Abstract: Fibromyalgia (FM) is a chronic pain disorder characterized by widespread pain and associated with unspecific symptoms. So far, no laboratory tests have been validated. The aim of the present study was to investigate the presence in saliva of potential diagnostic and/or prognostic biomarkers which could be useful for the management of FM patients. Specifically, we carried on our previous work by comparing the salivary profile of 30 FM patients with those of 30 healthy subjects, 30 subjects suffering migraine (model of non-inflammatory chronic pain), and 30 patients affected by rheumatoid arthritis (model of inflammatory chronic pain). For the proteomics analysis we applied 2-DE and SELDI-TOF-MS. The SELDI analysis allowed focusing our attention on two peaks which could correspond to orexigenic neuropeptide QRFP and peptidyl-prolyl cis-trans isomerase. From 2-DE we found serotransferrin (TRFE) and alpha-enolase (ENOA) differentially expressed in FM. Hence, we validated their expression by ELISA together with phosphoglycerate mutase I (PGAM1) and transaldolase, which were found in our previous work. Moreover, ROC curve was calculated to investigate the discriminative power of our biomarkers. The best performance was obtained by combining ENOA, PGAM1 and TRFE. We believe that this panel could be a useful tool in supporting clinicians in diagnosis.
To Editors

of Journal of Proteomics

Dear Bijar Ghafouri and Emmanuel Bäckryd,

We have the honour to submit for consideration, as an article for the special issue “Proteomics in chronic pain; investigating mechanistic markers of pain”, the research paper entitled “Putative predictive salivary biomarkers to distinguish fibromyalgic pain.”, by Ciregia et al. The work is a clinical biomarkers study and aims to investigate the presence in saliva of potential diagnostic and/or prognostic biomarkers which could be useful for the management of patients with Fibromyalgia. Study included validation on a different cohort of patients respect the discovery phase and all the subjects reflected the typical clinical situation required for the research. We believe that the obtained results could be a useful tool in supporting clinicians’ diagnosis and defining Fibromyalgia clusters and targeted treatment. Actually, our future perspective foresees to develop a simple, rapid and not invasive point-of-care test.

The material is original research, it has not been previously published and has not been submitted for publication elsewhere while under consideration. The final manuscript has been seen, reviewed and approved by all named authors. The authors declare no conflict of interest. If accepted, the work will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

Looking forward for your reply, we thank you for your consideration.

Sincerely Yours,

Dr Federica Ciregia

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SIGNIFICANCE

FM is one of the most common chronic pain condition which is associated with significant disability. The fibromyalgic pain is a peculiar characteristic of this disease and FM patients suffer from reduced quality of life, daily functioning and productivity. Considering the deep complexity of FM, the discovery of more objective markers is crucial for supporting clinical diagnosis. Therefore, the aim of the present study was the selection of biomarkers effectively associated with fibromyalgic pain which will enable clinicians to achieve an unambiguous diagnosis, and to improve approaches to patients’ management. We defined a panel of 3 salivary proteins which could be one of the criteria to be taken into account. Consequently, the identification of disease salivary biomarkers could be helpful in detecting FM clusters and targeted treatment. Actually, our future perspective foresees to develop a simple, rapid and not invasive point-of-care testing which will be of use during the diagnostic process. In addition, the present results can offer a clue for shedding light upon the complex entity of such a disease like FM.
**Highlights**

- The diagnosis of Fibromyalgia (FM) basically relies on the clinicians experience and no laboratory tests have been validated.

- We investigated the presence in saliva of eventual diagnostic and/or prognostic biomarkers which could be useful for the management of FM patients.

- A panel combing 3 proteins, ENOA, PGAM1 and TRFE, is proposed as an useful tool in supporting clinicians in diagnosis of FM.
Putative predictive salivary biomarkers to distinguish fibromyalgic pain.

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Fibromyalgia (FM) is a chronic pain disorder characterized by widespread pain and associated with unspecific symptoms. So far, no laboratory tests have been validated. The aim of the present study was to investigate the presence in saliva of potential diagnostic and/or prognostic biomarkers which could be useful for the management of FM patients. Specifically, we carried on our previous work by comparing the salivary profile of 30 FM patients with those of 30 healthy subjects, 30 subjects suffering migraine (model of non-inflammatory chronic pain), and 30 patients affected by rheumatoid arthritis (model of inflammatory chronic pain). For the proteomics analysis we applied 2-DE and SELDI-TOF-MS. The SELDI analysis allowed focusing our attention on two peaks which could correspond to orexigenic neuropeptide QRFP and peptidyl-prolyl cis-trans isomerase. From 2-DE we found serotransferrin (TRFE) and alpha-enolase (ENOa) differentially expressed in FM. Hence, we validated their expression by ELISA together with phosphoglycerate mutase I (PGAM1) and transaldolase, which were found in our previous work. Moreover, ROC curve was calculated to investigate the discriminative power of our biomarkers. The best performance was obtained by combining ENOA, PGAM1 and TRFE. We believe that this panel could be a useful tool in supporting clinicians in diagnosis.

FM is one of the most common chronic pain condition which is associated with significant disability. The fibromyalgic pain is a peculiar characteristic of this disease and FM patients suffer from reduced quality of life, daily functioning and productivity. Considering the deep complexity of FM, the discovery of more objective markers is crucial for supporting clinical diagnosis. Therefore, the aim of the present study was the selection of biomarkers effectively associated with fibromyalgic pain which will enable clinicians to achieve an unambiguous diagnosis, and to improve approaches to patients’ management. We defined a panel of 3 salivary proteins which could be one of the criteria to be taken into account. Consequently, the identification of disease salivary biomarkers could be helpful in detecting FM clusters and targeted treatment. Actually, our
future perspective foresees to develop a simple, rapid and not invasive point-of-care testing which will be of use during the diagnostic process. In addition, the present results can offer a clue for shedding light upon the complex entity of such a disease like FM.
Fibromyalgia (FM) is a chronic pain disorder characterized by widespread pain for at least 3 months. Other associated unspecific symptoms may be present, including fatigue, sleep disturbances, memory problems, irritable bowel syndrome, headache, and depression [1,2]. This condition is associated with significant disability: FM patients suffer from reduced quality of life, daily functioning and productivity. Hence, it involves losses in productivity, reduced work hours, absenteeism, disability, unemployment, early retirement, informal care and other costs [3].

The global mean prevalence of FM has been estimated to be 2.7%. In women, the mean prevalence is 4.2% and in men 1.4%, with a female-to-male ratio of 3:1 [2]. Weir et al. [4] reported an incidence rate of 6.88 new cases per 1,000 person/years for males and 11.28 new cases per 1,000 person/years for females.

The diagnosis of FM is a contentious issue due to the lack of laboratory testing. In 1990 the American College of Rheumatology (ACR) defined some criteria requiring that individuals have widespread pain (pain in the axial skeleton, above and below the waist, and on both sides of the body) as well as tenderness in 11 or more of 18 possible “tender points” [1, 5]. In 2010 and 2011 these criteria have been revised leading to a change in the concept of FM, excluding tender point site palpation as an essential diagnostic criterion to make the diagnosis [6, 7]. In particular, in 2011, patient-based FM criteria were defined and added to the physician-based criteria from 2010 [8]. Thereby, newer diagnostic criteria are entirely symptom-based and do not require counts of the number of tender points [1]. More recently, in 2016, a systematic review found consistency in sensitivity and specificity between the ACR 1990 diagnostic criteria and the 2010/2011 criteria [8].

The 2016 committee maintained the difference between patient and physician-criteria but added a “generalized pain” criterion to prevent the inclusion of regional pain syndromes in the FM [10]. However, despite the progress made, the diagnosis basically relies on the clinicians experience.

Hitherto, the etiology of FM has been elusive; although FM has usually been considered a non-inflammatory and non-autoimmune disease, many studies have focused on the inflammatory and
autoimmune hypothesis. Alterations in cytokine profiling, and presence of autoantibodies have been reported in patients with FM [11-16]. Nevertheless, up to date, no consistent validated markers have been found. In recent years, few works investigated miRNA in biofluids such as blood [17-19], saliva [18] and cerebrospinal fluid [20]. These studies proposed that miRNA can help in characterizing FM, but they were limited to a small number of FM patients and validation in larger study groups is needed.

Another area of investigation is genetic study, founded on the idea that genetic factors may predispose to FM in combination with environmental triggers (e.g. trauma, infections or emotional stress). The principal genes supposed to be a risk factor for FM are serotonin transporter (5-HTT), catechol-O-methyltransferase (COMT) and the dopamine receptor [21, 22]. But the selected polymorphisms are often associated with psychiatric disorders thereby they could be related to psychiatric comorbidities rather than to sole FM. Moreover, genetic results are often controversial and no specific candidate gene has been closely connected with FM. Since the actual biological function relies on dynamic population of proteins, there is increasing interest in the field of proteomics. Moreover, only the characterization of the proteins themselves can give insight into protein-protein interactions and functions. That the reason why, in the last years, we applied proteomic analysis in the discovery of biomarkers for many rheumatic diseases [23-25]. In 2009, we carried out a study on human whole saliva (WS) of patients affected by FM [26]. The aim was to identify the protein content of WS defining the differences between FM patients and healthy subjects. The use of saliva might enable the easy characterization of a non-invasively collected biological fluid, giving rise to a different approach in the diagnosis of FM. In this work we used two-dimensional electrophoresis (2-DE) to obtain the WS protein map of FM patients. Our study attested the potential usefulness of the proteomic characterization of human WS in distinguishing FM from healthy subjects [26].

Following these encouraging results, the focus of the present study has been to investigate the presence in WS of any eventual diagnostic and/or prognostic biomarkers which could be useful for
the management of FM patients. Specifically, we compared the profile of FM not only with healthy subjects (negative controls), but also with two different positive controls. Two models of chronic pain were selected: patients with migraine (as model of non-inflammatory regional chronic pain), and patients affected by rheumatoid arthritis (RA) (as model of inflammatory chronic pain).

The tools exploited for establishing the fingerprint profiles of WS were 2-DE and surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS). SELDI-TOF-MS is a high throughput technique, particularly appropriate for the investigation of low-molecular weight proteins (< of 20 KDa) with femtomole sensitivity, and the ability to examine native proteins which provides a complementary visualization technique of the 2-DE [27, 28].

Once identified, the panel of biomarkers was validated in a different cohort of patients affected by FM and controls, also examining the statistical correlation with the patients’ clinical features. The aim was the selection of markers effectively associated with FM which will enable clinicians to achieve an unambiguous diagnosis and improve approaches to patients’ management.

METHODS

Study design
In the present translational study, we conducted a proteome analysis of WS in patients suffering from FM. The global study design is shown in figure 1. There were 3 specified phases: collection, discovery and validation phases. First of all, we selected 30 FM patients, 30 healthy subjects, 30 patients suffering from RA, and 30 with migraine. The second “discovery” phase was aimed at characterizing the salivary proteomic profile of FM in comparison to positive and negative control groups. RA represents a model of chronic inflammatory disease, while migraine is a control as chronic pain with a non-inflammatory origin. Once a panel of biomarkers has been selected, in the “validation” phase we performed ELISA assay to assess the ability of these candidate proteins to differentiate FM patients from controls on a new cohort of subjects. Finally, we examined the statistical correlation of these selected proteins with patients’ clinical features.

Chemicals
CHAPS, urea, thiourea, glycerol, SDS, TEMED, ammonium persulfate, glycine, 30% acrylamide-
N,N,N-bisacrylamide, sodium chloride (NaCl), trizma base, dithiothreitol (DTT), and sodium
dihydrogen phosphate dehydrate (NaH$_2$PO$_4$) were from AppliChem (Darmstadt, Germany).
Iodoacetamide (IAA), Tween 20, bovine serum albumin (BSA), and HEPES were acquired from
Sigma-Aldrich (St. Louis, MO, USA). Coomassie Brilliant Blue G 250 was from Merck
(Darmstadt, Germany). IPGs pH 3–10 L, pharmalyte 3–10 and dry strip cover fluid were purchased
from GE Health Care Europe (Uppsala, Sweden). Trifluoroacetic acid (TFA), acetonitrile (ACN),
and sodium acetate from J.T. Baker (Center Valley, PA, USA). Ruthenium II tris
(bathophenanthroline disulfonate) tetrasodium salt was from SunaTech Inc. (Suzhou, P. R. China).
Ethanol, phosphoric acid (H$_3$PO$_4$) were from Romil (Cambridge, UK).

**Patients**

A total of 180 patients were consecutively recruited from the Rheumatology Unit at University-
Hospital of Pisa. Sixty patients with a diagnosis of FM [mean age 49.85 ± 12.5 years, (mean ± SD);
51 females and 9 males], made according to the ACR criteria for the disease, 60 patients affected by
RA (the patients fulfilled the ACR criteria for RA; mean age 45.38 ± 13.23 years; 52 females and 8
males), and 60 patients with migraine (diagnosis according to the International Classification of
Headache Disorders; mean age 46.38 ± 14.32 years; 46 females and 14 males) were enrolled in the
study. Sixty healthy subjects, with similar mean age (42.57 ± 6.22; 40 females and 20 males),
similar demographic characteristics and with no severe headaches that interfered with their daily
activities were included as controls. Ninety patients and 30 healthy subjects were included in the
discovery phase, the remaining were included in the validation phase.

This study was approved by the local Ethics Committee, and an informed consensus was obtained
for diagnostic or clinical purposes.

**Clinical assessment of patients**
For diagnosis of FM, patients performed a rheumatologic visit with routine clinical evaluation of medical history. The clinical assessment of FM patients was made on the basis of the following criteria:

- Fibromyalgia Impact Questionnaire (FIQ);
- Tenderness at tender points evaluated by digital pressure;
- Visual analogue scale (VAS) for minor symptoms of FM (fatigue, headache, sleep disturbances, gastro-intestinal symptoms and other symptoms), 0 indicates no symptoms whereas 10 is the worst condition;
- Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT fatigue) Scale (version 4);
- Health Assessment questionnaire (HAQ) to determine physical disability;
- Pittsburgh Sleep Quality Index questionnaire (PSQI) for assess sleep quality and disturbances;
- SF-36 questionnaire (Short Form with 36 questions), a well-documented, self-administered quality of life (QoL) scoring system.

For the diagnosis of RA patients, clinical assessment foresaw:

- Number of tender (TJC) and swollen joint count (SJC);
- 44 swollen joint count (SW44);
- General Health Status (GH);
- 28 non-graded joints (DAS28);
- modified Health Assessment Questionnaire (HAQ) score;
- joint deformities, extraarticular features, erosions.

The clinical assessment of migraine patients included:

- headache intensity on a VAS ;
- SF-36 questionnaire;
Migraine Disability Assessment Questionnaire (MIDAS).

Finally, psychiatric aspects of all patients were evaluated by means of:

- Structured Clinical Interview (SCID) for DSM-IV (26,27)
- Mood Spectrum Self-Report (MOODS-SR) lifetime version (28)
- Hamilton Depression Rating Scale (HAM-D) (29).

All data were collected according to the Good Clinical Practice.

Moreover, patients satisfied the inclusion criteria here listed: diagnosis of FM/RA/Migraine; aged between 18 and 65; patients of both sexes; acceptance of the protocol and signed informed consent.

Exclusion Criteria: patients aged > 65 and < 18; status of alleged or established pregnancy and lactation; lack or withdrawal of consent by the patient. Healthy subjects satisfied the inclusion and exclusion criteria here listed. Inclusion Criteria: healthy constitution; aged between 18 and 65; subjects of both sexes; acceptance of the protocol and signed informed consent. Exclusion criteria: patients aged > 65 and < 18; status of alleged or established pregnancy and lactation; presence of active rheumatic diseases, psychiatric disorders, infectious and/or unstable medical condition; Lack or withdrawal of consent by the subject.

**Laboratory tests**

The following blood tests, for FM patients, were adminstered: sedimentation rate, C reactive protein (CRP), thyroidal hormones and anti-thyroid antibody, serotonin, Ca\(^{2+}\), Mg\(^{2+}\), blood count, insulin-like growth factor 1, growth hormone, parathormone, 25-OH vitamin D, FAN antibodies, laboratory evaluation of spasmophilia. Blood tests for RA patients: rheumatoid factor (RA test), anti-cyclic citrullinated peptide antibody (anti-CCP), antinuclear antibody (ANA), CRP, sedimentation rate and blood count. RA patients will be characterized also by Joint X-rays.

**Whole saliva collection**

Salivary samples were collected from patients and controls with a saliva collector sponge (Surescreen Diagnostics LTD; Derby, UK). WS samples were collected early in the morning (between 8 and 11 a.m.) according to a standard protocol [29]. No evidence of oral pathologies or
inflammatory processes were observed. The saliva collected was immediately centrifuged at 17,000
2 g for 20 minutes at 4°C to yield clear samples. Samples were stored at -80°C. Protein amounts of
resulting supernatants were determined using the Bio-Rad DC-protein assay. BSA was used as a
standard.

Two-dimensional electrophoresis

2-DE was carried out as previously described [24]. We pooled samples according to their diagnosis,
6 pools for each group were prepared. Each pool contained 200 µg of proteins which were filled up
to 450 µl in rehydration solution. Immobiline Dry-Strips (GE Health Care Europe; Uppsala,
Sweden; 18 cm, linear gradient pH 3–10) were rehydrated overnight in the sample and then
transferred to the Ettan IPGphor Cup Loading Manifold (GE Healthcare) for isoelectrofocusing
(IEF). The second dimension (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis;
SDS-PAGE) was carried out by transferring the proteins to 12.5% polyacrylamide, running at 16
mA per gel and 10°C for about 16 h, using the Protean® Plus Dodeca Cell (BioRad, Hercules, CA,
USA). The gels were stained with Ruthenium II tris (bathophenanthroline disulfonate) tetrasodium
salt (RuBP). “ImageQuant LAS4010” (GE Health Care) was used for the acquisition of images. The
2-DE experiments were performed in triplicate. 2-DE SDS-PAGE standards (17.5-76 kDa, 4.5-8.5
pH; from BioRad) were used for calibration. The analysis of images was performed using the Same
Spot (v4.1, TotalLab; Newcastle Upon Tyne, UK) software. The spot volume ratios between the
different conditions were calculated using the average spot normalized volume of the three
biological replicates. The software included statistical analysis calculations. The protein spots of
interest were cut out from the gel and identified by nano-liquid chromatography electrospray
ionization tandem mass spectrometry (NanoLC-ESI-MS/MS) analysis.

NanoLC-ESI-MS/MS Analysis by LTQ-Orbitrap Velos analysis.

The gel pieces were destained in 100% EtOH during 2 hours. Subsequently, they were rehydrated
with 100 µl of 50 mM ammonium bicarbonate for 15 min and dehydrated with 100 µl of 50 mM
ammonium bicarbonate in 30% AcN for 15 min.
The gel pieces were then dried for 30 minutes in a Centrivap vacuum centrifuge (Labconco, Kansas City, USA). The dried pieces of gel were rehydrated for 45 min at 4°C in 20 μl of trypsin porcine (Sigma) solution (6.25 ng/μl in 50 mM ammonium bicarbonate) and then incubated at 37°C overnight. Extraction of the peptides was performed with 20 μl of 1% TFA for 30 min at room temperature with occasional shaking. The TFA solution containing the proteins was transferred to a polypropylene tube. A second extraction of the peptides was performed with 20 μl of 0.1% TFA in 50% AcN for 30 min at room temperature with occasional shaking. The second TFA solution was pooled with the first one. The volume of the pooled extracts were dried completely and finally resuspended in CH₃CN/FA 50%/0.1%.

LC-ESI-MS/MS was performed on a linear trap quadrupole (LTQ) Orbitrap Velos from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters. Peptides were trapped on a home-made 5 μm 200 Å Magic C18 AQ (Michrom) 0.1 × 20 mm pre-column and separated on a home-made 5 μm 100 Å Magic C18 AQ (Michrom) 0.75 × 150 mm column with a gravity-pulled emitter. The analytical separation was run for 23 min using a gradient of H₂O/FA 99.9%/0.1% (solvent A) and CH₃CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0–5 min 95% A and 5% B, then to 65% A and 35% B at 6 min, and 20 % A and 80 % B at 7 min at a flow rate of 220 nL/min. For MS survey scans, the orbitrap (OT) resolution was set to 60000 and the ion population was set to 5 × 10⁵ with an m/z window from 400 to 2000. For protein identification, up to five precursor ions were selected for collision-induced dissociation (CID). For MS/MS in the LTQ, the ion population was set to 1 × 10⁴ (isolation width of 2 m/z), while as for MS/MS detection in the OT, it was set to 1 × 10⁵ (isolation width of 2 m/z). The normalized collision energies were set to 35% for CID.

**Protein identification**

Peak lists were generated from raw orbitrap data using the embedded software from the instrument vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script [30]. The peaklist files were searched against the
UniProtKB/Swiss-Prot database using Mascot (Matrix Sciences, London, UK). Human taxonomy was specified for database searching. The parent ion tolerance was set to 10 ppm. Variable amino acid modifications were oxidized methionine and fixed amino acid modifications were carbamidomethyl cysteins. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. 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.

ELISA

The levels of Serotransferrin (TRFE), Alpha-enolase (ENO), and phosphoglycerate mutase I (PGAM1) were detected in WS by enzyme-linked immunosorbent assay (ELISA) kits (Uscn life science Inc.) according to the manufacturer’s instructions. The lower limit of detection of these assays was less than 0.051 ng/ml, 0.128 ng/ml, and 0.55 ng/ml for TRFE, ENO, and PGAM1 respectively. The ELISA kit for transaldolase (TALDO) (MyBioSource; San Diego, CA, USA) has a detection range of 0.625 ng/ml - 20 ng/ml with sensitivity of 0.1 ng/ml.

The dilutions of WS were 1:750, 1:2, 1:2, and 1:10 for TRFE, ENO, PGAM1, and TALDO respectively.

SELDI-TOF-MS

We used Protein Chip Arrays (BioRad) CM10, which captures proteins with positive surface charges. Aliquots of WS (corresponding to 20 μg) were mixed (2:3 v/v) with denaturing buffer solution (9 M urea, 2% CHAPS) and incubated for 30 min before loading onto Protein Chip arrays. Each chip was prepared according to manufacturer’s instructions. We first washed the chips two times with the binding buffer (100 mM sodium acetate pH 4 for CM10) then we applied on the spots the sample. After an incubation of 1 h, under constant agitation, the chips were washed three times with the specific binding buffer, twice with 150 μL of HEPES (10 mM, pH 7.0) and then air-dried for 5 min. Finally 1 μL of a 50% saturated solution of sinapinic acid (SPA) in 50% ACN, 1% TFA was applied twice to each spot to facilitate desorption and ionization. The chips were read
on a ProteinChip SELDI reader (Personal Edition, BioRad) using an automated protocol (laser energy 3500 nJ; matrix attenuation 1000; focus mass 10 kDa; acquired mass range from 0 to 100 kDa). Analysis of the spectra was carried out using Protein Chip data manager software 3.5. Spectra were visually examined and poor quality spectra were excluded from further analysis.

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

OFFGEL

The OFFGEL High Resolution kit pH 3–10 (Agilent Technologies) was used for pI-based protein preparative isoelectric focusing (IEF) in solution. WS protein samples (800 µg of proteins) were solubilized in a Protein OFFGEL fractionation buffer supplied by the manufacturer (containing 8 M urea, 2 M thiourea, 1% DTT, 12% glycerol, and 1.2% buffer with ampholytes), and aliquots were evenly distributed in a 24-well 3100 OFFGEL Fractionator (Agilent Technologies) tray according to supplier instructions. We applied a preset program (separation limits: 8000 V, 200 mW, and 50 µA; starting voltage, 200–350 V; ending voltage, 2000–4200 V; after the application of 64 kVh, the protein separation zones were maintained at constant voltage). The liquid fractions were recovered, and pH of each fraction was measured. Then the 24 OFFGEL fractions were assayed by SELDI-TOF to search the presence of peaks of interest.
**Statistical analysis**

**2-DE**

A comparison between FM and controls was performed. The significance of the differences of normalized volume for each spot was calculated by the software Progenesis Same Spot including the Analysis of variance (ANOVA test). The protein spots with $p < 0.05$ were cut out from the gel and identified by NanoLC-ESI-MS/MS analysis.

**ELISA**

Comparisons between groups were performed using the Mann-Whitney U test for non-normal data. Linear regression analysis was used to determine the correlation among levels of different biomarkers. Logistic regression was used to determine the weight given to each biomarker and then to calculate a specific formula to provide a combined risk index \[\text{[31]}\]. To estimate whether this biomarker combination might increase their performance in FM detection, receiver operating characteristic (ROC) curves were plotted, and the areas under curves (AUC) were calculated with their 95% confidence intervals using standard techniques to evaluate sensitivity and specificity of each marker and their combination. Statistical analyses were performed with SPSS (Statistical Package for the Social Science update for 10.1. Chicago, IL: SPSS Inc., 2000.).

**Clinical correlations**

To determine the statistical correlations among putative biomarkers and clinical parameters, the Spearman’s rank correlation coefficient, a non-parametric measure of correlation based on data ranks was calculated. A $p$-value $< 0.05$ was considered significant. Clinical correlations were performed with SPSS. The evaluated clinical parameters were: Fibromyalgia Impact Questionnaire (FIQ) and Revised Fibromyalgia Impact Questionnaire (FIQR), Functional Assessment of Chronic Illness Therapy-Fatigue Scale (FACIT), the number of tender points, pain visual analogic scale (VAS).

**SELDI-TOF-MS**
The data of SELDI-TOF-MS were analyzed by univariate (Mann-Whitney) and further multivariate analysis. The univariate analysis determines if the intensity of a peak is significantly different in the experimental group spectrum as compared to controls; p-values associated with every peak were calculated using the Mann-Whitney test (significant when < 0.05). Subsequently, in order to extract potentially relevant peaks, among peaks found significant by the univariate analysis, we used multivariate analysis (classification and regression tree algorithm). The classes of comparison were FM vs healthy subjects, FM vs migraine, and FM vs migraine plus healthy.

RESULTS

Two-dimensional electrophoresis and validation

Fig. 2 illustrates a representative 2-DE image of WS. The quality of the gels was assessed by the software Same Spot which includes the SpotCheck function as previously described [32]. Normalized spot volumes were analyzed by the ANOVA test to detect the proteins which were significantly related with FM. These protein spots were chosen for excision and identified by NanoLC-ESI-MS/MS analysis. The analysis of 2-DE profiles allowed us to find 17 spots with a different expression in FM respect to RA, 19 spots from the comparison of FM with migraine, and 23 in FM respect to healthy subjects. In particular, we found 5 spots differentially expressed solely in FM. Four spots were identified as TRFE and the other as ENOA (fig. 3). The list of identified proteins, with statistical analysis, molecular weight (MW), isoelectric point (pI), coverage, and score values of NanoLC-ESI-MS/MS is given in tables 1 and 2. We indicated also proteins whose identification has been derived from previous works characterizing WS proteomics map [[24], [25], [26]].

ELISA assays were used to validate in WS the expression changes of 4 proteins: TRFE, ENOA, PGAM1, and TALDO. PGAM1, and TALDO were proteins found up-regulated in WS of FM patients in our previous work [26].
The mean values of TRFE, ENOA, and PGAM1 were statistically different in FM respect to controls (healthy subjects and migraine). On the other hand, the increased expression in WS of FM was not confirmed for TALDO (figure 4, table 3).

ROC curves were calculated to assess the clinical potential of our selected proteins to distinguish FM from control samples (healthy and migraine). The areas under the ROC curves (AUC) were calculated for each protein individually, showing if each marker alone can discriminate FM. Moreover, using a logistic regression analysis, we investigated if the discriminative power of each marker could be potentially increased by the combination of different markers. With this purpose, we tested all the different combinations in order to select the best association of biomarkers useful to discriminate control from FM samples. ENOA was found as the most differentiating biomarker, with AUC of ROC curve of 0.738. However its discriminative power was increased if ENOA was combined with other proteins. In particular, the best performance in diagnosis was obtained by combining ENOA, PGAM1 and TRFE as shown in table 4 and figure 5 which illustrates the ROC curve obtained by combining all three biomarkers (AUC 0.792).

**Clinical correlations**

No statistically significant correlation was detected between our putative biomarkers expression and any of the following FM clinical parameters: FIQ, FIQR, FACIT, the number of tender points, and pain VAS.

**SELDI-TOF-MS**

Figure 6 reports a representative protein profile of WS by using CM10 ProteinChips in the molecular range of 0–50 kDa. Since SELDI-TOF-MS is often criticize for its poor reproducibility, it’s mandatory to perform at the same time all the experiments with the same chip for the different classes. In addition the use of quality controls (QC) is highly recommended. QC is a well-characterized pool of samples processed alongside the experimental samples in order to calculate coefficient of variation (CV) for peak intensities and mass accuracy as a measure of reproducibility of the SELDI-TOF-MS analyses. The QC samples were applied randomly on different chips in
order to avoid any artefact due to experimental handling. The CV (the standard deviation of the series divided by the mean of the series) was calculated using multiple protein peaks selected over the experiments. In this study the CV was 24.5% for peak intensity, and 0.008% for mass accuracy with the CM10 chips. Our CVs indicated acceptable reproducibility of the spectra. Peak detection with the ProteinChip data manager software 3.5 resolved a total of 116 peaks on CM10 in the m/z ratio between 2000 and 100,000. Each spectra was thus described by 116 input variables where each variable correspond to the peak intensity for the given m/z.

We selected the most discriminating peaks for each comparison: 2 peaks in the comparison of FM patients vs healthy subjects, 4 peaks for FM patients vs migraine patients, 4 peaks for FM patients vs migraine patients plus healthy subjects, and 3 peaks for FM patients vs RA patients. Tables 5 illustrates the most discriminating peaks obtained with logistic regression for each comparison; in the table we report the predictions of these statistical analysis with the error rate.

The identification of the peaks of interest was launched by carrying out a separation of the proteins with OFFGEL. This technique allows the separation of the sample proteins, according to their isoelectric point (pI) and to collect them in liquid fractions. Therefore, by processing our WS samples, the OFFGEL fractionation allowed us to focus our attention on 2 peaks: v21 (m/z 4548) with a pI of 5.18, and v83 (m/z 13288) with a pI of 6.3. Further studies are mandatory to identify these peaks, but through the use of TagIdent we could hypothesize their identity. TagIdent is a tool from Expasy which allows the generation of a list of proteins close to a given pI and MW (http://web.expasy.org/tagident/). The peak v21 could be the orexigenic neuropeptide QRFP (MW 4522, pI 5.11), while the v83 seems to be the peptidyl-prolyl cis-trans isomerase (PPIA; MW 13208, pI 6.3).

**DISCUSSION**

Considering the deep complexity of FM, the discovery of more objective markers could support clinicians’ diagnosis. Therefore, with the present work, we aimed to carry out our proteomic study on FM patients by characterizing WS. Respect to our previous work [26], we decided to extend the
analysis to both positive and negative controls, and therefore we added RA and migraine patients as positive control subjects. Indeed, the clinicians can differentiate a patient with RA from one with FM but we were interested in examining the putative inflammatory component in FM by including a model of chronic inflammatory pain. In conjunction, it was more complete to also compare FM WS pattern with a model of non-inflammatory chronic pain such as migraine. Therefore we compared WS proteins of FM patients with sex- and age-matched control subjects.

The most relevant observation from 2-DE analysis was the peculiar up-regulation in FM of TRFE which was validated by ELISA. We identified more protein spots corresponding to TRFE because different variants are known. TRFE is the main blood iron-binding protein which delivers iron to sites of storage and utilization from those of absorption and heme degradation. Hence, TRFE reflects the need of tissues for iron; in fact an iron deficiency in rats causes the fall in ferritin and the rise of TRFE in the brain [33]. Some studies hint at a role of iron in FM’s pathophysiology, since it is a cofactor in serotonin and dopamine production [34]. Actually, in another disease in which the dopaminergic system is involved, the restless legs syndrome, high level of TRFE in patients’ cerebrospinal fluid were found, in spite of levels similar to those of normal controls in serum [35, 36]. To the best of our knowledge, no association was found between serum levels of TRFE and FM [37] and ours, is the first study reporting evidence of an association between salivary TRFE levels and FM. It has been demonstrated that TRFE can be secreted by parotid cells so its detection in WS is not due to blood contamination [38]. Its role in saliva has not yet been investigated but lactoferrin, a salivary analogue of TRFE, has bacteriostatic effects [39, 40] and therefore it has been proposed that TRFE could have a similar antimicrobial action due to its iron-sequestering properties [38].

Another protein found up-regulated in WS from patients, respect to controls was ENOA. This is the enzyme involved in the penultimate step of glycolysis. Beyond its well-known enzymatic role in metabolism, ENOA is a multifunctional protein with several functions diverging from its original role in the glycolytic pathway. Indeed, it can act as a plasminogen receptor promoting metastatic
invasion in many types of cancers and is involved in cell-matrix adhesion, survival, and senescence [41-44]. Moreover, specific auto-antibodies anti-ENOA were detected in connective tissue diseases [45] which arise the question of a generic association of this biomarker with different inflammatory and autoimmune pathologies [46-49]. Indeed, when we analyzed ENOA levels by ELISA in WS from RA patients we observed its increase respect to controls (healthy subjects plus migraine patients). Thus, while this could potentially sustain the autoimmune nature of FM, on the other side, it shows ENOA is not a specific biomarker. This explains the reason for searching a combination of biomarkers instead of a single one. In fact, its discriminative power was increased if ENOA was combined with TRFE and PGAM1.

PGAM1 is the enzyme catalyzing the step of glycolysis preceding ENOA. We found its up-regulation in FM patients respect to healthy subjects, confirming our previous results, but also respect to migraine subjects, by both 2-DE and ELISA. Autoantibodies against anti-PGAM1 were found in sera from patients with various neurological diseases; therefore PGAM1 specificity is questionable and it has been defined as a nonspecific marker of autoimmune diseases of the central nervous system [50, 51]. Hence, this knowledge is quite interesting considering the neurological feature of FM but further sustains the importance of a combination of multiple biomarkers in defining FM.

TALDO is the enzyme whose increase was typical for FM patients respect to healthy subjects, in our previous study [26]. This enzyme takes part to the pentose-phosphate pathway which is related to the production of NADPH. Oxidative stress has been often been considered as a major factor in pathophysiology of FM [52, 53], even if its role remains elusive [54]. Therefore, the up-regulation of TALDO can be interpreted as an attempt of increasing the NADPH production in order to reduce oxidative damage of tissues. This increase has been actually confirmed in the present work by 2-DE. On the other hand, its alteration was not found in the comparison between FM and migraine patients. It is worth to notice that ELISA totally corroborated these results. Indeed, figure 4D and table 3 show the results from the comparison of TALDO levels in WS from FM patients respect to
controls (healthy subjects plus migraine patients). However, the TALDO level in healthy subjects is much lower (signal intensity in healthy = 39.6 vs signal intensity in FM = 117.6) and the p-value respect to FM patients becomes 0.0062, strengthening our previous study. So, this result reduces the reliability of TALDO as FM biomarker, because it did not allow distinguishing FM patients from migraine patients. At the same time the finding could let to speculate that the alteration of TALDO is typical of a non-inflammatory chronic pain such as migraine, and therefore also of FM, whose inflammatory component has always been debated. Finally, none of the candidate proteins showed a statistical correlation with the patients’ clinical features (e.g. FIQ, VAS, tender points), as already previously found for TALDO and PGAM1 [26].

In addition to 2-DE for the first time, we analyzed protein expression profiles obtained by SELDI-TOF-MS for each sample, with the attempt of providing a complementary visualization technique to 2-DE. Therefore, by processing our WS samples with SELDI-TOF-MS and by fractionation with OFFGEL, we focused our attention on 2 peaks: v21 and v83. The peak v21 could be the orexigenic neuropeptide QRFP, while the v83 seems to be the PPIA. On the other hand, in our previous work, we detected in FM saliva the up-regulation of cyclophilin A, another protein with analogue peptidylprolyl cis-trans-isomerase activity. Concerning the neuropeptide QRFP, its role has been recently reviewed and it is has been pointed out that QRFP mRNA and/or binding sites are enriched in the parafascicular thalamic nucleus, the locus coeruleus, the dorsal raphe nucleus and the parabrachial nucleus, which are involved in pain transmission [55]. Moreover, data indicated that this peptide is involved in the regulation of glucose homeostasis [55, 56]. Therefore its potential up-regulation in FM deserves further study. This hypothesis clearly need to be validated using with complementary techniques.

In conclusion, we performed proteomic analysis of WS by using complementary approaches and overcoming the limit of our previous work. Indeed, we have now extended the analysis to a big cohort of patients including both negative and positive controls, instead of only healthy subjects. Nevertheless, we could support the results of the previous work and also propose new biomarkers
which can collectively contribute in defining a panel of salivary proteins allowing to distinguish fibromyalgic pain with good sensitivity and specificity. We believe that this panel could be a useful tool in supporting clinicians’ diagnosis and defining FM clusters and targeted treatment. Actually, our future perspective foresees to develop a simple, rapid and not invasive point-of-care test. This device could also be worthwhile for population screening and to characterize fibromyalgic pain. In addition, together with mandatory future experiments to validate SELDI analysis, the present results can offer a clue for a better knowledge of such a complex disease like FM.

ACKNOWLEDGMENTS

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FIGURES CAPTIONS

Figure 1. Study design. The graphical representation of experimental design.

Figure 2. 2-DE image of WS. Representative image of 2-DE proteomic pattern of WS from a FM pool. A total of 200 μg of proteins was separated by 2-DE using 18 cm pH 3-10-L strips and 12.5% SDS-PAGE. Identified spots are encircled.

Figure 3. 2-DE enlarged images and analysis of TRFE, ENOA. Histograms of the normalized volume (mean ± SEM) obtained by 2-DE analysis of proteins found up-regulated in WS of FM patients in respect to all control subjects (A), and enlarged images of these proteins (B). TRFE: n° 69, 84, 1466, 1472; ENOA: n° 343. M: migraine.

Figure 4. Validation of ENOA, TRFE, PGAM1, TALDO. ELISA results for ENOA (A), TRFE (B), PGAM1 (C), TALDO (D): the bar graph shows the mean ± SEM of signal intensity. Statistical significance of the differences was calculated by Mann-Whitney U test (*p ≤ 0.05, **p ≤ 0.01 ***p ≤ 0.001).

Figure 5. ROC curve. Receiver operating characteristic curve (ROC) obtained from the combination of ENOA, TRFE, PGAM1, in WS.

Figure 6. SELDI-TOF. Representative protein profile of WS by SELDI-TOF-MS using CM10 ProteinChips in the molecular range of 0–50 kDa.
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Table 2: Statistical analysis of proteins which were significantly different in WS of FM patients respect to controls. RA: Rheumatoid Arthritis; M: Migraine.

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<td>+2.3</td>
<td>0.0009</td>
<td>+1.6</td>
<td>1.30e-06</td>
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<td>p-value</td>
<td>log2FoldChange</td>
<td>FDR Adjusted p-value</td>
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<td>1472</td>
<td>Serotransferrin</td>
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<td>+2.1</td>
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<td>Serum albumin</td>
<td>P02788</td>
<td>n.s.</td>
<td>0.0085</td>
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<td>P02788</td>
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<td>*Cystatin-SN</td>
<td>P01037</td>
<td>n.s.</td>
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<td>2.0e^-05</td>
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<td>*Cystatin-SN</td>
<td>P01037</td>
<td>n.s.</td>
<td>n.s.</td>
<td>0.02</td>
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<td>1425</td>
<td>*Calgranulin-A</td>
<td>P05109</td>
<td>n.s.</td>
<td>0.03</td>
<td>+1.4</td>
<td>n.s.</td>
<td>0.003</td>
<td>+1.7</td>
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* Proteins whose identification has been derived from previous works characterizing WS proteomics map (ref. 24, 25, 26).
Table 3. Statistical analysis of signal intensity of selected WS proteins obtained by ELISA. Controls: healthy subjects plus migraine patients.

<table>
<thead>
<tr>
<th>ELISA signal intensity (M±SEM)</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>FM</strong></td>
<td><strong>Controls</strong></td>
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<tr>
<td>Serotransferrin</td>
<td>3456±411</td>
</tr>
<tr>
<td>Alpha-enolase</td>
<td>3.2 ± 0.56</td>
</tr>
<tr>
<td>PGAM1</td>
<td>3.72 ± 1.11</td>
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<tr>
<td>TALDO</td>
<td>117.56±21.5</td>
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</table>
Table 4. Comparison between sensitivities and specificities of single biomarkers and their combination.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUC</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>Cut-off ng/ml</th>
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<tbody>
<tr>
<td>TRFE</td>
<td>0.699</td>
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<td>57</td>
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<td>TRFE, ENOA, PGAM1</td>
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<td>FM vs M m/z</td>
<td>FM vs RA m/z</td>
<td>FM vs H+M m/z</td>
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<td>V101</td>
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Error rate%  
17.7%   16.1%   24.1%   12.3%
PROTEOME ANALYSIS

COLLECTION

FM MIGRAINE
n=30  n=30

RA HEALTHY
n=30  n=30

DISCOVERY

2-DE
+ NanoLC-ESI-MS/MS

VALIDATION

ELISA
New cohort of subjects

ROC
Best markers combination
Conflict of Interest
Click here to download Conflict of Interest: coi_Ciregia.pdf
*Conflict of Interest
Click here to download Conflict of Interest: coi_Giacomelli.pdf
*Conflict of Interest
Click here to download Conflict of Interest: coi_Piga.pdf
*Conflict of Interest
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*Conflict of Interest

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*Conflict of Interest
Click here to download Conflict of Interest: coi_Lucacchini.pdf
Conflict of Interest

Click here to download Conflict of Interest: coi_Bazzichi.pdf