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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.

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Cover Letter

Pisa, November 9th 2017

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.

Graphical Abstract

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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, PGAM1and TRFE, is proposed as an useful tool in supporting clinicians in diagnosis of FM.

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1 **Putative predictive salivary biomarkers to distinguish fibromyalgic pain.**

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19 **ABSTRACT**

20 Fibromyalgia (FM) is a chronic pain disorder characterized by widespread pain and associated with
21 unspecific symptoms. So far, no laboratory tests have been validated. The aim of the present study
22 was to investigate the presence in saliva of potential diagnostic and/or prognostic biomarkers which
23 could be useful for the management of FM patients. Specifically, we carried on our previous work
24 by comparing the salivary profile of 30 FM patients with those of 30 healthy subjects, 30 subjects
25 suffering migraine (model of non-inflammatory chronic pain), and 30 patients affected by
26 rheumatoid arthritis (model of inflammatory chronic pain). For the proteomics analysis we applied
27 2-DE and SELDI-TOF-MS. The SELDI analysis allowed focusing our attention on two peaks
28 which could correspond to orexigenic neuropeptide QRFP and peptidyl-prolyl cis-trans isomerase.
29 From 2-DE we found serotransferrin (TRFE) and alpha-enolase (ENOA) differentially expressed in
30 FM. Hence, we validated their expression by ELISA together with phosphoglycerate mutase I
31 (PGAM1) and transaldolase, which were found in our previous work. Moreover, ROC curve was
32 calculated to investigate the discriminative power of our biomarkers. The best performance was
33 obtained by combining ENOA, PGAM1 and TRFE. We believe that this panel could be a useful
34 tool in supporting clinicians in diagnosis.

35 **SIGNIFICANCE**

36 FM is one of the most common chronic pain condition which is associated with significant
37 disability. The fibromyalgic pain is a peculiar characteristic of this disease and FM patients suffer
38 from reduced quality of life, daily functioning and productivity. Considering the deep complexity of
39 FM, the discovery of more objective markers is crucial for supporting clinical diagnosis. Therefore,
40 the aim of the present study was the selection of biomarkers effectively associated with
41 fibromyalgic pain which will enable clinicians to achieve an unambiguous diagnosis, and to
42 improve approaches to patients' management. We defined a panel of 3 salivary proteins which
43 could be one of the criteria to be taken into account. Consequently, the identification of disease
44 salivary biomarkers could be helpful in detecting FM clusters and targeted treatment. Actually, our

45 future perspective foresees to develop a simple, rapid and not invasive point-of-care testing which
46 will be of use during the diagnostic process. In addition, the present results can offer a clue for
47 shedding light upon the complex entity of such a disease like FM.

48 **INTRODUCTION**

49 Fibromyalgia (FM) is a chronic pain disorder characterized by widespread pain for at least 3
50 months. Other associated unspecific symptoms may be present, including fatigue, sleep
51 disturbances, memory problems, irritable bowel syndrome, headache, and depression [1,2].
52 This condition is associated with significant disability: FM patients suffer from reduced quality of
53 life, daily functioning and productivity. Hence, it involves losses in productivity, reduced work
54 hours, absenteeism, disability, unemployment, early retirement, informal care and other costs [3].
55 The global mean prevalence of FM has been estimated to be 2.7 %. In women, the mean prevalence
56 is 4.2 % and in men 1.4 %, with a female-to-male ratio of 3:1 [2]. Weir et al. [4] reported an
57 incidence rate of 6.88 new cases per 1,000 person/years for males and 11.28 new cases per 1,000
58 person/years for females.
59 The diagnosis of FM is a contentious issue due to the lack of laboratory testing. In 1990 the
60 American College of Rheumatology (ACR) defined some criteria requiring that individuals have
61 widespread pain (pain in the axial skeleton, above and below the waist, and on both sides of the
62 body) as well as tenderness in 11 or more of 18 possible "tender points" [1, 5]. In 2010 and 2011
63 these criteria have been revised leading to a change in the concept of FM, excluding tender point
64 site palpation as an essential diagnostic criterion to make the diagnosis [6, 7]. In particular, in 2011,
65 patient-based FM criteria were defined and added to the physician-based criteria from 2010 [8].
66 Thereby, newer diagnostic criteria are entirely symptom-based and do not require counts of the
67 number of tender points [1]. More recently, in 2016, a systematic review found consistency in
68 sensitivity and specificity between the ACR 1990 diagnostic criteria and the 2010/2011 criteria [8].
69 The 2016 committee maintained the difference between patient and physician-criteria but added a
70 "generalized pain" criterion to prevent the inclusion of regional pain syndromes in the FM [10].
71 However, despite the progress made, the diagnosis basically relies on the clinicians experience .
72 Hitherto, the etiology of FM has been elusive; although FM has usually been considered a non-
73 inflammatory and non-autoimmune disease, many studies have focused on the inflammatory and

74 autoimmune hypothesis. Alterations in cytokine profiling, and presence of autoantibodies have been
75 reported in patients with FM [11-16]. Nevertheless, up to date, no consistent validated markers have
76 been found. In recent years, few works investigated miRNA in biofluids such as blood [17-19],
77 saliva [18] and cerebrospinal fluid [20]. These studies proposed that miRNA can help in
78 characterizing FM, but they were limited to a small number of FM patients and validation in larger
79 study groups is needed.
80 Another area of investigation is genetic study, founded on the idea that genetic factors may
81 predispose to FM in combination with environmental triggers (e.g. trauma, infections or emotional
82 stress). The principal genes supposed to be a risk factor for FM are serotonin transporter (5-HTT),
83 catechol-O-methyltransferase (COMT) and the dopamine receptor [21, 22]. But the selected
84 polymorphisms are often associated with psychiatric disorders thereby they could be related to
85 psychiatric comorbidities rather than to sole FM. Moreover, genetic results are often controversial
86 and no specific candidate gene has been closely connected with FM.
87 Since the actual biological function relies on dynamic population of proteins, there is increasing
88 interest in the field of proteomics. Moreover, only the characterization of the proteins themselves
89 can give insight into protein-protein interactions and functions. That the reason why, in the last
90 years, we applied proteomic analysis in the discovery of biomarkers for many rheumatic diseases
91 [23-25]. In 2009, we carried out a study on human whole saliva (WS) of patients affected by FM
92 [26]. The aim was to identify the protein content of WS defining the differences between FM
93 patients and healthy subjects. The use of saliva might enable the easy characterization of a non-
94 invasively collected biological fluid, giving rise to a different approach in the diagnosis of FM. In
95 this work we used two-dimensional electrophoresis (2-DE) to obtain the WS protein map of FM
96 patients. Our study attested the potential usefulness of the proteomic characterization of human WS
97 in distinguishing FM from healthy subjects [26].
98 Following these encouraging results, the focus of the present study has been to investigate the
99 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

126 CHAPS, urea, thiourea, glycerol, SDS, TEMED, ammonium persulfate, glycine, 30% acrylamide-
127 N,N,N-bisacrylamide, sodium chloride (NaCl), trizma base, dithiothreitol (DTT), and sodium
128 dihydrogen phosphate dehydrate (NaH_2PO_4) were from AppliChem (Darmstadt, Germany).
129 Iodoacetamide (IAA), Tween 20, bovine serum albumin (BSA), and HEPES were acquired from
130 Sigma-Aldrich (St. Louis, MO, USA). Coomassie Brilliant Blue G 250 was from Merck
131 (Darmstadt, Germany). IPGs pH 3–10 L, pharmalyte 3–10 and dry strip cover fluid were purchased
132 from GE Health Care Europe (Uppsala, Sweden). Trifluoroacetic acid (TFA), acetonitrile (ACN),
133 and sodium acetate from J.T. Baker (Center Valley, PA, USA). Ruthenium II tris
134 (bathophenanthroline disulfonate) tetrasodium salt was from SunaTech Inc. (Suzhou, P. R. China).
135 Ethanol, phosphoric acid (H_3PO_4) were from Romil (Cambridge, UK).

136 **Patients**

137 A total of 180 patients were consecutively recruited from the Rheumatology Unit at University-
138 Hospital of Pisa. Sixty patients with a diagnosis of FM [mean age 49.85 ± 12.5 years, (mean ± SD);
139 51 females and 9 males], made according to the ACR criteria for the disease, 60 patients affected by
140 RA (the patients fulfilled the ACR criteria for RA; mean age 45.38 ± 13.23 years; 52 females and 8
141 males), and 60 patients with migraine (diagnosis according to the International Classification of
142 Headache Disorders; mean age 46.38 ± 14.32 years; 46 females and 14 males) were enrolled in the
143 study. Sixty healthy subjects, with similar mean age (42.57 ± 6.22; 40 females and 20 males),
144 similar demographic characteristics and with no severe headaches that interfered with their daily
145 activities were included as controls. Ninety patients and 30 healthy subjects were included in the
146 discovery phase, the remaining were included in the validation phase.

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

149 **Clinical assessment of patients**

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

153 – Fibromyalgia Impact Questionnaire (FIQ);
154 – Tenderness at tender points evaluated by digital pressure;
155 – Visual analogue scale (VAS) for minor symptoms of FM (fatigue, headache, sleep
156 disturbances, gastro-intestinal symptoms and other symptoms), 0 indicates no symptoms
157 whereas 10 is the worst condition;
158 – Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT fatigue) Scale (version
159 4);
160 – Health Assessment questionnaire (HAQ) to determine physical disability;
161 – Pittsburgh Sleep Quality Index questionnaire (PSQI) for assess sleep quality and
162 disturbances;
163 – SF-36 questionnaire (Short Form with 36 questions), a well-documented, self-administered
164 quality of life (QoL) scoring system.

165 For the diagnosis of RA patients, clinical assessment foresaw:

166 – Number of tender (TJC) and swollen joint count (SJC);
167 – 44 swollen joint count (SW44);
168 – General Health Status (GH);
169 – 28 non-graded joints (DAS28);
170 – modified Health Assessment Questionnaire (HAQ) score;
171 – joint deformities, extraarticular features, erosions.

172 The clinical assessment of migraine patients included:

173 – headache intensity on a VAS ;
174 – SF-36 questionnaire;

175 – Migraine Disability Assessment Questionnaire (MIDAS).

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

177 – Structured Clinical Interview (SCID) for DSM-IV (26,27)

178 – Mood Spectrum Self-Report (MOODS-SR) lifetime version (28)

179 – Hamilton Depression Rating Scale (HAM-D) (29).

180 All data were collected according to the Good Clinical Practice.

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

190 **Laboratory tests**

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

197 **Whole saliva collection**

198 Salivary samples were collected from patients and controls with a saliva collector sponge
199 (Surescreen Diagnostics LTD; Derby, UK). WS samples were collected early in the morning
200 (between 8 and 11 a.m.) according to a standard protocol [29]. No evidence of oral pathologies or

201 inflammatory processes were observed. The saliva collected was immediately centrifuged at 17,000
202 g for 20 minutes at 4°C to yield clear samples. Samples were stored at -80°C. Protein amounts of
203 resulting supernatants were determined using the Bio-Rad DC-protein assay. BSA was used as a
204 standard.

205 **Two-dimensional electrophoresis**

206 2-DE was carried out as previously described [24]. We pooled samples according to their diagnosis,
207 6 pools for each group were prepared. Each pool contained 200 µg of proteins which were filled up
208 to 450 µl in rehydration solution. Immobiline Dry-Strips (GE Health Care Europe; Uppsala,
209 Sweden; 18 cm, linear gradient pH 3–10) were rehydrated overnight in the sample and then
210 transferred to the Ettan IPGphor Cup Loading Manifold (GE Healthcare) for isoelectrofocusing
211 (IEF). The second dimension (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis;
212 SDS-PAGE) was carried out by transferring the proteins to 12.5% polyacrylamide, running at 16
213 mA per gel and 10°C for about 16 h, using the Protean® Plus Dodeca Cell (BioRad, Hercules, CA,
214 USA). The gels were stained with Ruthenium II tris (bathophenanthroline disulfonate) tetrasodium
215 salt (RuBP). "ImageQuant LAS4010" (GE Health Care) was used for the acquisition of images. The
216 2-DE experiments were performed in triplicate. 2-DE SDS-PAGE standards (17.5-76 kDa, 4.5-8.5
217 pH; from BioRad) were used for calibration. The analysis of images was performed using the Same
218 Spot (v4.1, TotalLab; Newcastle Upon Tyne, UK) software. The spot volume ratios between the
219 different conditions were calculated using the average spot normalized volume of the three
220 biological replicates. The software included statistical analysis calculations. The protein spots of
221 interest were cut out from the gel and identified by nano-liquid chromatography electrospray
222 ionization tandem mass spectrometry (NanoLC-ESI-MS/MS) analysis.

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

224 The gel pieces were destained in 100% EtOH during 2 hours. Subsequently, they were rehydrated
225 with 100 µl of 50 mM ammonium bicarbonate for 15 min and dehydrated with 100 µl of 50 mM
226 ammonium bicarbonate in 30% AcN for 15 min.

227 The gel pieces were then dried for 30 minutes in a Centrivap vacuum centrifuge (Labconco, Kansas
228 City, USA). The dried pieces of gel were rehydrated for 45 min at 4°C in 20 µl of trypsin porcine
229 (Sigma) solution (6.25 ng/µl in 50 mM ammonium bicarbonate) and then incubated at 37°C
230 overnight. Extraction of the peptides was performed with 20 µl of 1% TFA for 30 min at room
231 temperature with occasional shaking. The TFA solution containing the proteins was transferred to a
232 polypropylene tube. A second extraction of the peptides was performed with 20 µl of 0.1% TFA in
233 50% AcN for 30 min at room temperature with occasional shaking. The second TFA solution was
234 pooled with the first one. The volume of the pooled extracts were dried completely and finally
235 resuspended in CH_3CN/FA 50%/0.1%.
236 LC-ESI-MS/MS was performed on a linear trap quadrupole (LTQ) Orbitrap Velos from Thermo
237 Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters. Peptides were
238 trapped on a home-made 5 µm 200 Å Magic C18 AQ (Michrom) 0.1 × 20 mm pre-column and
239 separated on a home-made 5 µm 100 Å Magic C18 AQ (Michrom) 0.75 × 150 mm column with a
240 gravity-pulled emitter. The analytical separation was run for 23 min using a gradient of H_2O/FA
241 99.9%/0.1% (solvent A) and CH_3CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows:
242 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
243 a flow rate of 220 nL/min. For MS survey scans, the orbitrap (OT) resolution was set to 60000 and
244 the ion population was set to 5 × 105 with an m/z window from 400 to 2000. For protein
245 identification, up to five precursor ions were selected for collision-induced dissociation (CID). For
246 MS/MS in the LTQ, the ion population was set to $1 \times 10e^4$ (isolation width of 2 m/z), while as for
247 MS/MS detection in the OT, it was set to $1 \times 10e^5$ (isolation width of 2 m/z). The normalized
248 collision energies were set to 35% for CID.
249 **Protein identification**
250 Peak lists were generated from raw orbitrap data using the embedded software from the instrument
251 vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected
252 using an in-house written Perl script [30]. The peaklist files were searched against the

253 UniProtKB/Swiss-Prot database using Mascot (Matrix Sciences, London, UK). Human taxonomy
254 was specified for database searching. The parent ion tolerance was set to 10 ppm. Variable amino
255 acid modifications were oxidized methionine and fixed amino acid modifications were
256 carbamidomethyl cysteins. Trypsin was selected as the enzyme, with one potential missed cleavage,
257 and the normal cleavage mode was used. The mascot search was validated using Scaffold 3.6.0
258 (Proteome Software, Portland, OR). Only proteins matching with two different peptides with a
259 minimum probability score of 95% were considered identified.

260 **ELISA**

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

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

269 **SELDI-TOF-MS**

270 We used Protein Chip Arrays (BioRad) CM10, which captures proteins with positive surface
271 charges. Aliquots of WS (corresponding to 20 μg) were mixed (2:3 v/v) with denaturing buffer
272 solution (9 M urea, 2% CHAPS) and incubated for 30 min before loading onto Protein Chip arrays.
273 Each chip was prepared according to manufacturer's instructions. We first washed the chips two
274 times with the binding buffer (100 mM sodium acetate pH 4 for CM10) then we applied on the
275 spots the sample. After an incubation of 1 h, under constant agitation, the chips were washed three
276 times with the specific binding buffer, twice with 150 μL of HEPES (10 mM, pH 7.0) and then air-
277 dried for 5 min. Finally 1 μL of a 50% saturated solution of sinapinic acid (SPA) in 50% ACN,
278 1%TFA was applied twice to each spot to facilitate desorption and ionization. The chips were read

279 on a ProteinChip SELDI reader (Personal Edition, BioRad) using an automated protocol (laser
280 energy 3500 nJ; matrix attenuation 1000; focus mass 10 kDa; acquired mass range from 0 to 100
281 kDa). Analysis of the spectra was carried out using Protein Chip data manager software 3.5. Spectra
282 were visually examined and poor quality spectra were excluded from further analysis.
283 Pre-processing of data is required before analysis. These processing steps include: calibration,
284 baseline subtraction, normalization and peak detection. Calibration, carried out according to the
285 manufacturer's instructions, is necessary for mass accuracy. The software was externally calibrated
286 using All-in-One Protein Standard and All-in-one Peptide Standard (BioRad). Baseline subtraction
287 was achieved by using an algorithm that eliminates any baseline signal caused by matrix distortions.
288 Peak intensities were normalized between samples in each study group to the total ion current (TIC)
289 for avoiding the signal interference from SPA. Auto-detection of peaks was performed with
290 "expression difference mapping" (EDM) under the following conditions: signal/noise ratio of 3 or
291 higher for the first pass, 2 for the second pass, presentation in at least 10% of spectra for
292 identification, 0.1% mass window and mass range 2,000-100,000 Da. Peaks having a m/z ratio < 2
293 KDa were not used for analysis because they overlap with SPA signal.

294 **OFFGEL**

295 The OFFGEL High Resolution kit pH 3−10 (Agilent Technologies) was used for pI-based protein
296 preparative isoelectric focusing (IEF) in solution. WS protein samples (800 µg of proteins) were
297 solubilized in a Protein OFFGEL fractionation buffer supplied by the manufacturer (containing 8 M
298 urea, 2 M thiourea, 1% DTT, 12% glycerol, and 1.2% buffer with ampholytes), and aliquots were
299 evenly distributed in a 24-well 3100 OFFGEL Fractionator (Agilent Technologies) tray according
300 to supplier instructions. We applied a preset program (separation limits: 8000 V, 200 mW, and 50
301 µA; starting voltage, 200−350 V; ending voltage, 2000−4200 V; after the application of 64 kVh, the
302 protein separation zones were maintained at constant voltage). The liquid fractions were recovered,
303 and pH of each fraction was measured. Then the 24 OFFGEL fractions were assayed by SELDI-
304 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 [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

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

337 **RESULTS**

338 **Two-dimensional electrophoresis and validation**

339 Fig. 2 illustrates a representative 2-DE image of WS. The quality of the gels was assessed by the
340 software Same Spot which includes the SpotCheck function as previously described [32].
341 Normalized spot volumes were analyzed by the ANOVA test to detect the proteins which were
342 significantly related with FM. These protein spots were chosen for excision and identified by
343 NanoLC-ESI-MS/MS analysis. The analysis of 2-DE profiles allowed us to find 17 spots with a
344 different expression in FM respect to RA, 19 spots from the comparison of FM with migraine, and
345 23 in FM respect to healthy subjects. In particular, we found 5 spots differentially expressed solely
346 in FM. Four spots were identified as TRFE and the other as ENOA (fig. 3). The list of identified
347 proteins, with statistical analysis, molecular weight (MW), isoelectric point (pI), coverage, and
348 score values of NanoLC-ESI-MS/MS is given in tables 1 and 2. We indicated also proteins whose
349 identification has been derived from previous works characterizing WS proteomics map [[24], [25],
350 [26]].

351 ELISA assays were used to validate in WS the expression changes of 4 proteins: TRFE, ENOA,
352 PGAM1, and TALDO. PGAM1, and TALDO were proteins found up-regulated in WS of FM
353 patients in our previous work [26].

354 The mean values of TRFE, ENOA, and PGAM1 were statistically different in FM respect to
355 controls (healthy subjects and migraine). On the other hand, the increased expression in WS of FM
356 was not confirmed for TALDO (figure 4, table 3).
357 ROC curves were calculated to assess the clinical potential of our selected proteins to distinguish
358 FM from control samples (healthy and migraine). The areas under the ROC curves (AUC) were
359 calculated for each protein individually, showing if each marker alone can discriminate FM.
360 Moreover, using a logistic regression analysis, we investigated if the discriminative power of each
361 marker could be potentially increased by the combination of different markers. With this purpose,
362 we tested all the different combinations in order to select the best association of biomarkers useful
363 to discriminate control from FM samples. ENOA was found as the most differentiating biomarker,
364 with AUC of ROC curve of 0.738. However its discriminative power was increased if ENOA was
365 combined with other proteins. In particular, the best performance in diagnosis was obtained by
366 combining ENOA, PGAM1and TRFE as shown in table 4 and figure 5 which illustrates the ROC
367 curve obtained by combining all three biomarkers (AUC 0.792).

368 **Clinical correlations**

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

372 **SELDI-TOF-MS**

373 Figure 6 reports a representative protein profile of WS by using CM10 ProteinChips in the
374 molecular range of 0–50 kDa. Since SELDI-TOF-MS is often criticize for its poor reproducibility,
375 it's mandatory to perform at the same time all the experiments with the same chip for the different
376 classes. In addition the use of quality controls (QC) is highly recommended. QC is a well-
377 characterized pool of samples processed alongside the experimental samples in order to calculate
378 coefficient of variation (CV) for peak intensities and mass accuracy as a measure of reproducibility
379 of the SELDI-TOF-MS analyses. The QC samples were applied randomly on different chips in

380 order to avoid any artefact due to experimental handling. The CV (the standard deviation of the
381 series divided by the mean of the series) was calculated using multiple protein peaks selected over
382 the experiments. In this study the CV was 24.5% for peak intensity, and 0.008% for mass accuracy
383 with the CM10 chips. Our CVs indicated acceptable reproducibility of the spectra. Peak detection
384 with the ProteinChip data manager software 3.5 resolved a total of 116 peaks on CM10 in the m/z
385 ratio between 2000 and 100,000. Each spectra was thus described by 116 input variables where
386 each variable correspond to the peak intensity for the given m/z.
387 We selected the most discriminating peaks for each comparison: 2 peaks in the comparison of FM
388 patients vs healthy subjects, 4 peaks for FM patients vs migraine patients, 4 peaks for FM patients
389 vs migraine patients plus healthy subjects, and 3 peaks for FM patients vs RA patients. Tables 5
390 illustrates the most discriminating peaks obtained with logistic regression for each comparison; in
391 the table we report the predictions of these statistical analysis with the error rate.
392 The identification of the peaks of interest was launched by carrying out a separation of the proteins
393 with OFFGEL. This technique allows the separation of the sample proteins, according to their
394 isoelectric point (pI) and to collect them in liquid fractions. Therefore, by processing our WS
395 samples, the OFFGEL fractionation allowed us to focus our attention on 2 peaks: v21 (m/z 4548)
396 with a pI of 5.18, and v83 (m/z 13288) with a pI of 6.3. Further studies are mandatory to identify
397 these peaks, but through the use of TagIdent we could hypothesize their identity. TagIdent is a tool
398 from Expasy which allows the generation of a list of proteins close to a given pI and MW
399 (http://web.expasy.org/tagident/). The peak v21 could be the orexigenic neuropeptide QRFP (MW
400 4522, pI 5.11), while the v83 seems to be the peptidyl-prolyl cis-trans isomerase (PPIA; MW
401 13208, pI 6.3).
402 **DISCUSSION**
403 Considering the deep complexity of FM, the discovery of more objective markers could support
404 clinicians' diagnosis. Therefore, with the present work, we aimed to carry out our proteomic study
405 on FM patients by characterizing WS. Respect to our previous work [26], we decided to extend the

406 analysis to both positive and negative controls, and therefore we added RA and migraine patients as
407 positive control subjects. Indeed, the clinicians can differentiate a patient with RA from one with
408 FM but we were interested in examining the putative inflammatory component in FM by including
409 a model of chronic inflammatory pain. In conjunction, it was more complete to also compare FM
410 WS pattern with a model of non-inflammatory chronic pain such as migraine. Therefore we
411 compared WS proteins of FM patients with sex- and age-matched control subjects.
412 The most relevant observation from 2-DE analysis was the peculiar up-regulation in FM of TRFE
413 which was validated by ELISA. We identified more protein spots corresponding to TRFE because
414 different variants are known. TRFE is the main blood iron-binding protein which delivers iron to
415 sites of storage and utilization from those of absorption and heme degradation. Hence, TRFE
416 reflects the need of tissues for iron; in fact an iron deficiency in rats causes the fall in ferritin and
417 the rise of TRFE in the brain [33]. Some studies hint at a role of iron in FM's pathophysiology,
418 since it is a cofactor in serotonin and dopamine production [34]. Actually, in another disease in
419 which the dopaminergic system is involved, the restless legs syndrome, high level of TRFE in
420 patients' cerebrospinal fluid were found, in spite of levels similar to those of normal controls in
421 serum [35, 36]. To the best of our knowledge, no association was found between serum levels of
422 TRFE and FM [37] and ours, is the first study reporting evidence of an association between salivary
423 TRFE levels and FM. It has been demonstrated that TRFE can be secreted by parotid cells so its
424 detection in WS is not due to blood contamination [38]. Its role in saliva has not yet been
425 investigated but lactoferrin, a salivary analogue of TRFE, has bacteriostatic effects [39, 40] and
426 therefore it has been proposed that TRFE could have a similar antimicrobial action due to its iron-
427 sequestering properties [38].
428 Another protein found up-regulated in WS from patients, respect to controls was ENOA. This is the
429 enzyme involved in the penultimate step of glycolysis. Beyond its well-known enzymatic role in
430 metabolism, ENOA is a multifunctional protein with several functions diverging from its original
431 role in the glycolytic pathway. Indeed, it can act as a plasminogen receptor promoting metastatic

432 invasion in many types of cancers and is involved in cell-matrix adhesion, survival, and senescence
433 [41-44]. Moreover, specific auto-antibodies anti-ENOA were detected in connective tissue diseases
434 [45] which arise the question of a generic association of this biomarker with different inflammatory
435 and autoimmune pathologies [46-49]. Indeed, when we analyzed ENOA levels by ELISA in WS
436 from RA patients we observed its increase respect to controls (healthy subjects plus migraine
437 patients). Thus, while this could potentially sustain the autoimmune nature of FM, on the other side,
438 it shows ENOA is not a specific biomarker. This explains the reason for searching a combination of
439 biomarkers instead of a single one. In fact, its discriminative power was increased if ENOA was
440 combined with TRFE and PGAM1.
441 PGAM1 is the enzyme catalyzing the step of glycolysis preceding ENOA. We found its up-
442 regulation in FM patients respect to healthy subjects, confirming our previous results, but also
443 respect to migraine subjects, by both 2-DE and ELISA. Autoantibodies against anti-PGAM1 were
444 found in sera from patients with various neurological diseases; therefore PGAM1 specificity is
445 questionable and it has been defined as a nonspecific marker of autoimmune diseases of the central
446 nervous system [50, 51]. Hence, this knowledge is quite interesting considering the neurological
447 feature of FM but further sustains the importance of a combination of multiple biomarkers in
448 defining FM.
449 TALDO is the enzyme whose increase was typical for FM patients respect to healthy subjects, in
450 our previous study [26]. This enzyme takes part to the pentose-phosphate pathway which is related
451 to the production of NADPH. Oxidative stress has been often been considered as a major factor in
452 pathophysiology of FM [52, 53], even if its role remains elusive [54]. Therefore, the up-regulation
453 of TALDO can be interpreted as an attempt of increasing the NADPH production in order to reduce
454 oxidative damage of tissues. This increase has been actually confirmed in the present work by 2-
455 DE. On the other hand, its alteration was not found in the comparison between FM and migraine
456 patients. It is worth to notice that ELISA totally corroborated these results. Indeed, figure 4D and
457 table 3 show the results from the comparison of TALDO levels in WS from FM patients respect to

458 controls (healthy subjects plus migraine patients). However, the TALDO level in healthy subjects is
459 much lower (signal intensity in healthy = 39.6 vs signal intensity in FM = 117.6) and the p-value
460 respect to FM patients becomes 0.0062, strengthening our previous study. So, this result reduces the
461 reliability of TALDO as FM biomarker, because it did not allow distinguishing FM patients from
462 migraine patients. At the same time the finding could let to speculate that the alteration of TALDO
463 is typical of a non-inflammatory chronic pain such as migraine, and therefore also of FM, whose
464 inflammatory component has always been debated. Finally, none of the candidate proteins showed a
465 statistical correlation with the patients' clinical features (e.g. FIQ, VAS, tender points), as already
466 previously found for TALDO and PGAM1 [26].
467 In addition to 2-DE for the first time, we analyzed protein expression profiles obtained by SELDI-
468 TOF-MS for each sample, with the attempt of providing a complementary visualization technique to
469 2-DE. Therefore, by processing our WS samples with SELDI-TOF-MS and by fractionation with
470 OFFGEL, we focused our attention on 2 peaks: v21 and v83. The peak v21 could be the orexigenic
471 neuropeptide QRFP, while the v83 seems to be the PPIA. On the other hand, in our previous work,
472 we detected in FM saliva the up-regulation of cyclophilin A, another protein with analogue
473 peptidylprolyl cis-trans-isomerase activity. Concerning the neuropeptide QRFP, its role has been
474 recently reviewed and it is has been pointed out that QRFP mRNA and/or binding sites are enriched
475 in the parafascicular thalamic nucleus, the locus coeruleus, the dorsal raphe nucleus and the
476 parabrachial nucleus, which are involved in pain transmission [55]. Moreover, data indicated that
477 this peptide is involved in the regulation of glucose homeostasis [55, 56]. Therefore its potential up-
478 regulation in FM deserves further study. This hypothesis clearly need to be validated using with
479 complementary techniques.
480 In conclusion, we performed proteomic analysis of WS by using complementary approaches and
481 overcoming the limit of our previous work. Indeed, we have now extended the analysis to a big
482 cohort of patients including both negative and positive controls, instead of only healthy subjects.
483 Nevertheless, we could support the results of the previous work and also propose new biomarkers

484 which can collectively contribute in defining a panel of salivary proteins allowing to distinguish
485 fibromyalgic pain with good sensitivity and specificity. We believe that this panel could be a useful
486 tool in supporting clinicians' diagnosis and defining FM clusters and targeted treatment. Actually,
487 our future perspective foresees to develop a simple, rapid and not invasive point-of-care test. This
488 device could also be worthwhile for population screening and to characterize fibromyalgic pain. In
489 addition, together with mandatory future experiments to validate SELDI analysis, the present results
490 can offer a clue for a better knowledge of such a complex disease like FM.

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661 **FIGURES CAPTIONS**

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

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

666 **Figure 3. 2-DE enlarged images and analysis of TRFE, ENOA.** Histograms of the normalized
667 volume (mean ± SEM) obtained by 2-DE analysis of proteins found up-regulated in WS of FM
668 patients in respect to all control subjects (A), and enlarged images of these proteins (B). TRFE: n°
669 69, 84, 1466, 1472; ENOA: n° 343. M: migraine.

670 **Figure 4. Validation of ENOA, TRFE, PGAM1, TALDO.** ELISA results for ENOA (A), TRFE
671 (B), PGAM1 (C), TALDO (D): the bar graph shows the mean ± SEM of signal intensity. Statistical
672 significance of the differences was calculated by Mann-Whitney U test (*$p \leq 0.05$, **$p \leq 0.01$ ***p
673 ≤ 0.001).

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

676 **Figure 6**. **SELDI-TOF.** Representative protein profile of WS by SELDI-TOF-MS using CM10
677 ProteinChips in the molecular range of 0–50 kDa.

Table 1: Protein identification of differentially expressed proteins in WS of FM patients by MS/MS; molecular weight (MW); isoelectric point (pI); **a**: theoretical; **b**: observed.

n° spot	Protein	ID	gene	MW		pI		Matched peptides	Sequence Coverage%	Best ion score
				a	b	a	b			
334	Alpha-amylase 1	P04745	AMY1A	58	52	6.5	6.5	12	30	70.9
342	Alpha-amylase 1	P04745	AMY1A	58	50	6.5	5.9	7	19	66.9
349	Alpha-amylase 1	P04745	AMY1A	58	49	6.5	5.7	10	25	104.6
351	Alpha-amylase 1	P04745	AMY1A	58	49	6.5	7.2	10	26	78.0
353	Alpha-amylase 1	P04745	AMY1A	58	49	6.5	6.8	12	28	79.2
378	Alpha-amylase 1	P04745	AMY1A	58	44	6.5	6.6	10	25	78.3
343	Alpha-enolase	P06733	ENO1	47	50	7.0	6.0	15	40	109.4
1432	Glucosamine-6-phosphate isomerase 1	P46926	GNPDA1	33	34	6.4	6.5	3	13	67.3
812	Glutathione S-transferase P	P09211	GSTP1	23	28	5.4	4.9	4	27	100.0
1462	Heat shock protein beta-1	P04792	HSPB1	23	47	5.9	5.9	5	42	71.3
632	Ig alpha-1 chain C region	P01876	IGHA1	38	33	6.1	6.3	3	11	79.7
1428	Ig alpha-1 chain C region	P01876	IGHA1	38	34	6.1	6.1	2	6	68.6
1490	Ig kappa chain C region	P01834	IGKC	12	31	5.6	5.9	5	65	102.1
1487	Ig lambda-2 chain C regions	P0CG05	IGLC2	11	31	6.9	7.1	4	56	106.5
59	Polymeric immunoglobulin receptor precursor	P01833	PIGR	83	80	5.6	5.5	14	23	89.2
69	Serotransferrin	P02787	TF	77	78	6.8	6.1	20	31	98.3
84	Serotransferrin	P02787	TF	77	76	6.8	6.4	9	18	107.8
1466	Serotransferrin	P02787	TF	77	76	6.8	6.6	19	27	103.8
1472	Serotransferrin	P02787	TF	77	78	6.8	6.0	9	17	81.6
363	Serum albumin	P02768	ALB	69	47	5.9	5.5	16	34	93.6
365	Serum albumin	P02768	ALB	69	47	5.9	5.7	10	23	94.6

Table 2: Statistical analysis of proteins which were significantly different in WS of FM patients respect to controls. RA: Rheumathoid Arthritis; M: Migraine.

n°	Protein Name	ID	FM vs RA		FM vs Healthy		FM vs M		RA vs Healthy	
			p-value	fold	p-value	fold	p-value	fold	p-value	fold
334	Alpha-amylase 1	P04745	n.s.		0.0012	+1.6	0.0045	+1.6	0.0007	+1.9
342	Alpha-amylase 1	P04745	0.006	-1.5	0.0002	+1.8	0.0024	+1.4	$1.30e^{-06}$	+2.6
349	Alpha-amylase 1	P04745	0.04	-1.3	0.00025	+2.3	0.026	+1.6	$6.60e^{-07}$	+3.1
351	Alpha-amylase 1	P04745	0.0025	-2.1	0.0035	+2.5	n.s.		$5.30e^{-09}$	+5.3
353	Alpha-amylase 1	P04745	0.0054	-1.9	0.002	+2.0	0.026	+1.6	$4.60e^{-07}$	+3.9
378	Alpha-amylase 1	P04745	0.013	-1.7	0.00027	+2.4	0.0045	+1.8	$6.70e^{-07}$	+4.1
343	Alpha-enolase	P06733	0.03	+1.3	0.0013	+1.7	0.009	+1.6	n.s.	
1432	Glucosamine-6-phosphate isomerase 1	P46926	0.0011	-2.0	n.s.		0.012	-1.6	0.00002	-2.8
812	Glutathione S-transferase P	P09211	n.s.		0.005	+1.7	n.s.		0.00003	+2.1
1462	Heat shock protein beta-1	P04792	0.009	-1.7	n.s.		n.s.		0.00002	+2.7
632	Ig alpha-1 chain C region	P01876	0.000031	-3.2	n.s.		n.s.		$8.50e^{-09}$	+4.9
1428	Ig alpha-1 chain C region	P01876	0.000037	-2.0	n.s.		0.015	+1.4	$6.10e^{-06}$	+2.6
1490	Ig kappa chain C region	P01834	n.s.		0.0010	+1.5	0.0007	+1.6	0.0003	+2.3
1487	Ig lambda-2 chain C regions	P0CG05	n.s.		0.0012	+1.5	0.016	+1.4	n.s.	
1474	* Phosphoglycerate mutase 1	P18669	n.s.		0.032	+1.3	0.04	+1.3	n.s.	
532	*Transaldolase	P37837	n.s.		0.026	+1.4	n.s.		n.s.	
59	Polymeric immunoglobulin receptor precursor	P01833	0.007	+2	0.012	-1.5	n.s.		0.00001	-2.9
58	*Polymeric immunoglobulin receptor precursor	P01833	n.s.		0.014	-1.4	n.s.		$7e^{-06}$	-2.3
60	*Polymeric immunoglobulin receptor precursor	P01833	0.007	+1.8	0.003	-1.6	n.s.		$8.5e^{-05}$	-2.8
69	Serotransferrin	P02787	0.004	+2.1	0.000007	+2	$1.00e^{-06}$	+2.4	n.s.	
84	Serotransferrin	P02787	0.004	+2.1	0.0003	+2	$1.70e^{-06}$	+2.7	n.s.	
1466	Serotransferrin	P02787	0.0019	+2.3	0.0009	+1.6	$1.30e^{-06}$	+2.0	n.s.	

1472	Serotransferrin	P02787	0.003	+2.1	0.00012	+1.7	$9.00e^{-06}$	+2.1	n.s.	
363	Serum albumin	P02768	n.s.		0.0085	+2.4	0.008	+2.4	n.s.	
365	Serum albumin	P02768	0.0014	-2.1	0.00033	+2.3	0.039	+1.6	$3.80e^{-09}$	+4.8
1286	*Cystatin-SN	P01037	n.s.		0.001	-2	$2.0e^{-05}$	-2.1	0.00097	-2.2
1292	*Cystatin-SN	P01037	n.s.		n.s.		0.02	-1.5	0.0009	-1.7
1425	*Calgranulin-A	P05109	n.s.		0.03	+1.4	n.s.		0.003	+1.7

* 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.

	ELISA signal intensity (M±SEM)		p-value
	FM	**Controls**	**FM vs CTRL**
Serotransferrin	3456± 411	2052±250	0.005
Alpha-enolase	3.2 ± 0.56	1.1±0.15	0.0007
PGAM1	3.72 ± 1.11	0.98±0.32	0.0178
TALDO	117.56±21.5	130.26±20.1	n.s.

Table 4. Comparison between sensitivities and specificities of single biomarkers and their combination.

TRFE AUC 0.699					
Sensitivity %	71	68	68	65	55
Specificity %	57	60	63	63	73
Cut-off ng/ml	1812	1889	2065	2238	2562
ENOA AUC 0.738					
Sensitivity %	81	81	77	71	68
Specificity %	61	63	63	68	71
Cut-off ng/ml	0.95	1.01	1.05	1.25	1.36
PGAM1 AUC 0.683					
Sensitivity %	48	45	45	42	39
Specificity %	80	87	90	90	90
Cut-off ng/ml	1.40	1.56	1.62	1.79	1.93
Combining TRFE, ENOA, PGAM1 AUC 0.792					
Sensitivity %	84	81	74	71	52
Specificity %	63	67	73	77	93
Cut-off combined risk index	0.36	0.41	0.43	0.44	0.52

Table 5. Multivariate SELDI analysis

	FM vs H	m/z	FM vs M	m/z	FM vs RA	m/z	FM vs H+M	m/z
	V83	13288	*V15*	4132	*V18*	4353	*V19*	4423
	V107	31063	*V53*	7173	*V65*	10179	*V21*	4548
			V73	11308	*V116*	80857	*V54*	7354
			V101	23688			*V83*	13288
Error rate%	**17.7%**		**16.1%**		**24.1%**		**12.3%**	

Figure 1
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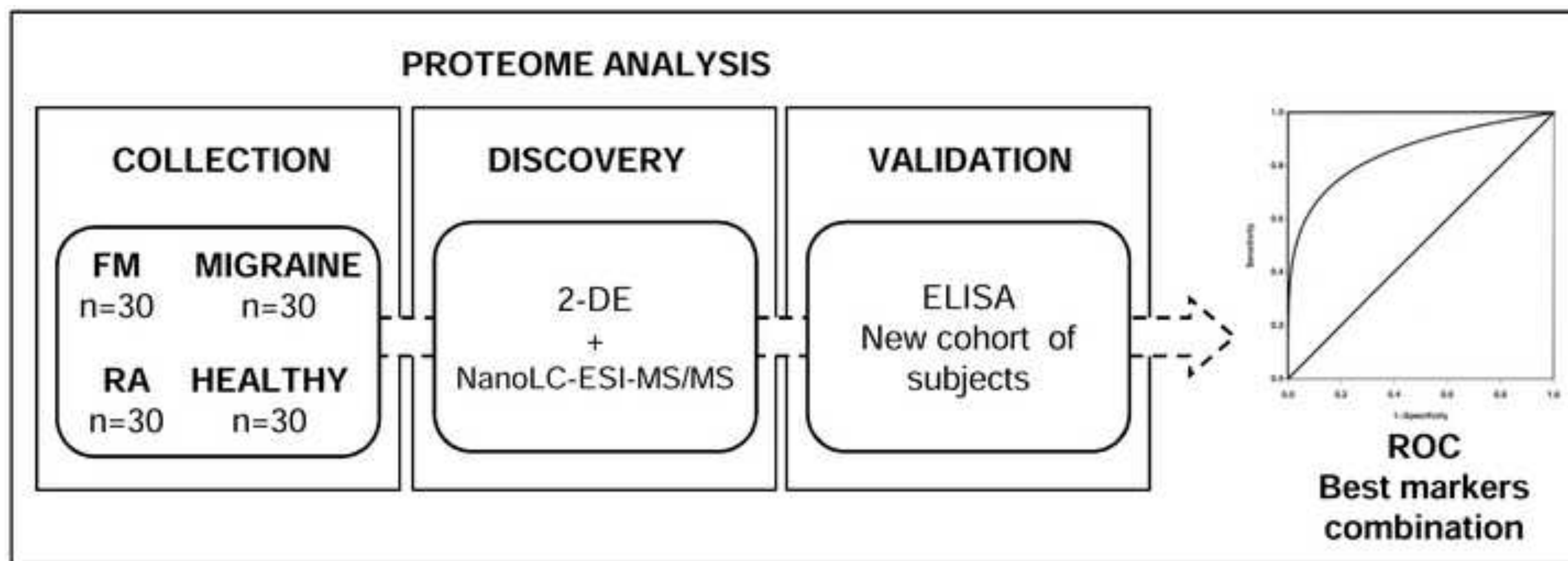

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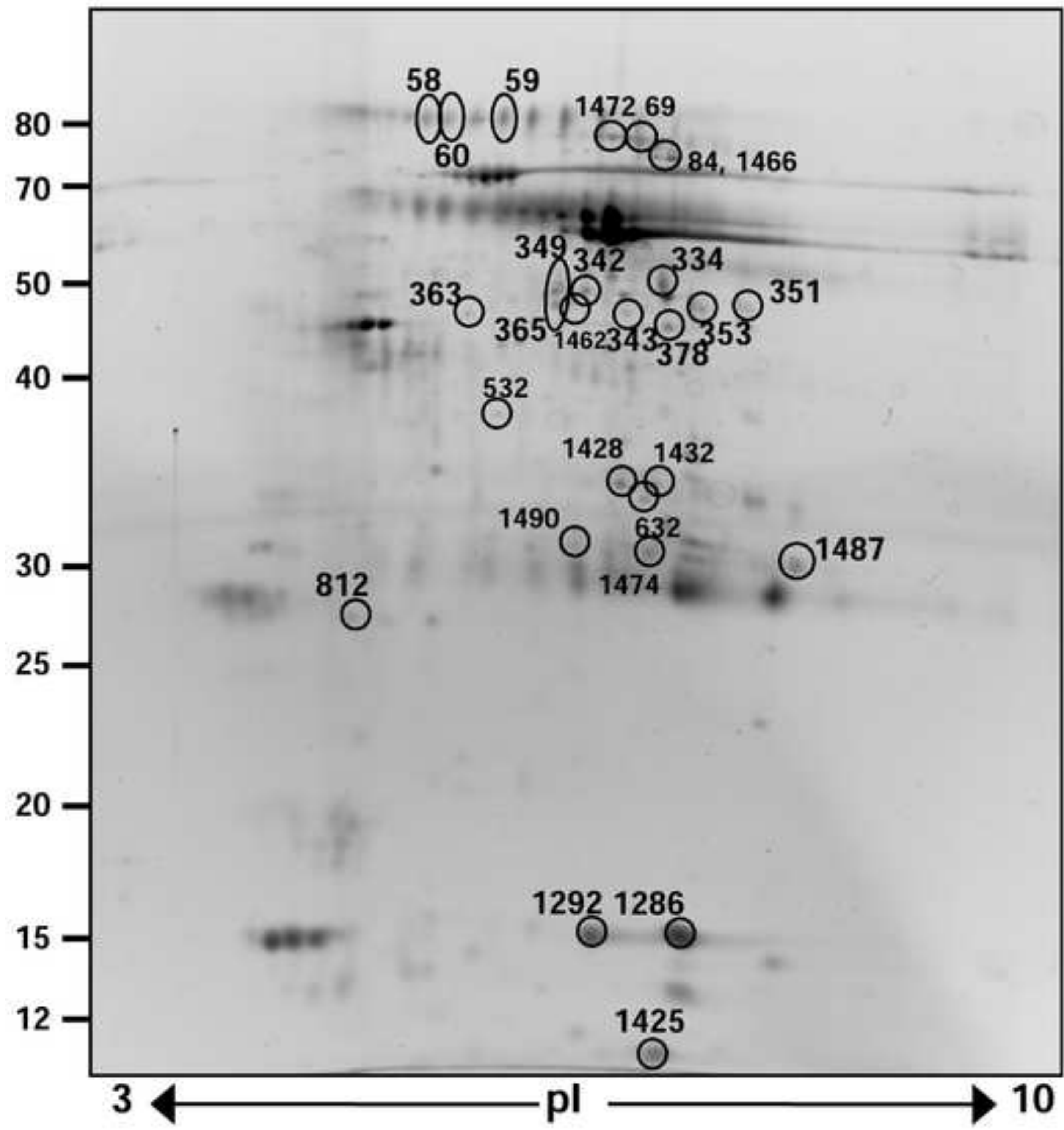

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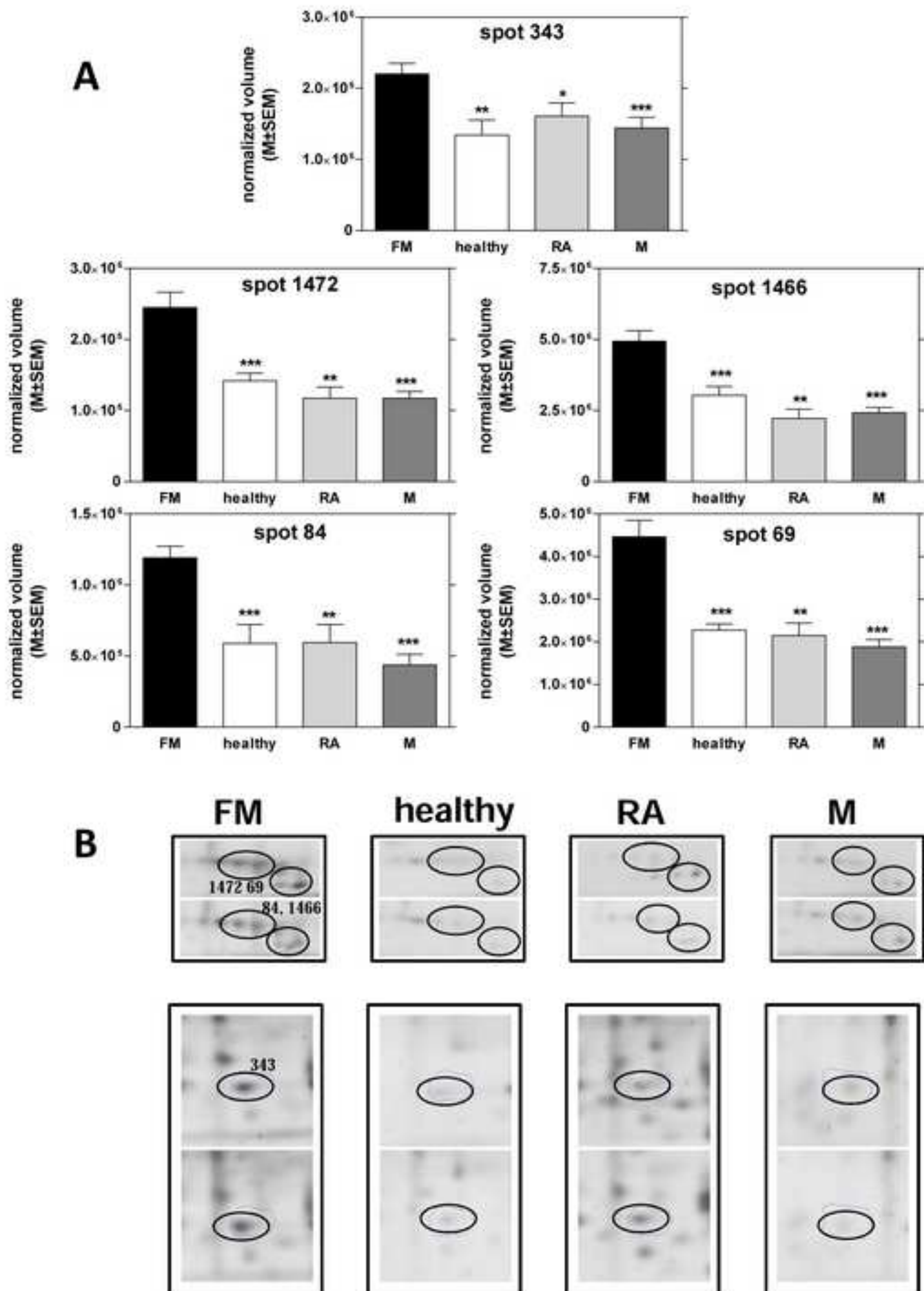

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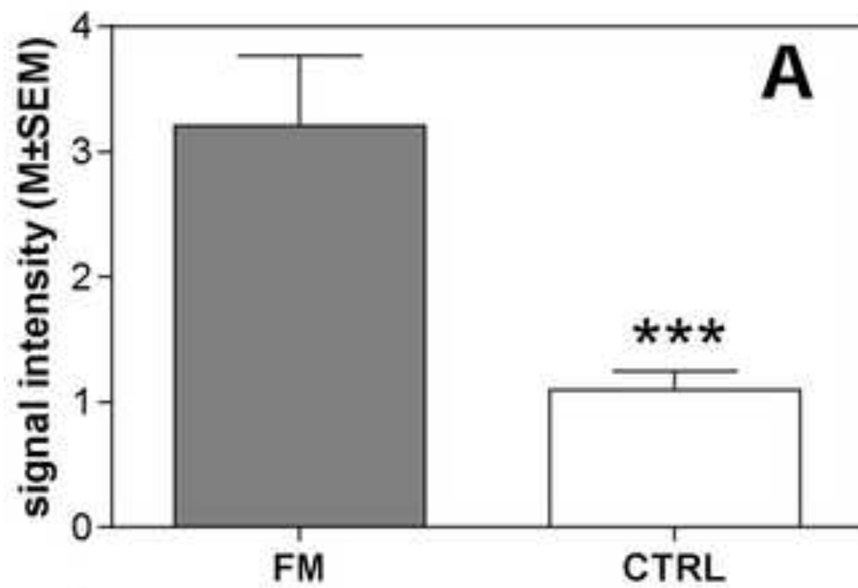

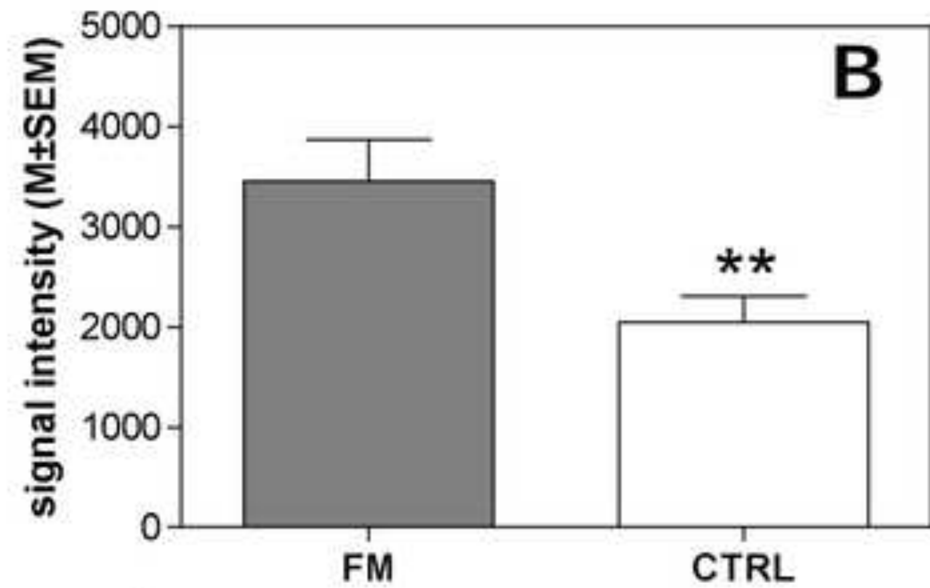

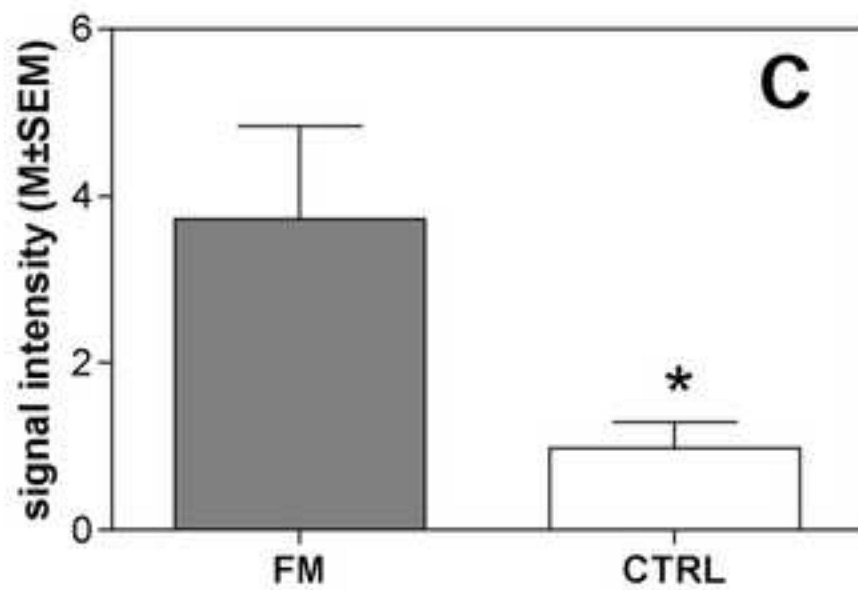

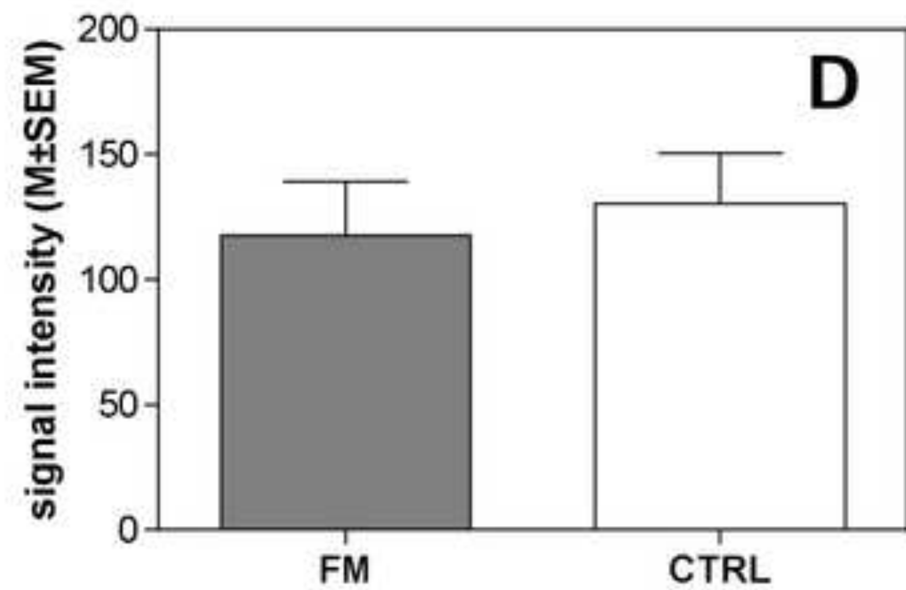

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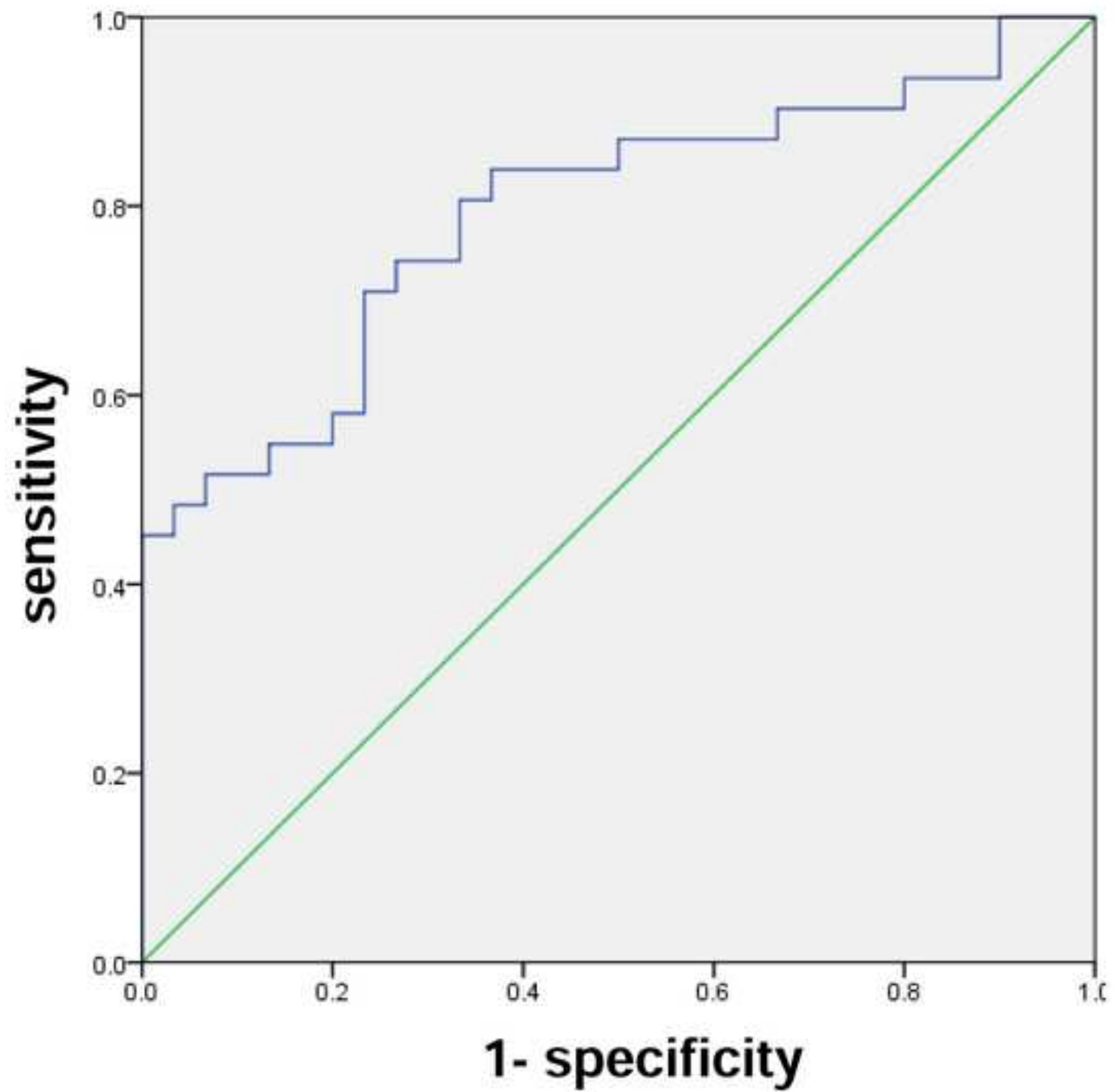

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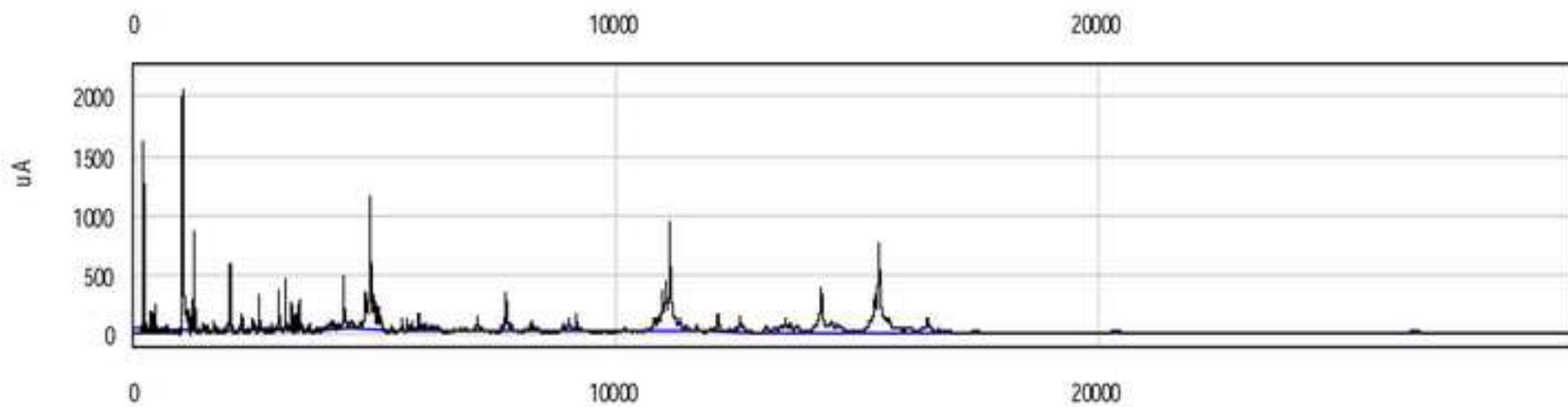

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