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

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ABSTRACT

Objective. *To study the effects of both balneotherapy and mud-bath therapy treatments in patients affected by primary fibromyalgia (FM) using rheumatological, psychiatric, biochemical and proteomic approaches.*

Methods. *Forty-one FM patients (39 females, 2 males), who fulfilled the American College of Rheumatology criteria received a 2-week thermal therapy programme consisting of 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. Comparative whole saliva (WS) proteomic analysis was performed using a combination of two-dimensional electrophoresis (2DE) and mass spectrometry techniques.*

Results. *We observed a 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 in BDNF concentrations was observed either after balneotherapy or mud-bath therapy when assayed after twelve weeks, while no significant change in oxytocin levels, ATP levels and serotonin transporter were detected. Significant differences were observed for phosphoglycerate mutase1 (PGAM1) and zinc alpha-2-glycoprotein 1 (AZGP1) protein expression.*

Conclusions. *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 longer lasting results.*

Introduction

Fibromyalgia syndrome (FM), as defined in the 1990 American College of Rheumatology (ACR) criteria (1), is a chronic, generalised pain condition with characteristic tender points on physical examination, often accompanied by a number of associated symptoms, such as fatigue, sleep disturbance, headache, irritable bowel syndrome and mood (2-7).

The pathophysiology and aetiology of FM still remain unclear. FM has a high prevalence in the general population (2–3%) and the condition is more common amongst women than men, while representing 30% of rheumatic diseases (8, 9). 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 (10). 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.

In the last decade, significant improvements have been made in the knowledge of the mechanisms involved in the altered pain threshold of FM patients (12-14), and new strategies of treatment have been developed or proposed. Different medical treatments are used to treat fibromyalgia. Generally, pharmacological compounds (non-steroidal anti-inflammatory drugs, adjuvant analgesic drugs, tricyclic anti-depressants and selective serotonin reuptake inhibitors) are prescribed to enhance the pain

threshold and to improve sleep (15, 16). Non-pharmacological interventions include psychotherapy, acupuncture, massage, physical therapy and balneotherapy (17-20). The recent guidelines suggest that the optimal treatment of FM consists in a multidisciplinary approach with a combination of pharmacological and non-pharmacological treatment modalities (21-27). Spa therapy comprises different therapeutic modalities including hydrotherapy, balneotherapy, physiotherapy, mud-pack therapy and exercise (28-31). Although several articles have been written on this theme, its role in modern medicine is still not clear.

Some studies have reported on spa therapy effect in FM, but only a few were paralleled by a biological evaluation. 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, in order to evaluate salivary biomarkers (32).

The present study describes the results of a multidisciplinary approach (rheumatological, psychiatric, biochemical and 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 (Italy) and the University of Pisa (Department of Pharmacy and Department of Clinical and Experimental Medicine, Division of Rheumatology and Division of Psychiatry) for the evaluation of the effects of balneotherapy and mud-bath therapy on 41 patients affected by fibromyalgia.

Methods

Patients

Forty-one primary fibromyalgia patients (39 female, 2 male) aged between 31 and 69 years participated in the study. The patients were contacted by letter or by phone to explain the nature of the study and give the first appointment date. 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. 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 randomised 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 ran for 12 consecutive sessions except Sundays at Montecatini Terme Spa (Italy, Salse-Sulphate-Alkaline waters, prevalently formed of sodium chlorides and sodium and magnesium sulphates [Na+, Cl-, Mg ++, So4–-], 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 medication, four took anti-depressants, one took anti-inflammatory drugs, four took analgesics, four took muscle relaxants and two took antiepileptics. Among the patients treated with mud-bath therapy, four did not take any medication, seven took anti-depressants, four took anti-inflammatory drugs, one took analgesics, and two took antiepileptics. The patients had been 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) (33, 34), 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) (35), the Health Assessment Questionnaire (HAQ) (36, 37) to determine physical disability and the SF-36 questionnaire (ShortForm with 36 questions) (38), a well-documented, self-administered quality of life (QoL) scoring system.

Trained psychiatrists have evaluated the patients using the Structured Clinical Interview Scale (SCID) for DSM-IV (39, 40). 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.

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

WS samples:
collection and preparation

Unstimulated WS samples were collected early in the morning (between 8 and 10 a.m) in standard conditions, and processed as previously described (41). WS samples from individual patients (corresponding to 200µg) were solubilised 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
Whole venous blood samples (30 ml) for platelet preparation, BDNF, oxytocin determination were drawn from overnight fasting subjects between 8:00 and 9:00 am at the division of Rheumatology, University of Pisa.

[3H]-Paroxetine binding assay
Serotonin transporter (SERT) binding parameters (maximal binding capacity, Bmax, fmol/mg protein; dissociation constant, Kd, nM), were evaluated in platelet membranes by measuring the specific binding of [3H]-paroxetine. The platelet preparation and the binding assay were carried out as described by Giannaccini *et al*. (42).

Oxytocin determination
Aliquots of plasma (6 ml) obtained as previously described (43) 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 S.p.A., Italy) 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 and the remaining lyophilised samples were stored at -80°C.
Quantitative determination of oxytocin in samples was measured by using a competitive oxytocin Enzyme Immunoassay kit (EIA kit, Enzo Life Sciences, Inc.) according to the manufacturer's instructions. 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 neutralisation 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). The assay was performed essentially as previously described (44).

2DE analysis
Twenty randomly selected samples were pooled according to thermal treatment (balneotherapy or mud-bath therapy) and to each time of collection (T0, T1 and T2). Then, 2DE analysis was performed in triplicate according to protocols previously described (41, 45). The gels were stained with Ruthenium II tris (bathophenanthrolinedisulfonate) tetrasodium salt (SunaTech Inc., China) and the images analysed as described (45).

Mass spectrometry analysis and protein identification
Gels pieces destaining, peptide extraction, LC ESI MS/MS were performed exactly as previously described (46).
The mascot search was validated using Scaffold 3.6.0 (Proteome Software, Portland, OR). Only proteins matching with two different peptides with a minimum probability score of 95% were considered identified.
Enzyme linked-Immunosorbent Assay of transaldolase (TALDO1), phosphoglycerate mutase1 (PGAM1) and zinc α-2-glycoprotein 1 (AZGP1) proteins
The concentration of TALDO1, PGAM1 and 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 are shown in Table I. Analysing the data all together (balneo + mud-bath treatment) we showed significant improvement of FIQ (p<0.05) and VAS pain (p<0.01) after two weeks of treatment (Table II).
The clinical characteristics of the patients allocated to mud-bath therapy or balneotherapy are shown in Table III and Table IV, respectively. No statistically significant difference of demographic or clinical characteristics between the two groups of patients, assigned either to balneotherapy or to

Table I. Demographic and baseline characteristics of fibromyalgia patients.

	Balneotherapy group (n=20)	Mud-bath group (n=21)
Sex	1M, 19F	1M, 20Fa
Age (years) (mean±SD)	54.00 ± 7.22 (42–68 ys)	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 (1–30)
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

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

Table II. Results of outcome measures of patients analysed all together (balneotherapy plus mud-bath therapy) (median, min-max).

	T0 (baseline)	T1 (2 weeks)	T2 (12 weeks)	Friedman	
Pain (VAS)	8 (3-10)*	6 (0-10)*	7 (1-10)	13.020	$p<0.01$
Fatigue (VAS)	8 (0-10)	7 (0-10)	8 (0-10)	3.417	NS
Anxiety (VAS)	6 (1-9)	5 (0-10)	5.2 (1-10)	5.285	NS
Depression (VAS)	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	$p<0.05$
TP	18 (4-18)	18 (2.9-18)	18 (2-18)	0.568	NS
SF-36 sub-items					
PF-physical functioning	55 (5-85)	55 (15-95)	55 (15-90)	4.950	NS
PR 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

mud-bath therapy, was observed at the beginning of the study.

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

The group of patients allocated to balneotherapy (Table IV) showed a significant improvement of VAS pain which decreased from T0 to T1 (7.5 (3–10) *vs*. 5.0 (1–10) ($p<0.05$). They showed a slight improvement of the following clinical parameters: VAS fatigue, FIQ values, VAS anxiety and VAS depression which slightly decreased from T0 to T1 and then returned to values close to the initial value at T2. Tender points count did not change from T0-T1-T2. The SF36 domains tended to increase from T0 to T1 and then returned to values close to the initial value at T2 (except for the domain emotional role that tend to increase also at T2). Also FACIT tended to decrease from T0 to T1. HAQ values remained unchanged during the three observation.

In this group of patients the frequency of characteristic symptoms remain unchanged after thermal treatment. The personal comments of patients were the following: 21% of patients reported improvement in symptoms at both T1 and T2, 21% patients only at T1, 21% patients only at T2, and 37% of patients did not report any effects. In particular, the 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 during 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).

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

	T0 (baseline)	T1 (2 weeks)	T2 (12 weeks)	Friedman	
Pain (VAS)	8 (3-10)*	7 (1-9)	6 (1-9)*	8.708	$p<0.05$
Fatigue (VAS)	9 (2-10)	8 (1-10)	8 (3-10)	2.696	NS
Anxiety (VAS)	6 (1-9)	5 (0-10)	5.2 (1-10)	0.394	NS
Depression (VAS)	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	$p<0.05$
Tender points count	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
PR 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

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

	T0 (baseline)	T1 (2 weeks)	T2 (12 weeks)	Friedman	
Pain (VAS)	7.5 (3-10)*	5.0 (0-10)*	7.5 (1-10)	8.394	$p<0.05$
Fatigue (VAS)	8 (0-10)	7.0 (0-10)	8 (0-10)	1.069	NS
Anxiety (VAS)	8 (1-10)	5 (0-10)	7 (0-10)	7.298	NS
Depression (VAS)	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
Tender points count	17.5 (8-18)	18 (10-18)	18 (8-18)	2.178	NS
SF-36 sub-items					
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

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

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

Table V summarises results of the biochemical parameters in relation with treatment and response within time. Nonetheless, the high variability of biochemical parameters is supported by the high values of standard deviation (SD). However, a significant decrease of neuropeptide concentrations was observed both after balneotheraphy 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 to time.

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

A comparative proteomic analysis was performed on WS samples using 2DE followed by nano LC-ESI-MS/MS analysis. The computational analysis of 2DE gel images of pools from balneotherapy and mud-bath therapy before and after treatment showed a significant difference of expression of four spots. Figure 1 shows a representative 2DE WS image where the spots of interest are circled. Enlarged images of these spots are represented for balneotherapy and mud-bath therapy pools at three times (T0, T1, T2). The differentially expressed protein spots were subjected to nano LC-ESI-MS/MS analysis and identified. A list of identified proteins, MW, pI, score and coverage values of MS/MS, are shown in Table VI. Among proteins responsive

Table V. Results of biochemical evaluations of patients assigned to balneotherapy or mud-bath therapy.

	Balneotherapy			Mud-bath therapy		
	T0 (mean ± SD)	T1 (mean ± SD)	T2 (mean ± SD)	T0 (mean ± SD)	T1 (mean ± SD)	T2 (mean ± SD)
Oxytocin (pg/ml)	14.2 ± 7.25	15.28 ± 8.4	16.5 ± 8.19	17.56 ± 5.9	17.7 ± 6.9	14.99 ± 4.8
BDNF s (pg/ml)	10450 ± 1493	10260 ± 1739	9362 ± 1849*	9838 ± 2261	9087 ± 2634	8524 ± 2492**
SERT Bmax (fmol/mg)	912 ± 334	1011 ± 466	913 ± 471	1122 ± 443	1201 ± 371	1165 ± 473
SERT, Kd (nM)	0.035 ± 0.008	0.06 ± 0.07	0.08 ± 0.07	0.048 ± 0.044	0.05 ± 0.05	0.08 ± 0.06
ATP (fmol/plt)	0.021 ± 0.008	0.026 ± 0.009	0.02 ± 0.006	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 (*$p<0.05$; **$p<0.01$).

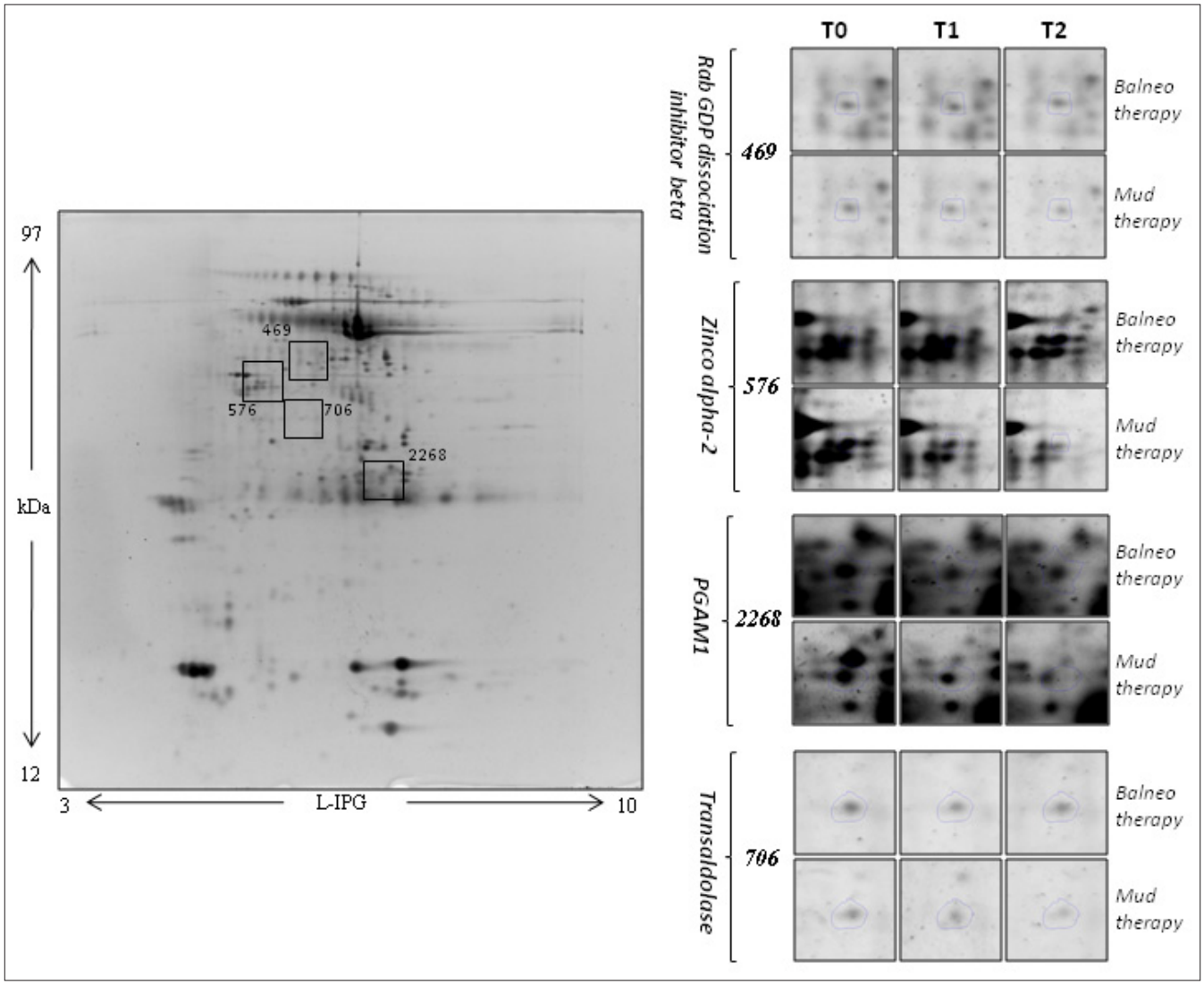

Fig. 1. Representative 2DE gel map of WS where the spots of interest are circled. Enlarged images of these spots are represented for balneotherapy and mud-bath therapy pools at three times (T0, T1, T2).

Table VI. MS/MS data of protein spots differentially expressed in WS of FM patients following thermal treatments.

Spot no.	Accession no.	Protein	Gene name	MW		pI		Matched peptides	Coverage (%)	Best Ion Score	Balneotherapy fold variation			Mud-bath therapy fold variation		
				obs	th.	obs	th.				T1/T0	T2/T1	T2/T0	T1/T0	T2/T1	T2/T0
590	P50395	Rab GDP dissociation inhibitor beta	GDI2	56	51	6.0	6.1	8	19	70	1.4	1.2	1.1	1.4	1.4	1.3
703	P25311	Zinc-alpha-2-glycoprotein	AZGP1	48	34	5.6	5.7	2	12	64.9	1.2	1.4	1.2	1.2	2.4	1.8
935	P37837	Transaldolase	TALDO1	41	37	6	6.3	7	22	76.4	1.2	1.2	1.4	1.2	1.5	1.4
1330	P18669	Phosphoglycerate Mutase 1	PGAM1	34	29	6.7	6.6	2	5	45.7	1.3	1.3	1	1.2	1.1	1.4

to treatment, PGAM1, TALDO were previously described by us as potential biomarkers of FM (19, 33) and zinc alpha 2 glycoprotein resulted the most responsive to treatment.

Validation of TALDO1, PGAM1 and AZGP1 by ELISA kit analysis

The ELISA kit analysis was used to validate the different expression of TALDO1, PGAM1 and AZGP1 in WS samples of patients distinguished both for type and time of treatment. The concentration of the specific protein was determined using a standard calibration curve (Table VII). Overall

Table VII. ELISA assays of PGAM 1, TALDO1 and AZGP1 in WS of FM patients.

PGAM1	Balneotherapy			Mud-bath therapy		
Times 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)	0.103	0.398	1.000	0.126	0.236	**0.053**
TALDO1	Balneotherapy			Mud-bath therapy		
Times 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)	0.079	0.092	0.702	0.735	0.456	0.259
AZGP1	Balneo therapy			Mud-bath therapy		
Times 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 ttest (*p*-values)	0.475	0.073	0.135	0.691	**0.034**	**0.015**

statistical analysis confirms proteomic results.

Discussion

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 a 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 the 12th week pain values returned near to the initial values, similarly a slight improvement of fatigue and of quality of life occurred.

Discordant results were reported in literature about the long lasting effects of balneotherapy (47-51). Buskila *et al*. (47), evaluated the effectiveness of balneotherapy on patients with fibromyalgia (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. Neuman *et al*. (48) 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 were shorter. Moreover, as observed by Evcick *et al*. (49) the extension of balneotherapy time treatment from one to three weeks led to positive effects 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 is concerned, our results agree with those published by Fioravanti *et al*. (50) in a multicentre single blind randomised clinical trial study where the effects of a cycle of 12 mud-packs and thermal bath treatment over a period of 2 weeks was analysed 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 characterised 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 such as the Beck Depression Inventory Index, which evaluates only depression (52, 53).

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 (54-57). 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 (58-60). Oxytocin is known to have anti-nociceptive and analgesic effects as well as anxiolytic and sedative effects. In addition, anti-anxiety and anti-depression effects have been documented. Differences of serum oxytocin expression in FM patients has been reported by Anderberg *et al*. (53) which suggested that depressed FM patients have a reduced level of neuropeptide with respect to non-depressed FM patients. No significant change of oxytocin concentration after balneo and mud-bath therapy was observed in our patients. However, among the patients who underwent balneotherapy, a significant increase of oxytocin level was evidenced for those who presented affective disorders (data not shown). This response was shown immediately after the two weeks of treatment suggesting a positive effect of spa therapy. As BDNF is concerned, different roles have been hypothesised in FM and other chronic pain conditions suggesting a pivotal role of this neuromodulator in these conditions. Increased BDNF serum levels were shown in FM patients with respect to healthy subjects (58-61). 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 (62, 63). In a previous study (64), we reported a decrease in the density and rate of platelet SERT in patients with FM with respect to healthy subjects and we also proposed a specific role for SERT in the pathogenesis of FM. 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. Previously, a reduction of ATP levels has been observed in platelets (44) suggesting a bioenergetic alteration in FM. Our results confirmed a reduced basal level of ATP concentration in platelets of FM patients with respect to healthy subjects. Nevertheless, no significant change of ATP concentrations was observed after both thermal treatments.

Previously, we performed a comparative proteomic analysis of FM whole saliva, by combining 2DE and MALDI-TOF-MS (32). 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 (32, 46). 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 and nano-LC-ESI/MS/MS spectrometry techniques 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. 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 AZGP1. In fact, a reduction of the expression values toward normal values was found in WS of the patients treated with mud-balneo therapy, particularly at 12th 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 (65). The mechanism may be involved in mediating the effects of AZGP1 in relation to increased energy utilisation. All in all we can see the protein upregulation 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 (recommended) for patients suffering from fibromyalgia. On the other hand, respect to the biological effects, the mud-balneo 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 AZGP-1which usually resulted up regulated in FM patients (46, 60, 61).

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 (47, 51) 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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