures	Follow-up	Results
Buskila [77]	A: 24 B: 24	A: Bal. (20 min daily for 10 days at 37 °C) B: No treatment	VAS FIQ, TPC, Dolorimeter, FDI	3 months	Significant between group improvement in pain and TPC in favour of A, still seen after 3 months
Neumann[78]	A: 24 B: 24	A: Bal. (20 min daily × 10 days at 37 °C) B: No treatment	SF36, AIMS, VAS	3 months	Significant improvement in most subscales of the SF36 for both groups. The improvement in physical components of the QoL index lasted 3 months, whereas improvement in measures of psychological well-being was of shorter duration. Subjects in group A reported greater and longer lasting improvement than subjects in the group B
Evcik[79]	A: 22 B: 20	A: Bal. (20 min × 5days/week for 3 weeks at 36 °C) B: No treatment	VAS, FIQ, TPC, BDI	6 months	The group A showed statistically significant improvement in TPC, VAS, FIQ and BDI at the end of the therapy and this improvement persisted at 6 months except for BDI
Dönmez[80]	A: 16 B: 14	A: Spa therapy (thermal pool baths 20 min × 6 days/week for 2 weeks at 36 ± 1 °C, pressurizzed shower at 37°C or classical massage for 15min each on altermate days) B: No treatment	VAS, FIQ, TPC, BDI	9 months	Significant improvement in pain, TPC and FIQ for group A. The pain and TPC results persisted for up to one month and the FIQ results for up to 6 months
Ardiç[76]	A: 12 B: 12	A: Bal. (20 min × 5 days/week for 3 weeks at 37 °C) B: No treatment	VAS, TPC, FIQ, BDI	3 weeks	Statistically significant improvement in VAS, BDI, TPC and FIQ was only found in group A at the end of the treatment cycle
Fioravanti[72]	A: 40 B: 40	A: Mud-packs (15 min daily for 2 weeks at 45 °C) and baths (10 min daily for 2 weeks at 37 °C–38 °C) B: No treatment	FIQ, TPC, VAS, AIMS, HAQ	16 weeks	In group A, a significant improvement in all parameters was recorded after mud-pack therapy and after 16 weeks
Özkurt[81]	A: 25 B: 25	A: Bal. (20 min twice/day for 2 weeks at 36 °C ± 1 °C) B: No treatment	VAS, FIQ, BDI, PGA, IGA, SF-36, TPC	3 months	Statistically significant improvement was recorded in group A for all outcome parameters at the end of the treatment cycle and after 3 months, except for BDI and IGA
Bazzichi[82]	A:20 B:21	A: Bal. (20min/day for 2 weeks at 38 °C) B: mud-pack (10min daily at 47 °C) + Bal. (10min daily at 38 °C) for 2 weks	FIQ, SF-36, HAQ, FACIT, other biological parameters	12 weeks	Group B showed a significant improvement of the VAS pain and SF-36. Group A showed a slight improvement of VAS pain and FIQ. Significant decrease of BDNF in both group. Group B showed a decrease of PGAM1 and aZGP1 protein.

Abbreviations: Bal, Balneotherapy; VAS, Visual Analogue Scale; PAS, Pressure Algometric Scores; FIQ, Fibromyalgia Impact Questionnaire; TPC, Tender Point Count; FDI, Functional Disability Index; SF36, Short Form-36; AIMS, Arthritis Impact Measurement Scales; BDI, Beck Depression Inventory; HAQ, Health Assessment Questionnaire; PGA, Patient global assessment; IGA, Investigator's Global Assessment. FACIT, Functional Assessment of Chronic Illness Therapy-Fatigue Scale

Although not totally proven, alpha1-antitrypsin (AAT) gene deficiency (AATD) are suspected to contribute to FM pathogenesis. One study focused on the effects of AAT on two women AATD and affected by FM, showed a possible relationship between AAT deficiency and the disease itself, it was observed that a therapy aimed at increasing the levels of AAT could effectively control symptoms of FM [83], [84]. The non-pharmacological therapies have certainly not proven the effectiveness of the practices which, however, are still widely exploited for the benefits it can bring, but despite this, intensive testing is still underway to try to understand the mechanisms that generate the FM and which impact the different therapeutic profiles have on it.

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 [85]. The proteome was originally defined as the complete protein complement expressed by a genome [85]. 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 [86]. 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. 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 [87].

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 [88].

Sample preparation.

Preparation of samples for 2-D PAGE involves solubilization, denaturation and reduction to completely break up the interactions between the proteins [89]. 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 [90]. 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 [91]. 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 [90]. 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 [92]. 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 [90]. 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 [90],[93].

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 [94]. 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 [94]. The use of commercially prepared IPG DryStrip, introduced by Bjellqvist et al. [95]. and Gorg et al. [96] eliminates these variations. The pH gradient is immobilized by covalent incorporating Immobiline® acrylamide 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 [94], [97]. Further, pI resolution to 0.01 pH unit can be achieved [95]. 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 [94].

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 [96] 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 [98]. IAA is added to the equilibration buffer in order to remove excess DTT (responsible for the "point streaking" in silver stained patterns) [99]. 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 [90]. 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 [100], [101]. Today, among the variety of methods used for protein detection after gel electrophoresis, fluorescent methods offer an interesting compromise, especially for

detection linearity and for compatibility with mass spectrometry [102]. Stained spots excised from the electrophoresis gel can be identified by mass spectrometry.

Mass Spectrometry, nanoLC-ESI-MS/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 the increase in sensitivity (until 1000fold) and the speed of analysis. Today, the Nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC–MS/MS) has become an essential tool in the field of proteomics, due to your enormous analytical advantages when dealing with sample-limited situations. Tandem mass spectrometry has been used as a microscale de novo sequencing tool for peptides because collision-induced dissociation (CID) followed by product ion scanning provides systematic fragment information of amino acid sequences. Further improvement in peptide sequencing sensitivity was accomplished by the development of nano electrospray combined with a peptide sequence tag approach for protein identification in databases. Because even one peptide is sufficient to identify a unique protein, this approach is more powerful for protein identification in proteome-scale experiments than the peptide fingerprinting approach where several peptide masses from one protein are used for identification [103]. For proteomic analysis, proteins are normally first cleaved into peptides by enzymatic digestion (i.e., usually by trypsin) and subsequently, separated by reversed phase (RP) Nano LC before data-dependent MS/MS analysis [104]. The typical microcolumns for nanoLC are prepared using RP materials with a 3-10μm diameter packed into fused silica capillaries with a 12-100μm diameter. In ESI-

MS, a spray needle is used as a restrictor for packed particles to prepare a fritless column, to minimize the post-column dead volume [103]. Generally, smaller colums at a lower flowrate combined with real nanoelectrospray conditions give higher sensitivity. For peptides, in combination with C18 stationary phases, acidic conditions are usually used: Trifluoroacetic acid (TFA) is one of the most popular reagents because of higher peak capacity with smaller peak width while Acetonitrile or Methanol have been used as an organic solvent in off-line infusion. Finally, because of the low flowrate, and the small size ranges of proteomic samples (<100µl), trap columns are useful to reduce the injection time. 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.

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 [105] 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 [105]. 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 [106]. 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[106]. 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 [105], [107]. 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 [105]. 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 [107].

Proteomic Biomarkers and FM

In the last few years, several attempts have been carried out to identify specific biological markers in FM, but so far no tests have proven to be of diagnostic validity. Recently, our research group focused its attention on the proteomic profiling of whole saliva (WS) in FM patients respect to healthy controls, in order to evaluate salivary biomarkers [20]. Proteomic may be defined as a large spectrum study of the expression and identification of proteins expressed in one tissue or organism [42], [43]. This approach has already been used to explore biomarkers in different disorders, such as systemic sclerosis [44]-[46], Sjögren's syndrome [47]-[49], or as research tool in thyroid neoplasms [50]-[52] or FM [17], [20], [82]. In this case we observed the exclusive and

significant over expression of two enzymes in FM saliva: transaldolase and phosphoglycerate mutase I (PGAM1). 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) [53]. Many evidences have shown that oxidative stress and nitric oxide may play an important role in FM pathophysiology [54], but it is still unclear whether oxidative stress abnormalities documented in FM are the primary or secondary [55], [56]. 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, was differently expressed in FM patients [57], [20]. PGAM1 is an enzyme of the glycolytic pathway that catalyzes the conversion of 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG). This enzyme also promotes glycolysis and ATP production via the Citric Acid Cycle and the electron transport system, through its substrate and product (3PG and 2PG, respectively) modulates two other biosynthetic pathways derived from glycolysis: the oxidative branch of the pentose phosphate pathway and the serine biosynthesis pathway.

AIM

The present study describes the results of a multidisciplinary approach (rheumatological, psychiatric, biochemical, proteomic) of a research project entitled "Effects of balneotherapy treatment at Montecatini Terme spa on biochemical markers in fibromyalgia patients" funded by the Foundation for Scientific Thermal Research (FORST) and conducted in collaboration between the Montecatini spa (PT, Italy) and the University of Pisa (Department of Pharmacy and Department of Clinical and Experimental Medicine, Division of Rheumatology) for the evaluation of the effects of balneotherapy and mud-bath therapy on 41 patients affected by FM.

METHODS

Patients

Forty-one primary FM patients (39 female, 2 male) aged between 31 and 69 years participated in the study. The patients enrolling was conducted in steps: in the first step we consulted about 300 medical records in order to individuate those FM patients without circulatory problems or heart disease or other conditions which influence the inclusion in the study and who lived in areas adjacent to the spa. The patients were contacted by letter or by phone to explain them the nature of the study and give a first date of appointment. The response rate of patients with this first contact was 30%. The percentage of patients who were found unsuitable or that after the first visit did not come back was 10%. At the first visit the patients were clinically classified by a rheumatologist according to the 1990 ACR criteria [1], which include the following: pain for more than 3 months from all of the four body quadrants, axial skeletal pain and pain upon digital palpation of at least 11 out of 18 specific bilateral points. The exclusion criteria were represented by the presence of contraindication to any form of balneotherapy such as a peripheral vascular disease; having taken balneotherapy in the last 12 months; pregnancy or nursing. The demographic characteristics of 41 FM patients have been summarized in Table 2. During this first visit, the rheumatologist administered the clinical questionnaires. After fulfilling the inclusion criteria and obtaining the written informed consent, the patients were randomly assigned to balneotherapy treatment. The patients were randomized using a computer-generated random number list by an independent investigator, and allocated to either the mud-bath therapy or balneotherapy treatment. Almost all the selected patients came from areas near the spa and continued to live at home and carry out their daily routines, with the exception of two patients who preferred to stay in hotels close to the spa. Daily

treatment lasted 12 consecutive sessions except Sundays at MontecatiniTerme spa (PT, Italy, Salse-Sulphate-Alkaline waters, prevalently formed of sodium chlorides and sodium and magnesium sulphates [Na^{+}, Cl^{-}, Mg^{++}, So^{4--}], fixed residue at 180°C: 19.2 g/l). Twenty patients were treated with balneotherapy for 20 minutes a day and the other 20 patients were treated with mud-bath therapy. The mud was applied on the body surface at a temperature of 47°C for 10 min daily in the morning, followed by immersion in thermal water at 38°C for 10 min. After the spa therapy, a period of rest for both groups in a hot bathrobe followed. The entire daily treatment lasted an hour. Among the patients treated with balneotherapy, four did not take any medicines, four took antidepressants, one took anti-inflammatory drugs, four took analgesics, four took muscle relaxants and two took antiepileptics. Instead, among the patients treated with mud-bath therapy, four did not take any medicines, seven took antidepressants, four took anti-inflammatory drugs, one took analgesics, and two took antiepileptics. The patients were receiving drug treatment for at least three months, the doses were stable and no new drugs were added during the spa treatment.

Clinical evaluations

At baseline (T0), after 2 weeks (T1) and after 12 weeks (T2) the patients were clinically evaluated using the following outcome measures: the Fibromyalgia Impact Questionnaire (FIQ), the evaluation of tenderness at tender points by digital pressure, a 10-cm visual analogue scale (VAS) for pain and tiredness (0 indicates no symptoms whereas 10 is the worst condition), the presence of absence of minor symptoms of FM (fatigue, headache, sleep disturbances, gastro-intestinal symptoms and other symptoms), the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT fatigue) Scale (version 4), the Health Assessment questionnaire (HAQ) to determine physical

disability and the SF-36 questionnaire (ShortForm with 36 questions), a well-documented, self-administered quality of life (QoL) scoring system.

The FIQ (Fibromyalgia Impact Questionnaire) [107]-[109], is a questionnaire of 10 questions that assess the physical, occupational, depression, anxiety, sleep, pain, stiffness, fatigue and well-being in patients with FM. The higher scores indicate a worse quality of life. The HAQ ("Health Assessment Questionnaire") [110], [111] is used for patients with various rheumatic diseases: disability is assessed by 8 categories of questions designed to verify if the patient has difficulty in dressing, arising, eating, walking, bathing, reaching objects, shaking objects and doing activities.

The FACIT-Fatigue Scale questionnaire (Functional Assessment of Chronic Illness Therapy-Fatigue Scale) [112] is a collection of questions related to the quality of life aimed at chronic disease management. It consists of 13 questions that investigate the fatigue, the ability to perform everyday skills, the need for sleep, the need of help to perform daily activities, and how physical state affects the psychic state.

The health status questionnaire SF-36 [113] is a generic, multi-dimensional questionnaire articulated through 36 questions assembled in 8 different scales. The 36 questions relate conceptually to 8 health domains: PF-physical functioning (10 questions), RP physical role (4 questions), ER emotional role (3 questions), BP bodily pain (2 questions), GH general health (5 questions), VT vitality (4 questions), SF social functioning (2 questions) and MH mental health (5 questions). The higher scores are related to better health. Trained psychiatrists have evaluated the patients using the Structured Clinical Interview Scale (SCID) for DSM-IV [114], [115]. For the entire period of the study, the patients were recommended not to modify their pharmacological treatment, while the use of other drugs were not permitted. If serious adverse events occurred, the patients was excluded from the study.

Table 3. Tests of clinical evaluation used to classify each patient

Specific tests for evaluating FM
➢ Fibromyalgia Impact Questionnaire (FIQ)
➢ finger pressure of tender points
➢ Visual analogue scale (VAS) for minor symptoms in FM (e.g., fatigue, sleep disorders, migraine, stiffness in the morning, gastro-intestinal symptoms).
➢ Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT fatigue) Scale (version 4)
Evaluation of other parameters
➢ Health Assessment questionnaire (HAQ) to determine the physical disability
➢ Pittsburgh Sleep Quality Index questionnaire (PSQI) to determine quality and sleep disorders.
➢ SF-36 questionnaire (Short Form with 36 questions), a well-documented, self-administered quality of life (QoL) scoring system.
Assessment of Psychiatric aspects
➢ Structured Clinical Interview (SCID) for DSM-IV
➢ Experiences in Close Relationships (ECR)
➢ Jealousy questionnaire (QUEGE)

The study protocol has been approved by the Ethics Committee of the "Azienda Ospedaliera Universitaria Pisana".

Whole saliva samples: collection and preparation

Unstimulated whole saliva (WS) samples were collected early in the morning (between 8 and 10 a.m) in standard conditions, i.e. all the subjects were asked to be on an empty stomach, without having assumed any drinks or any kind of food (including gum or candies) since the night before. In order to minimize the degradation of the proteins, the samples were processed immediately and kept on ice during the process. Between 1-3 ml saliva was obtained from each subject. To remove the debris and the cells, a centrifugation at 14000 g for 30 min at 4 °C, was performed and the protein amount of resulting supernatants was estimated using a DC protein assay from Bio-Rad. Bovine serum albumin (BSA) was used as a standard. WS samples from individual patients (corresponding to 200µg) were solubilized with rehydration solution (7M Urea, 2M

thiourea, 4% CHAPS, 60 mM DTT, 0.002% bromophenol blue) filled up to 400µl and supplemented with 1.2% IPG Buffer pH3-10.

Blood samples preparation

Whole venous blood samples (30 ml) were drawn from overnight fasting subjects between 8:00 and 9:00 am at the division of Rheumatology, University of Pisa. The amounts of blood (5ml) were collected into serum tubes, and centrifuged at 200 x g for 15 minutes at 20-25°C for BDNF serum determination. The remainders were collected in vacutainers containing EDTA (1 mg/ml) as anticoagulant and processed as follows:

a) 10 ml was transferred to centrifuge tubes containing aprotinin (Sigma, Milan, Italy) (0.6 TIU/ml of blood) protease inhibitor then centrifuged at 1,500 x g for 15 minutes at 4°C and the resulting plasma was collected and kept at –80 °C until the oxytocin assay.

b) 10 ml was centrifuged at 200 x g for 10 minutes at 20-25°C (RT) obtaining platelet-rich plasma (PRP) and platelets were counted automatically with a flux cytometer (Cell-dyn 3500 system; Abbott, Milano, Italy). For [^{3}H]paroxetine binding, platelets were precipitated by centrifugation at 10000 x g for 10 minutes at 4 °C whereas for ATP assay, aliquots of PRP were centrifuged at 200 x g for 20 minutes at room temperature:

c) 5ml was centrifuged at 1,500 x g for 15 minutes at 4°C and the resulting plasma was collected and kept at –80 °C until the BDNF plasma assay.

Platelet membrane preparation.

At the time of the assay, the platelets were re-suspended in 10 volumes (*w:v*) ice-cold 5 mMTris-HCl buffer (pH 7.4) containing 5 mM EDTA and protease inhibitors (benzamidine 160μg/ml, bacitracine 200μg/ml; trypsine soy inhibitor 20μg/ml). After the homogenization by Ultraturrax, the samples were centrifuged at 48,000 g for 15 minutes at 4°C. The pellets were washed twice in 10 volumes (*w:v*) ice-cold 50 mMTris-HCl buffer (pH 7.4) and the resulting membrane pellets were suspended in the [^{3}H]paroxetine binding assay buffer (50 mMTris-HCl buffer pH 7.4, 120 mMNaCl and 5 mMKCl). The protein content was determined by the Bradford's method (Bio-rad), using γ-globulins as the standard.

[^{3}H]-Paroxetine binding assay

The SERT binding parameters (maximal binding capacity, B_{max}, fmol/mg protein; dissociation constant, K_d, nM) were evaluated in platelet membranes by measuring the specific binding of [^{3}H]-paroxetine. The [3H]-paroxetine Bmax represents the specific density (number) or the degree of SERT protein expression on platelet membranes of each enrolled subject, while KD being the main index of ligand-to-protein affinity [116]. Saturation experiments were carried out as follows: 100 μl of membranes (50–100 μg proteins/tube) were incubated in assay buffer (50 mM Tris-HCl, 5 mM KCl, 120 mM NaCl, pH 7.4) with five increasing concentrations of [^{3}H]-paroxetine, (0.08 -1.5 nM) in a final volume of 2 ml. Non-specific binding was performed, for each [3H]-paroxetine concentration point, in the presence of 10 μM fluoxetine, as displacer. Incubation was performed for 60 min at 22-24°C and halted by rapid filtration using Wathman GF/C glass fiber filters in a Brandell filtration apparatus. The filters were

washed three times with 5 ml ice-cold buffer assay and radioactivity was counted with a scintillation β-counter Packard 1600 TR. Specific binding was obtained by subtracting residual binding in the presence of 10 μM fluoxetine from total binding. The experiments was performed in duplicate.

Oxytocin Determination

Aliquots of plasma (6 ml) were immediately acidified with 6 ml of HCl 0.1 N and centrifuged at 48,000 x g for 10 minutes at 4°C. C-18 Sep-pack columns (Waters) were equilibrated by washing with 10 ml of methanol followed by 20 ml of water. Acidified plasma solutions were loaded into the equilibrated C-18 Sep-pack columns. Then the columns were washed slowly with 10 ml of 4% acetic acid followed by 2 ml of methanol and the washing liquids were discarded. Oxytocin was then eluted with methanol, the eluates were evaporated in a centrifugal concentrator under vacuum (Speedvac) and the remaining lyophilized samples were stored at –80°C.

Quantitative determination of Oxytocin in samples was measured by using a competitive Oxytocin Enzime Immunoassay kit (EIA kit, Enzo Life Sciences) according to the manufacturer's instructions. The optical density was read at 405 nm with a microplate reader (Victor Wallac, Perkin Elmer).The Lower Limit of Detection of these assays was less than 12 pg/ml.

Plasma and serum BDNF determination

The BDNF was measured in plasma and serum samples with commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions

(Promega, Emax ® ImmunoAssay System, Wallisellen, Switzerland). To measure the amount of total BDNF, plasma acidification and subsequent neutralization of the samples were followed before proceeding with the ELISA protocol, according to instruction. The absorbance was measured at 450 nm. The minimum of detection was of 15.6pg/ml of BDNF.

Intracellular adenosine 5'-triphosphate (ATP) determination

The ATP was released from platelet suspension using an Adenosine 5′-Triphosphate Bioluminescent Somatic Cell Assay Kit which follows the luciferin–luciferase reaction (Sigma-Aldrich St. Louis, MO). In the Eppendorf tubes, aliquots of platelet suspension (100 cells/50 μL) were incubated with 100 μL somatic cell ATP releasing reagent, 50 μL of sterile distilled water or 50 μL (200 ng) ATP standard solution (ATPstd). For luminescence analysis, a volume (100 μL) of these solutions was transferred into a 96 multi-well plate (OptiPlate™, Perkin-Elmer). After the addition of 100 μL of ATP assay mix, containing luciferin and luciferase, the luminescence light emission was directly measured by a Wallac 1420 Multilabel Counter (Perkin-Elmer, Inc.,CA) using a software-programmed for luminometry.

The sample platelet ATP amount (ATPs) was calculated by the equation:

$$ATPs = (ATP_{std}) \times (L_s)/L_{(s+std)} - L_{(s)}$$

where ATP_{std} was the ATP known concentration (in moles), added as internal standard, (I.S).; $L_{(s)}$ was the light emitted by "blank" sample without I.S., and $L_{(s+std)}$ was the light emitted by sample containing I.S.

2-D analysis

In a first phase, twenty randomly selected samples were pooled according to thermal treatment (balneotherapy or mud-bath therapy) into three groups corresponding to the three times of sample collection and 2-D analysis was performed in triplicate. Isoelectrofocusing (IEF) was carried out by using 18 cm Immobiline Dry-Strips (GE Healthcare) with a linear, pH 3–10, gradient. 200 µg of proteins were filled to 400 µl with rehydration buffer supplemented with 1,2% (v/v) IPG Buffer, pH 3–10 (GE Healthcare). IEF was performed at 16 °C on an EttanIPGphor II apparatus (GE Health Care) according to the following schedule: the samples were applied by in-gel rehydration for 24 h using the Reswelling Tray for the passive rehydration, then the Immobiline Dry-Strips were transferred onto Manifold and were applied a constant voltage at 200V for 2:30h and then at 300V for 1h. At this point the voltage was linearly increased from 300 to 5000 for 3h, and then the proteins were focused for up to 75 000Vh at a maximum voltage of 8000 V for 8:30h. 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 (21x18x0.15cm) and electrophoresis was performed using the PROTEAN Plus Dodeca Cell system (Bio-Rad) with constant amperage at 96mA until the dye front reached the bottom of the gel (about 1h) and then at 192mA over-night (about 14h) at 15°C.

Staining and image analysis

The gels were stained with Ruthenium II tris (bathophenanthrolinedisulfonate) tetrasodium salt (SunaTech Inc.). Briefly, after electrophoresis, the gels were fixed in 1% phosphoric acid (v/v) and 30% ethanol for 1h at room temperature and then were first stained overnight with 1 mM ruthenium complex (RuBP) in 1% phosphoric acid and 30% ethanol. Afterwards, the gels were destained for 4–6 hours in 1% phosphoric acid and 30% ethanol and rinsed in water prior to acquisition on fluorescence [102] by "ImageQuant LAS4010" (GE-Healthcare). The images were analyzed with the ProgenesisSameSpot (Nonlinear Dynamics) software. This software generates 2DE analyses which are robust and accurate. The gels were aligned to place all spots in exactly the same location, and then spot detection produced a complete data set since all gels contain the same number of spots, each matched to its corresponding spot on all gels. A comparison between three classes was performed. The software included statistical analysis calculations such as Anova p-value and False Discovery Rate (q-values). The gels were compared and protein spots with a >2-fold spot quantity change, $P < 0.05$ and q value < 0.05 (herein referred to as ''differentially expressed proteins'') were selected, were excised from the gel and were sent to Proteomic Core in Geneva (Switzerland) to identify the proteins using nanoLC-ESI-MS/MS.

Enzyme linked-Immunosorbent Assay of Transaldolase, Phosphoglicerate Mutase1 and Alpha-2-Glycoprotein1 Zinc Binding proteins

The concentration of Transaldolase (TALDO1), Phosphoglicerate Mutase1 (PGAM1) and Alpha-2-Glycoprotein1, Zinc Binding (aZGP1) was determined in all WS samples of individual patients (40 patients) at each time of collection (T0, T1, T2) using a commercial ELISA kit (USCN Life Science Inc., Wuhan, China) according to the

manufacturer's instruction. The limit of detection of each kit was 0.056ng/mL, 1.5ng/mL and 0.57ng/mL for TALDO1, aZGP1 and PGAM1, respectively. Amounts of 100 µl of no diluted WS samples were assayed except for PGAM1 where we operated a 1:2 dilution in phosphate buffer saline PBS 20mM pH 7.15, of WS samples. Absorbance values were measured spectrophotometrically at a wavelength of 450nm by Wallac Victor 2, 1420 label (Perkin Elmer).

Statistical Analyses

Clinical data were represented as median (minimum and maximum). The data were evaluated using non-parametric statistical methods. Friedman's test for repeated measures with post hoc test for multiple comparison was used to compare the baseline values and the other time points values in each treatment. Mann-Whitney U test was used to compare the independent groups. Pearson's chi-squared test was used to compare the frequency of specific symptoms. A p-value of less than 0.05 was considered statistically significant. Anova test has been used to explore quantitative differences in the protein expression between T0, T1 and T2 whole saliva samples. The significance of the differences was expressed by p-value <0.05.

The concentration of specific proteins in the samples by ELISA kits was determined by comparing the O.D. of the samples to the standard curve. The significance of the differences (p-value < 0.05) was calculated by Student *t* test for paired data.

RESULTS

Clinical assessment

The demographic characteristics and the treatment regimes of the patients enrolling in the study are shown in Table 4.

Table 4. Demographic and baseline characteristics of 41 FM patients.

	Balneotherapy group (N=20)	**Mud bath group (N=21)**
Sex	1M, 19F	1M, 20F*
Age (years) (mean±SD)	54.00±7.22 (42-68)	52.81±10.26 (31-69)
Height (cm) (mean±SD)	161.66±5.78 (150-176 cm)	162.30±5.37 (155-174 cm)
Weight (kg)) (mean±SD)	69.84± 15.53 (51-107 kg)	69.55±12.60 (55-100 kg)
Duration of symptoms (years) (mean±SD)	11.40±8.24	11.65±7.85
Married	16	13
Divorced	0	3
Widow	2	1
Unmarried	2	4
Occupational status	12 working, 5 housewife, 3 retired	13 working, 6 housewife, 2 retired
Hormonal status	25 post menopausal	11 post menopausal
Smoker	7	3

*1 of these patients left the study after T1 because of a diagnosis of breast cancer.

Analyzing data all together (balneotherapy plus mud-bath therapy treatment) we showed significant improvement of FIQ ($p<0.05$) and VAS pain ($p<0.01$) after two weeks of treatment and the data are reported in Table 5.

Table 5. Results of outcome measures of patients analyzed all together (balneotherapy+mud-bath therapy) (median, min-max).

	T0(baseline)	T1 (2 weeks)	T2 (12 weeks)	Friedman	p
VAS					
Pain	8 (3-10)*	6 (0-10)*	7 (1-10)	13.020	P<0.01
Fatigue	8 (0-10)	7 (0-10)	8 (0-10)	3.417	NS
Anxiety	6 (1-9)	5 (0-10)	5.2 (1-10)	5.285	NS
Depression	5 (0-9)	4 (0-9)	3 (1-10)	3.872	NS
FIQ	63.2 (18.1-98.0)*	54.10 (4.4-93.2)*	58.4 (6.6-93.7)	5.902	P<0.05
TPi	18 (4-18)	18 (2.9-18)	18 (2-18)	0.568	NS
SF-36 subitems					
PF-physical functioning	55 (5-85)	55 (15-95)	55 (15-90)	4.950	NS
RP physical role	0 (0-100)	0 (0-100)	0 (0-100)	9.812	NS
BP bodily pain	30 (0-72)	32 (0-61)	32 (0-100)	3.333	NS
GH general health	35 (10-86)	37 (0-92)	35 (0-82)	3.315	NS
VT vitality	40 (0-85)	35 (0-90)	35 (0-90)	0.727	NS
SF social functioning	50 (0-100)	50 (0-100)	50 (0-100)	1.141	NS
ER emotional role	0 (0-100)	33 (0-100)	33 (0-100)	3.282	NS
MH mental health	60 (4-100)	60 (4-100)	56 (0-92)	3.081	NS
FACIT	25 (4-51)	23 (0-48)	24.5 (0-45)	4.637	NS
HAQ	0.7 (0-2)	0.7 (0-4)	0.7 (0-2)	0.797	NS

TPi = Tender Points count

The clinical characteristics of patients allocated to mud-bath therapy or balneotherapy are shown in Table 6 and Table 7, respectively. No statistically-significant difference of demographic or clinical characteristics between the two groups of patients, assigned to balneotherapy or to mud-bath therapy, was observed at the beginning of the study.

The patients who received only mud-bath therapy (Tab. 6) showed a significant improvement of the VAS pain (T0: 8 (3-10)vs T2: 6 (1-9) $p<0.05$), FIQ values (T0: 70 (18.1-81.8) vs T2: 58.4 (12.4-87.4), $p<0.05$) and of the domain "physical role" of the SF-36 questionnaire (0 (0-75) vs 0 (0-100) vs 25 (0-100), $p<0.05$) after 12 week thermal program. VAS fatigue slightly decreased from T0 to T1 and to T2. The SF-36 domains "bodily pain", "emotional role", "social functioning" and "physical role" tended to increase from T0 to T1 to T2, suggesting an improvement following the mud-bath treatment. FACIT and HAQ did not change during the three times T0, T1, T2 .

In this group of patients it has been observed a significant reduced percentage of the frequency of tingling (T0: 76%, T1: 43%, T2: 33%, T0 vs T1, $P<0.05$; T0 vs T2 ,$P<0.01$).

The personal comments of patients treated with mud-bath were: 58% of patients reported improvement in symptoms at both T1 and T2, 16% patients only at T1, 5% patients only at T2, 21% of patients did not reported effects. In particular, this group of patients reported improvement in pain, asthenia, muscle stiffness and sleep, feeling more smoothly even if the present pain, pain relief in some parts of the body with persistence in others.

Table 6. Results of outcome measures of patients assigned to mud-bath therapy (median, min-max).

	T0(baseline)	T1 (2 weeks)	T2 (12 weeks)	Friedman	p
VAS					
Pain	8 (3-10)*	7 (1-9)	6 (1-9)*	8.708	P<0.05
Fatigue	9 (2-10)	8 (1-10)	8 (3-10)	2.696	NS
Anxiety	6 (1-9)	5 (0-10)	5.2 (1-10)	0.394	NS
Depression	5 (0-9)	4 (0-9)	3 (1-10)	0.184	NS
FIQ	70 (18.1-81.8)*	59.4 (9.6-78.3)	58.4 (12.4-87.4)*	7.238	P<0.05
TP	18 (4-18)	16 (4-18)	16 (2-18)	5.920	NS
SF-36 subitems					
PF-physical functioning	55 (5-85)	50 (15-80)	55 (15-90)	5.787	NS
RP physical role	0 (0-75)*	0 (0-100)	25 (0-100)*	11.31	p<0.05
BP bodily pain	22 (10-51)	30 (10-61)	32 (22-84)	4.964	NS
GH general health	35 (20-86)	40 (0-92)	35 (20-82)	0.591	NS
VT vitality	40 (15-85)	35 (5-85)	35 (1-80)	1.333	NS
SF social functioning	50 (25-100)	50 (0-100)	50 (12-100)	0.268	NS
ER emotional role	33 (0-100)	33 (0-100)	33 (0-100)	2.000	NS
MH mental health	64 (24-100)	60 (24-100)	60 (16-84)	1.684	NS
FACIT	25 (10-37)	22 (2-48)	23 (6-38)	1.675	NS
HAQ	0.7 (0.1-2.0)	0.8 (0.1-4.0)	0.6 (0.1-1.7)	2.658	NS

TPi = Tender Points count

The group of patients allocated to balneotherapy (Tab. 7) showed a significant improvement of VAS pain which decrease from T0 to T1 (8 (3-10) vs 7 (1-9) ($p<0.05$). They showed a slight improvement of the following clinical parameters: VAS fatigue, FIQ values, VAS anxiety and VAS depression which slightly decrease from T0 to T1 and then return to values close to the initial value at T2. Tender points count did not change from T0-T1-T2. The SF36 domains tend to increase from T0 to T1 and then return to values close to the initial value at T2 (except for the domain emotional role

that tend to increase also at T2). Also FACIT tend to decrease from T0 to T1. HAQ values remained unchanged in the three times of observations.

In this group of patients the frequency of characteristic symptoms remain unchanged after thermal treatment. The personal comments of patients are: 21% of patients reported improvement in symptoms at both T1 and T2, 21% patients only at T1, 21% patients only at T2, 37% of patients did not reported any effects. In particular, patients reported improvement in pain and sleep, feeling more smoothly despite the presence of pain (when persisted) and a reduction of cramping and headaches. The percentage of specific symptoms of the disease did not change at the three times in the group of patients treated with balneotherapy.

Psychiatric evaluations revealed 7 patients assigned to mud-bath therapy were suffering from psychiatric comorbidity (35%) of which 1 with current disease (panic) and 6 with lifetime disease (3 panic, 3 depression). In the group of patients assigned to balneotherapy 9 patients were suffering from psychiatric comorbidity (45%) of which 2 with current disease (1 panic and 1 depression) and 7 with lifetime disease (4 panic, 2 depression, 1 obsessive compulsive disorder).

Balneotherapy and mud-bath therapy were well tolerated in all patients. Only one patients, belonging to mud-bath group, left the study after the weeks treatment because the onset of breast cancer.

Table 7. Results of outcome measures of patients assigned to balneotherapy (median, min-max).

	T0(baseline)	**T1 (2 weeks)**	**T2 (12 weeks)**	**Friedman**	**p**
VAS					
Pain	7.5 (3-10)*	5.0 (0-10)*	7.5 (1-10)	8.394	P<0.05
Fatigue	8 (0-10)	7.0 (0-10)	8 (0-10)	1.069	NS
Anxiety	8 (1-10)	5 (0-10)	7 (0-10)	7.298	NS
Depression	6.5 (0-10)	5 (0-10)	6 (0-10)	5.938	NS
FIQ	61.8 (31.7-98.0)	47.3 (4.4-93.2)	61.2 (6.6-93.7)	3.900	NS
TPi	17.5 (8-18)	18 (10-18)	18 (8-18)	2.178	NS
SF-36 subitems					
PF-physical functioning	62.5 (25-85)	60 (25-95)	58 (15-90)	1.909	NS
RP physical role	0 (0-100)	0 (0-100)	0 (0-37.5)	0.889	NS
BP bodily pain	32 (0-72)	41 (0-61)	30.5 (0-100)	5.265	NS
GH general health	33.5 (10-76)	37 (15-72)	30.5 (0-67)	5.083	NS
VT vitality	37.5 (0-80)	35 (0-90)	35.5 (0-90)	0.113	NS
SF social functioning	50 (0-100)	56 (0-75)	50 (0-100)	1.279	NS
ER emotional role	0 (0-100)	33 (0-100)	36.5 (0-100)	0.571	NS
MH mental health	52 (4-92)	58 (4-96)	52 (0-92)	4.111	NS
FACIT	24.5 (4-51)	23 (0-43)	25 (0-45)	3.143	NS
HAQ	0.5 (0-1.5)	0.6 (0-1.5)	0.7 (0-2.1)	5.000	NS

TPi = Tender Points count

Determination of biochemical parameters: evaluation of oxytocin, BDNF, ATP and serotonin transporter levels during therapy

Tables 8a and 8b resume results of biochemical parameters in relation with treatment and response on time. Biochemical evaluations of patients assigned to mud-bath therapy are reported in table 8a, those relating to the balneotherapy are in the table 8b. Nonetheless the high variability of biochemical parameters as showed from high values of SD, however a significant decrease of neuropeptide concentrations were observed both after balneotherapy and mud-bath therapy when assayed after twelve weeks. As far as SERT is concerned a small decrease of affinity of about two fold of specific radioligand was observed at T2 with respect T0 time.

Table 8a. Results of biochemical evaluations of patients assigned to mud-bath therapy (mean ± sd).

	MUD-BATH THERAPY		
	T0	**T1**	**T2**
Oxytocin *(pg/ml)*	17.56± 5.9	17.7 ± 6.9	14.99±4.8
BDNF s *(pg/ml)*	9838±2261	9087±2634	8524±2492****
SERT B_{max}*(fmol/mg)*	1122± 443	1201 ± 371	1165 ± 473
SERT, K_d*(nM)*	0.048±0.044	0.05 ± 0.05	0.08± 0.06
ATP *(fmol/plt)*	0.025±0.008	0.021±0.006	0.024± 0.006

Significant differences observed between T2 *vs* T0 are based on Wilcoxon non parametric matched pairs test (*p<0.05; ** p<0.01)

Table 8b. Results of biochemical evaluations of patients assigned to balneotherapy (mean ± sd).

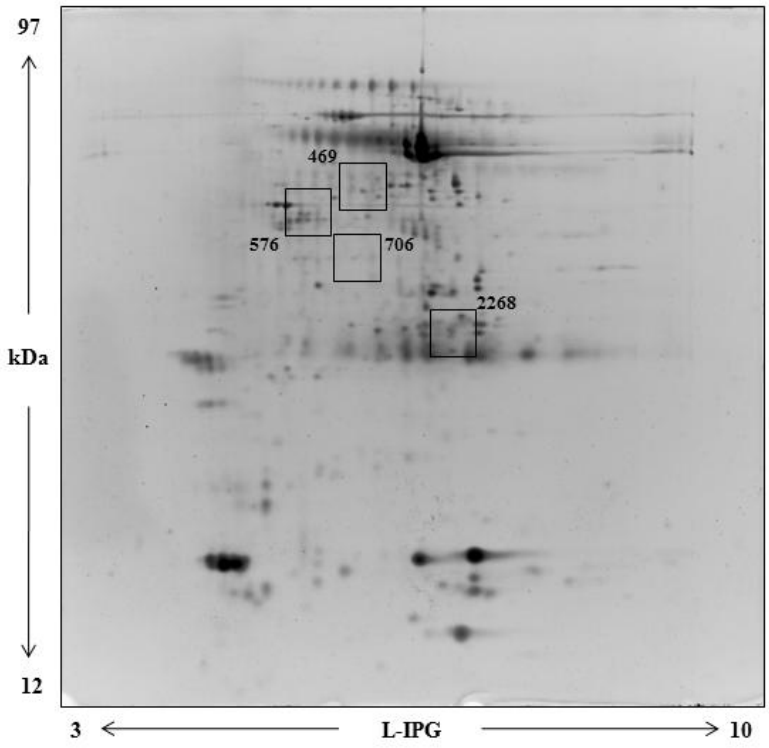
	BALNEOTHERAPY		
	T0	**T1**	**T2**
Oxytocin *(pg/ml)*	14.2 ± 7.25	15.28 ± 8.4	16.5±8.19
BDNF s *(pg/ml)*	10450±1493	10260±1739	9362±1849*****
SERT B_{max}*(fmol/mg)*	912 ± 334	1011±466	913± 471
SERT, K_d*(nM)*	0.035±0.008	0.06±0.07	0.08± 0.07
ATP *(fmol/plt)*	0.021 ± 0.008	0.026±0.009	0.02±0.006

Significant differences observed between T2 *vs* T0 are based on Wilcoxon non parametric matched pairs test (*p<0.05; ** p<0.01)

Identification of proteins (responsive) to balneotherapy and mud-bath therapy treatment.

A comparative proteomic analysis was performed on WS samples using 2-DE followed by nanoLC-ESI-MS/MS analysis. The computational analysis of 2DE gel images of pools from balneotherapy and mud-bath theraphy before and after treatment showed a significant difference of expression of four spots. Figure 1A shows a representative 2DE WS image where the spots of interest are circled. Moreover, in Figure 1B are represented the enlarged images of these spots for balneotherapy and mud-bath therapy pools at three time (T0, T1, T2).

1A)



1B)

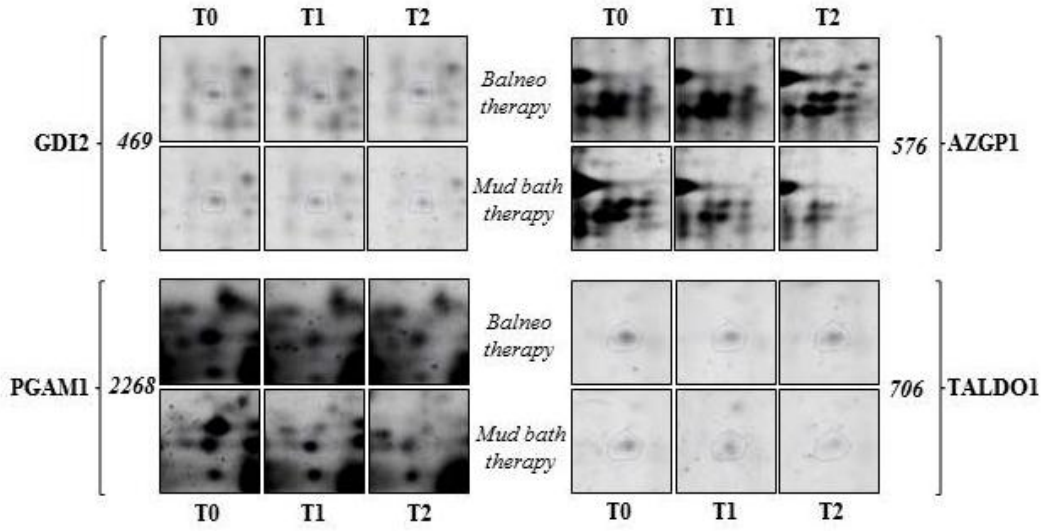


Figure 1. A) Representative 2-DE gel map of salivary proteins in FM patient. A total of 200 µg proteins were separated by 2-DE using 18 cm pH 3–10L strip and 12.5% SDS-PAGE. Proteins were detected by Ruthenium staining. The map was analyzed by the ProgenesisSameSpot (Nonlinear Dynamics) software. **B)** The enlarged images represented the protein spot differentially expressed in Balneotherapy and Mud-bath therapy for three time of treatment (T0,T1 and T2). Spot numbers indicate all the proteins differentially expressed identified by nanoLC-ESI-MS/MS and refer to the number reported in Table 8.

The differentially expressed protein spots were subjected to nano LC-ESI-MS/MS analysis and identified. A list of identified proteins, Molecular Weight, Isoelectric point, score and coverage values of MS/MS, are shown in Table 9a. Among proteins responsive to treatment, PGAM1, TALDO1 and RabGDP were previously described by

us as potential biomarkers of FM [17], [20] and zinc alpha 2 glycoprotein resulted the most responsive to treatment. Instead, in Table 9b are reported the fold variation of each protein spot

Table 9a. MS/MS data of protein spots differentially expressed in whole saliva of FM patients following thermal treatments.

# Spot	ID. No	Protein Name	Gene name	MW		pI		Mat. pep.	Cov. (%)	B. I. S.
				ob.	th.	ob.	th.			
469	P50395	Rab GDP dissociation inhibitor beta	GDI2	56	51	6.0	6.1	8	19	70
576	P25311	Zinc-alpha-2-glycoprotein	AZGP1	48	34	5.6	5.7	2	12	65
706	P37837	Transaldolase	TALDO1	41	37	6	6.3	7	22	76
2268	P18669	Phosphoglycerate Mutase 1	PGAM1	34	29	6.7	6.6	2	5	46

ID No =Accession Number; MW = Molecular Weight; pI = Isoelectric point; ob = observed; th= theorical; Mat. Pep. = Matched peptides; Cov. = Coverage; B.I.S. =Best Ion Score

Table 9b. Fold Variation expressed as ratio

# Spot	Balneotherapy			Mud-bath therapy		
	T1/T0	T2/T1	T2/T0	T1/T0	T2/T1	T2/T0
469	1.4	1.2	1.1	1.4	1.4	1.3
576	1.2	1.4	1.2	1.2	2.4	1.8
706	1.2	1.2	1.4	1.2	1.5	1.4
2268	1.3	1.3	1	1.2	1.1	1.4

Validation of Transaldolase (TALDO1), Phosphoglicerate Mutase1 (PGAM1) and Alpha-2-Glycoprotein1, Zinc Binding (aZGP1) by ELISA Kit analysis

ELISA Kit analysis was used to validate the difference in expression of proteins of interest of Transaldolase (TALDO1), Phosphoglicerate Mutase1 (PGAM1) and Alpha 2-Glycoprotein1, Zinc Binding (aZGP1) in WS samples of individual patients distinguished for type and time of treatment; the concentration of the specific protein was determined using a standard calibration curve. The results are showed in Table 10.

Table 10. ELISA assays of Phosphoglicerate Mutase 1, Transaldolasi and Zinc-alpha 2 glycoprotein in WS of FM patients.

Phosphoglicerate Mutase 1	**BALNEOTHERAPY**			**MUD-BATH THERAPY**		
Time of treatment	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**
Mean (OD)	27,74	19,42	24,52	35,98	33,68	17,72
SEM	6,27	4,59	6,25	6,92	7,88	4,35
Time Ratio	**T0/T1**	**T1/T2**	**T0/T2**	**T0/T1**	**T1/T2**	**T0/T2**
Paired t test (p values)	0,10	0,39	1,00	0,13	0,24	0,05*

Transaldolase	**BALNEOTHERAPY**			**MUD-BATH THERAPY**		
Time of treatment	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**
Mean (OD)	0,06	0,17	0,09	0,22	0,25	0,36
SEM	0,03	0,07	0,03	0,08	0,05	0,15
Time Ratio	**T0/T1**	**T1/T2**	**T0/T2**	**T0/T1**	**T1/T2**	**T0/T2**
Paired t test (p values)	0,08	0,09	0,70	0,73	0,46	0,26

Zinc-alpha-2 glycoprotein	**BALNEOTHERAPY**			**MUD-BATH THERAPY**		
Time of treatment	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**
Mean (OD)	20,85	22,84	18,84	22,19	17,52	14,49
SEM	9,05	8,79	7,88	5,72	4,65	5,04
Time Ratio	**T0/T1**	**T1/T2**	**T0/T2**	**T0/T1**	**T1/T2**	**T0/T2**
Paired t test (p values)	0,47	0,07	0,13	0,69	0,03*	0,01**

The figure 2 shows the histogram of the means±SEM of the concentrations of the proteins of interest in WS of FM patients between the three time of collection.

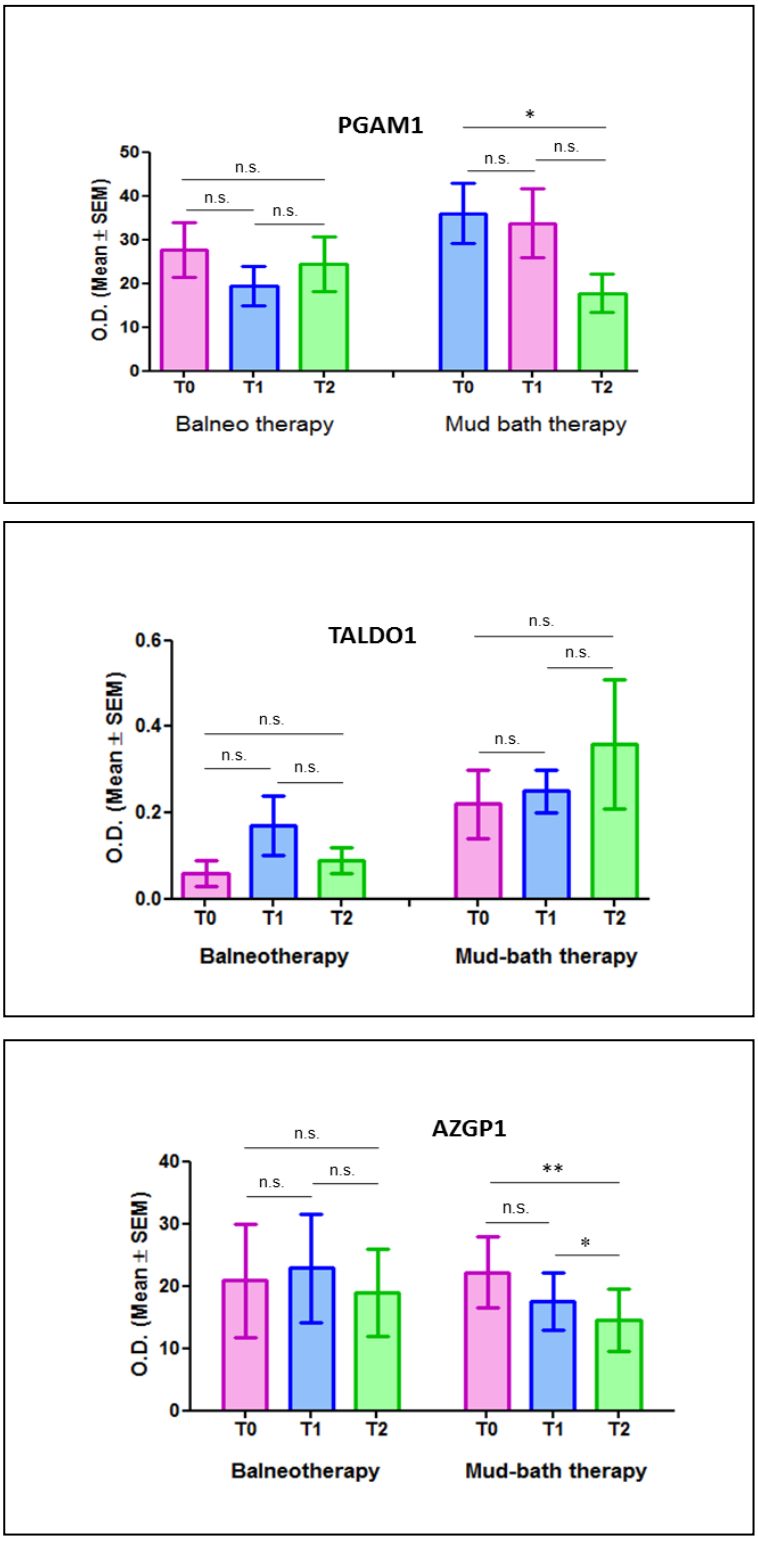


Figure 2. Each bar represents the mean±SEM of the mean of each protein concentrations in WS of FM patients at each time (T0,T1,T2). Significant differences are based on t test; (*p<0.05, **p<0.01, ***p<0.001).

DISCUSSION

FM is a chronic non-inflammatory musculoskeletal disorder, characterized by a generalized pain condition often accompanied by a wide range of other unspecific symptoms, such as fatigue, sleep disturbance, headache, mood disorders, anxiety, depression, cognitive dysfunction, sexual dysfunction and autonomic complaints like irritable bowel syndrome. FM is more common amongst women than men and affects between 2% and 4% of the general population. In 1990 the American College of Rheumatology (ACR) established criteria for classification of FM [1], and more recently Wolfe and colleagues [41] have proposed new diagnostic criteria. The establishment of these guidelines for diagnosis has permitted the performance of research studies by investigators in many countries contributing to the understanding of the mechanisms that underlie in FM [14]. A plethora of objective markers has been shown to be abnormal in FM, and some of these may even have characteristics of a biomarker because they are not only abnormal in patients versus controls, but also change in response to changes in symptoms, but at present no specific markers have been found [13]. The problem lies in the presence of too many data, often controversial, rather than in a lack of data [17]. The mechanisms underlying the FM are still unclear and largely unknown, and as a direct consequence, the diagnosis is very difficult. The difficulty 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. Then, to make a diagnosis of FM, the patient, in addition to reporting a widespread pain for at least 3 months and a painful response due to digital pressure of 11 of the 18 tender points, must be subjected to the Fibromyalgia Impact Questionnaire (FIQ) (Table 1) that gives a good idea of the full range of symptoms that are regularly found in FM. Therefore, the diagnosis of FM is basically

clinical [13] and the lack of easily accessible laboratory measures makes difficult to collect under the term of FM, patients presenting with homogeneous features and prognosis [39], [40]. At present, although proteomic studies have shown alterations in the expression of certain proteins in the saliva of FM patients compared to controls [20], there are no specific markers of FM. Several studies have identified alterations in the serum or plasma concentration of some neuromodulators/neurotransmitters, such as Oxytocin [22], BDNF [26]-[30], SERT [19] and ATP [34], [35] in patients with FM than in healthy subjects. The complexity of the disease means that there isn't a therapeutic treatment standards. The treatment of FM symptoms requires a multimodal approach that has to consider somatic aspects (i.e., pain onset, location, quality, quantity, duration), emotional aspects (i.e., mood and anxiety), cognitive aspects (i.e., coping styles, beliefs about pain), and environmental aspects (i.e., social context and patient relationships). From the pharmacological point of view, categories of drugs most commonly used are: analgesics, antidepressants, muscle relaxants and anti-epileptics. The current recommendations suggest that the optimal treatment of FM requires a multidisciplinary approach with a combination of non-pharmacological and pharmacological treatment modalities tailored according to pain intensity, function, associated features, such as depression, fatigue and sleep disturbances, decided through discussion with the patient, so the main strategy is symptom management [59]-[62]. Among non-pharmacological interventions the most used are physical therapy, psychotherapy, acupuncture, massage, and balneotherapy [63]-[66]. In the context of complementary medicine, the spa therapy, which includes hydrotherapy, balneotherapy, physiotherapy, mud-pack therapy and exercise [67]-[69], is known to be one of the most recognized therapeutic strategies for the treatment of rheumatic (as well as dermatological) disorder to pain alleviation [70]. The efficacy of this treatment for these indications underscores the applicability of spa therapy for the treatment of FM [71] but despite the

long history and popularity, there are not many randomized controlled trials that demonstrate their effect on FM patients [71], and only a few were paralleled by a biological evaluation. Therefore, the aim of this work was to understand the impact that two different types of thermal treatment (balneotherapy and mud-bath therapy) have on patients with FM, through the application of a multiple approach: the classical evaluation of clinical type of patients (before, after and three months after treatment), it has also made an assessment of biochemical and proteomic type.

Overall, our results indicate that both spa treatment are beneficial in patients affected by FM. In general the percentage of patients who self-reported positive effects of spa therapy was higher in the group treated with mud-bath than those treated with balneotherapy. In particular an improvement of FIQ and a decrease of pain were evidenced. The comparison of two different spa treatments showed that both mud-bath and balneotherapy were able to relieve the pain although with different effect span. In fact, mud-bath therapy showed significant improvement of the pain, the FIQ and the domain "physical role" which persist up to 12 weeks. Instead balneotherapy, showed a significant reduction of the pain immediately after the two weeks of treatment, but at 12 weeks pain values returned near to initial values, similarly slight improvement of fatigue and quality of life occurred (Tab. 5, 6, 7).

Discordant results were reported in literature about the long lasting effects of balneotherapy [72], [77], [78], [79], [117]. Buskila et al. [77], evaluate the effectiveness of balneotherapy on patients with FM at the Dead Sea. All participants stayed for 10 days at a Dead Sea spa. The improvement was especially notable in the treatment group and it persisted even after 3 months. Neumann and colleagues [78] observed an improvement in physical aspects of QoL of FM patients treated with balneotherapy which lasted usually 3 months, but on psychological measures the improvements was shorter. Moreover, as observed by Evcik et al [79], the extension of balneotherapy time

treatment from one to three weeks led to positive effect which persisted even after 6 months not only for the FIQ, but also for the pain and the tender point count.

As mud-bath-therapy as concerned our results agree with those published by Fioravanti et al. [72] in a multicentric single blind randomized clinical trial study where the effects of a cycle of 12 mud-packs and thermal bath treatment over a period of 2 weeks was analyzed on 40 FM patients. The researchers recorded a significant improvement of FIQ, tender point count, VAS for minor symptoms and HAQ after thermal therapy and after 16 weeks.

In addition, FM patients enrolled in our study have been carefully characterized and evaluated by trained psychiatrists using the Structured Clinical Interview Scale (SCID) for DSM-IV, detecting the presence of psychiatric comorbidity (lifetime and current) in about 40% of FM patients. Published works are lacking of psychiatric evaluations and anyway, when present, they are carried out through self-assessment instrument like the Beck Depression Inventory Index, which evaluate only depression [22], [81].

The mechanisms by which spa therapies relieve symptoms are not fully understood, although they probably include thermal, mechanical, chemical and immunomodulatory effects. Moreover, thermal therapy brings about a decrease of stress sensation, relaxation and a sense of well-being which have been related to an increase of serum levels of pituitary hormones and endogenous opiates such as endorphins [70], [118], [119], [120]. In spite of this, there are different reports which discuss about the role of neuropeptides and neurotransmitters in the pathophysiological mechanisms responsible for the symptoms in the FM. Among these, the neuropeptide oxytocin and the neurotrophic factor BDNF have been studied in FM patients with a particular attention to their correlation with features such as anxiety and depression. Oxytocin is known to have anti-nociceptive and analgesic effects as well as anxiolytic and sedative effects, in addition an anti-depressive and an anti-stress effects have been documented.

Differences of serum oxytocin expression in FM patients has been reported by Anderberg et al. [22] which suggested that depressed FM patients have reduced level of neuropeptide with respect to non-depressed FM patients. No significant change of oxytocin concentration after balneotherapy and mud-bath therapy was observed in our patients. As BDNF is concerned, different roles have been hypothesized in FM and other chronic pain conditions suggesting a pivotal role of this neuromodulator in these conditions. Recent findings have suggested an involvement of BDNF as a neuromediator of hyperalgesia and spinal central sensitization. Increased BDNF serum levels were shown in FM patients with respect healthy subjects, but there are different and controversial reports which discuss about the role of BDNF in the pathophysiological mechanisms responsible for the symptoms in the FM, particularly anxiety and depression [29], [30], [26], [28]. Our results indicate that spa therapy reduced the BDNF serum concentration by about 10–15% only after 12 weeks from the treatment. We believe that further studies are needed to clarify if these neuropeptides could be indicators of the beneficial thermal effects. Additionally, among the neurotransmitters, it has recently been suggested that altered serotonergic neuronal function might be related to the pathophysiology of FM [121], [122]. In a previous study [19], it was reported a decrease in the density and rate of platelet SERT in patients with FM respect to healthy subjects and it was also proposed a specific role for SERT in the pathogenesis of FM. A reduced density and rate of SERT are consistent with previous observations indicating that levels of 5-HT are altered in patients with FM [19]. In this study comparable mean values of Kd and Bmax were found in FM patients, but no significant changes both in terms of affinity and of the total number of SERT were observed after thermal treatment. Finally, from the biochemical point of view, in this study no significant change of ATP concentrations was observed after both thermal treatments. All biochemical data are summarized in Table 8a and 8b.

Previously, our group performed a comparative proteomic analysis of FM WS, by combining 2DE and MALDI-TOF-MS [20]. The most relevant observation which emerged from the data analysis was the exclusive and significant over-expression of TALDO1 and PGAM1 in FM samples with respect to healthy subjects suggesting that this could be involved in limiting oxidative damage to tissues [20], [17]. Based upon all these observations, in this study we evaluated if the change of protein expressions can occur in response to thermal treatments. Using a combination of 2DE (Fig. 1A, 1B) and nano-LC-ESI/MS/MS spectrometry techniques (Tab. 9a, 9b) we compared FM WS protein profiles of a randomized pool of patients at different times from each treatment. Only four proteins showed appreciable change (ratio range from 1.1 to 2.4) of their expression values in response to treatment. The quantitative evaluation and the significance of these protein expression variations were determined by using the specific ELISA kit (Tab. 10; Fig.2). We observed a high variability of protein concentrations in both treatment groups and also in different times. Significant differences were observed only for PGAM1 and Zinc-alpha 2 glycoprotein (AZGP1). In fact, a reduction of the expression values toward normal values was found in WS of the patients treated with mud-bath therapy, particularly at 12^{th} week suggesting an improvement of FM patients. A role of the adipokine AZGP1 in the activation of AMP kinase, an important regulator of energy metabolism, in human skeletal muscle cells has emerged [123]. The mechanism may be involved in mediating the effects of AZGP1 in relation to increased energy utilization. All in all we can see the protein up regulation as a response to an increase of the oxidative stress, responsive to mud bath treatment.

In conclusion, our results show that the thermal treatment might have a beneficial effect on the specific symptoms of the disease, in particular while balneotherapy gives results that in most patients occur after the end of the treatment, but which are no longer noticeable after 3 months, the mud-bath treatment gives more lasting results. Indeed, a

combined treatment of balneotherapy plus mud-bath treatment is advisable for patients suffering from FM. On the other hand, respect to the biological effects, the mud-bath therapy appears to be the best treatment producing a statistical significant reduction of level both of neuropeptide, such as BDNF, and proteins, such as PGAM1 and AZGP1 which usually resulted up regulated in FM patients [17], [26], [28]. In our study other factors which could contribute to the clinical improvement after a spa treatment, such as the pleasant scenery and the absence of work duties [77], [117] were not considered. In fact, the patients (except two) did not stay in the spa, but they were resident in areas surrounding it, continued their work activities and the time that the patients spent in the spa center was limited to the treatment. In this way the observed improvement of symptoms was only dependent on the spa therapy itself.

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