Journal of Pharmaceutical and Biomedical Analysis 184 (2020) 113165

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Proteomic serum profile in menstrual-related and post menopause migraine

Elisa Bellei [a], Cecilia Rustichelli [b,*], Stefania Bergamini [a], Emanuela Monari [a], Carlo Baraldi [c], Flavia Lo Castro [d], Aldo Tomasi [a], Anna Ferrari [c]

[a] Department of Surgery, Medicine, Dentistry and Morphological Sciences with Transplant Surgery, Oncology and Regenerative Medicine Relevance, University of Modena and Reggio Emilia, via del Pozzo, 71, 41124 Modena, Italy
[b] Department of Life Sciences, University of Modena and Reggio Emilia, via G. Campi, 103, 41125 Modena, Italy
[c] Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Via del Pozzo, 71, 41124 Modena, Italy
[d] School of Pharmacology and Clinical Toxicology, University of Modena and Reggio Emilia, Via del Pozzo, 71, 41124 Modena, Italy

ARTICLE INFO

Article history:
Received 10 October 2019
Received in revised form 6 February 2020
Accepted 9 February 2020
Available online 21 February 2020

Keywords:
Serum protein
Menstrual-related migraine
Post menopause migraine
Proteomics
2-DE
LC–MS/MS

ABSTRACT

The aim of this pilot study was to analyze the serum proteomic profile of women suffering from menstrual-related migraine (MM group, n = 15) and migraine in post-menopause (PM group, n = 15) in comparison with non-headache control females (C group, n = 15). Serum samples were subjected to two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry (MS) analysis for protein identification. Based on 2D-gel maps and PDQuest 2-D software, 13 differentially expressed spots, corresponding to 12 unique proteins identified by Liquid Chromatography-Electrospray Ionization-Quadrupole-Time of Flight/tandem mass spectrometry (LC-ESI-QToF-MS/MS), were detected in the MM and PM groups *vs* C group. Five inflammatory and regulatory of vascular integrity proteins (prothrombin, serum amyloid P-component, Ig kappa chain C region, apolipoprotein A-I, serum amyloid A-4 protein) were found deregulated in both MM and PM groups compared to C group; MM group showed the upregulation of other inflammatory protein fragments (inter-alpha-trypsin inhibitor heavy chain H4 and complement C4-A) compared to C group; PM group, in comparison with C group, displayed a noteworthy upregulation of transthyretin and other deregulated proteins (tetranectin, alpha-1-antitrypsin, haptoglobin, apolipoprotein A-IV) playing a role in anti-inflammatory and reparative processes. In conclusion, proteomic analysis was able to reveal differences in protein expression between migraine sufferers and non-headache women; as in other neurological diseases characterized by neuroinflammation, the serum proteome of migraine women presents an abundance of proteins indicative of cellular damage, oxidative stress and inflammation. This relevant inflammatory status, if confirmed in larger series, could represent a target for menstrual-related migraine treatment.

1. Introduction

More than 70 % of women suffering from migraine have menstrual-related attacks, *i.e.* from two days before to three days after the onset of menstrual flow. The third edition of the International Classification of Headache Disorders (ICHD-3) [1] includes menstrual migraine in the appendix, dividing it into "pure menstrual migraine", when the attacks are exclusively related to the menstrual cycle (in at least three consecutive menstrual cycles), and in "menstrual-related migraine", when the attacks occur both during and outside the menstrual period. The evidence on menstrual migraine is still limited. The decrease in estrogen concentration after being exposed to high levels is considered the main trigger [2], however the mechanisms that mediate this action are

Abbreviations: MM, menstrual-related migraine; PM, post menopause migraine; THRB, prothrombin; A1AT, alpha-1-antitrypsin; APOA4, apolipoprotein A-IV; SAMP, serum amyloid P-component; IGKC, Ig kappa chain C region; APOA1, apolipoprotein A-I; SAA4, serum amyloid A-4 protein; TETN, tetranectin; HPT, haptoglobin; TTHY, transthyretin; ITIH4, inter-alpha-trypsin inhibitor heavy chain H4; CO4A, complement C4-A; 2-DE, two-dimensional gel electrophoresis; LC-ESI-QToF-MS/MS, liquid chromatography-electrospray ionization-quadrupole-time of flight/tandem mass spectrometry; LC-ESI-QO-MS/MS, liquid chromatography/electrospray ionization-quadrupole-orbitrap/tandem mass spectrometry; CNS, central nervous system; CSF, cerebrospinal fluid.

* Corresponding author.
E-mail address: cecilia.rustichelli@unimore.it (C. Rustichelli).

https://doi.org/10.1016/j.jpba.2020.113165

not known. Menstrual-related attacks are longer, more disabling, more resistant to the treatment, and tend to reappear more than those that are not menstrual-related. Unfortunately, the treatment of menstrual migraine is the same as that for "regular migraine" and often inadequate [3].

Migraine is a complex condition, which involves a combination of genetic, environmental and lifestyle factors [4]. Proteomics studies the expression and function of proteins, together with the pathophysiological processes in which they are involved, helping to define more precisely the pathogenesis of diseases. Therefore, the proteomic approach could be appropriate to study a multifactorial disease such as migraine. Indeed, while the genome is constant, the proteome is continuously modulated by genome-environment interactions [5].

The aim of this pilot study was to analyse the serum proteome of women suffering from menstrual-related migraine and post menopause migraine in comparison with non-headache women, to search and identify differentially expressed proteins as potential biomarkers of menstrual migraine, which could be informative on the biological pathways that contribute to this complex disorder.

2. Material and methods

2.1. Chemical and reagents

Protease Inhibitor Cocktail, ProteoPrep® Immunoaffinity albumin and IgG depletion Kit, Urea, Thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Dithiotreitol (DTT), BSA, Iodoacetamide, Tetramethylethylenediamine (TEMED), Tris, Ammonium Persulfate, SDS, Potassium Tetrathionate, Potassium Acetate, Glycerol, and Silver Nitrate were purchased from Merck KGaA. Ampholytes pH 3–10, Ready IPG Strip™ pH 3–10, Acrylamide/bis solution 29:1, Tris-Glycine-SDS buffer, pH 8.3, and Precision Plus Protein™ Standards All Blue were from Bio-Rad Laboratories. Acetonitrile (ACN), Formic Acid (FA), Acetic Acid and Ethanol were of LC–MS purity grade (Carlo Erba Reagents). The ultra-pure water was purified by a Milli-Q Plus185 system from Millipore (Merck KGaA).

2.2. Subjects and procedures

Three groups participated in the study (Table 1): MM group, composed of 15 women diagnosed with menstrual-related migraine without aura according to the diagnostic criteria of the ICHD-3, appendix, A1.1.2 [1], aged between 18 and 45 years, who were not taking hormonal contraceptives; PM group, comprising 15 women in menopause (spontaneous amenorrhea for at least 12 months, not induced by a medical condition), diagnosed with migraine without aura [1] and who had suffered from menstrual-related migraine before menopause, aged no more than 65 years, who were not taking hormone replacement therapy; C group, 15 non-headache women (controls), aged between 18 and 45, who were not taking hormonal contraceptives. The inclusion criteria comprised the absence of major acute medical or psychiatric comorbidities, normal liver and kidney function and, for the C group, no more than 3 days of tension-type headache per month and no diagnosis of any other type of primary or secondary headache, according to the diagnostic criteria of ICHD-3 [1]. Women who were taking migraine prophylaxis and who were unable to understand the purposes of the study were excluded. The migraine women were enrolled among the patients consecutively afferent, for the first time, at the Headache Center of the University Hospital of Modena; non-headache women were patients' friends. All women provided their written consent to participation in the study, that was conducted in accordance with the ethical principles of the Helsinki Declaration, last edition (2013) and approved by the Ethics Committee of the Province of Modena (protocol n. 86/16).

Table 1
Characteristics of the studied subject groups (n = 15 in each group).

Variable	MM group N (%)	PM group N (%)	C group N (%)
Mean age ± SD (years)	34.8 ± 7.1	57.0 ± 5.0[a]	29.2 ± 7.2
Age range (years)	21÷45	51÷65	22÷45
BMI ± SD	23.1 ± 2.5	24.1 ± 4.1	23.3 ± 4.2
Smoker	2 (13)	1 (7)	3 (20)
Alcohol consumption	5 (33)	7 (47)	4 (27)
Coffee consumption	13 (87)	10 (67)	6 (40)
Varied diet	14 (93)	12 (80)	14 (93)
Vegetarian/vegan diet	1 (7)	2 (13)	0 (0)
Years of migraine ± SD	16.8 ± 8.5	33.1 ± 13.5[b]	0 (0)
Migraine days/3 months ± SD	25.7 ± 17.2	27.3 ± 19.2	0 (0)
Analgesic intake/3 months ± SD	27.6 ± 17.2	28.4 ± 17.4	0 (0)
Acute migraine treatment			
Triptans	9 (60)	8 (53)	0 (0)
NSAIDs	8 (53)	9 (60)	0 (0)
Analgesic combination	2 (13)	2 (13)	0 (0)
Comorbidities			
Hypertension	1 (7)	4 (27)	1 (7)
Allergic rhinitis	3 (20)	3 (20)	4 (27)
Hypercholosterolemia	1 (7)	3 (20)	0 (0)
Thyroiditis	1 (7)	4 (27)	1 (7)
Concomitant treatment			
Antihypertensive agents	1 (7)	4 (27)	1 (7)
Antihistamines	3 (20)	3 (20)	4 (27)
Statins	1 (7)	3 (20)	0 (0)
Levothyroxine	1 (7)	4 (27)	1 (7)

[a] PM *vs* MM group, P < 0.01; PM *vs* C group, P < 0.001; one-way ANOVA test.
[b] PM *vs* MM group, P < 0.001; one-way ANOVA test.

In women of fertile age, blood for proteomic analysis was taken between the seventh and tenth day, starting from the first day of menstruation and, in migraine women, at least two days after the last migraine attack. For each woman, during a medical examination, a form was compiled for the collection of personal data, life habits, clinical history, and for the patients also the history and characteristics of migraine.

2.3. Sample collection and preparation

Venous blood (10 mL) was collected in the morning from migraine patients and non-headache controls at fast and allowed to clot at room temperature for 1 h; serum was subsequently obtained by centrifugation at 2000 x g for 10 min at +4 °C. To preserve proteins, serum was added with a protease inhibitor cocktail prior to be divided into aliquots and stored at −80 °C until proteomic analysis. For this purpose, the complexity of serum was reduced by the selective depletion of the two most abundant serum proteins using the ProteoPrep® Immunoaffinity albumin and IgG depletion Kit, according to the manufacturer's protocol. For each group of patients, as well as for the control group, serum samples were combined to form 5 pools/group. Total protein content of each pool was measured spectrophotometrically at λ = 595 nm, using BSA as standard for the calibration curve.

2.4. Two-dimensional gel electrophoresis (2-DE) and image analysis

The obtained serum samples were subjected to 2-DE analysis. For the first-dimension separation, a total of 100 μg of protein was diluted with a rehydration buffer composed of 6 M urea, 2 M thiourea, 4% CHAPS, 25 mM DTT, 0.2 % ampholytes pH 3–10, and then loaded onto 17 cm, immobilized pH gradient strips (Ready IPG Strip™), pH range 3-10. IEF was conducted at +20 °C, first performing an actively rehydration of the strips at 50 V for 12 h,

then increasing the voltage at 250 V for 15 min, ramping up to 10.000 V for 3 h, and finally focusing to reach 75.000 V-hours. The IEF was followed by the equilibration of the strips in 1% DTT and 2.5 % Iodoacetamide in equilibration buffer (6 M urea, 50 mM Tris−HCl pH 8.8, 30 % glycerol, 2% SDS). The second-dimension separation was performed by SDS-PAGE on 8–16 % polyacrylamide gradient gel (Acrylamide/bis solution 29:1, 1.5 M Tris−HCl, pH 8.8, 10 % SDS, 1% TEMED, 10 % ammonium persulfate), using 1X Tris-Glycine-SDS, pH 8.3 as running buffer during the electrophoretic run. Finally, proteins were stained with a silver nitrate staining protocol, compatible with MS detection. Briefly, the gels were incubated in a fixing buffer solution (40 % ethanol/10 % acetic acid) for 4 h, then they were first washed in 40 % ethanol and subsequently in ultra-pure deionized water. After being sensitized by the enhancer solution (0.3 % potassium tetrathionate, 0.5 M potassium acetate, 30% ethanol), the gels were covered with a 0.2 % silver nitrate solution, incubated for 75 min in the dark and then developed, terminating the process with the addition of the blocker solution (4% Tris, 2% acetic acid).

Each gel image was acquired by a calibrated densitometer, series GS-800 (Bio-Rad Laboratories, Hercules, CA, USA), and analyzed by the PDQuest 2-D image analysis software, version 7.3.1 (Bio-Rad Laboratories, Hercules, CA, USA). This software detects the spot stain intensity, as optical density (OD), and the spot area, providing a final value expressed as "spot volume", that is the product of OD value per area (OD x mm2). The 2D gel images were compared to detect differentially expressed protein spots among the different groups. The fold-changes of spot abundance were achieved as ratio between the values of spot volume measured in both patients' groups *vs* controls.

2.5. MS protein identification

Proteins were extracted from the spot by an "in-gel" trypsin digestion protocol, fully reported in Supplementary Material. Afterward, peptides were undoubtedly identified using two different LC–MS/MS procedures. In the first approach, analyses were performed using an Agilent 1200 series LC system coupled to an Agilent 6520 Q-TOF mass spectrometer (LC-ESI-QTof-MS/MS) (Agilent, Waldbronn, Germany). The peptide mixtures were dissolved in 5% aqueous FA and loaded (4.0 μL) onto a reversed-phase 40 nL enrichment Chip (G4240-62001, Agilent) with a mobile phase of water-ACN (97:3, v/v; +0.1 FA) at a flow of 3.0 μL/min. The precolumn was then switched in-line with the nanoflow pump and the peptides were analyzed on a ZORBAX 300 SB-C18 (43 mm×75 μm ID; 5 μm particle size, Agilent) analytical column with a mobile phase of (A) water-ACN (97:3, v/v; +0.1 FA) and (B) water-ACN (5:95, v/v; +0.1 FA) using the following gradient program: 0 to 0.5 min, isocratic at 3% (B); 0.5–17 min, linear gradient from 3 to 25 % (B); 17–20 min, linear gradient from 25 to 35 % (B); 20–21 min, linear gradient from 35 to 95 % (B); 21–25 min, isocratic at 95 % (B); 25–26 min, linear gradient from 95 to 3% (B). The equilibration period between each run was 13 min long; total run time: 39 min. The flow rate was set at 400 nL/min. The ESI source operated in positive ionization mode; the drying gas (nitrogen) flow and temperature were 6 L/min and 350°C, respectively, the capillary voltage was set at 1950 V and the fragmentor voltage was 160 V. MS and MS/MS spectra were acquired in Auto MS/MS mode set with the following parameters: MS scan, *m/z* 300–1700 uma at 2 spectra/s and MS/MS scan, *m/z* 50–1700 uma at 3 spectra/s. Data were collected in a centroid mode with a precursor isolation window set at 4 uma. The 5 most intense multi-charged ions were selected for MS^2 nitrogen-promoted collision-induced dissociation with active exclusion on with 1 repeat and release after 0.17 min. Precursors with charge state +1 were excluded and the preferred charge state was: 2, 3, >3, unknown; the proper collision energy was calculated as follows: $3.6 \times (m/z)/100 + 2$. The Agilent MassHunter Workstation Acquisition software version B.05.00 (B2043) was used for instrument control, data acquisition, qualitative and quantitative data analysis. The instrument was calibrated with a standard mix prior each analysis batch to ensure mass accuracies at or below the 3 ppm.

Replicated spots were cut from different gels and further analyzed by LC-ESI-QO-MS/MS using an UHPLC-MS QExactive™ (Thermo Fisher Scientific, Reinach, Switzerland) to confirm protein identification; the optimized experimental conditions and the results are reported in Supplementary Material. Protein identifications were obtained by MASCOT search engine (version 2.4) against the UniProt knowledgebase database (UniProtKB).

2.6. Data and statistical analysis

All collected data, made anonymous, were entered into a specific database. Descriptive analysis of all variables was conducted. Statistical analysis was carried out by StataIC 13 software. The continuous variables normally distributed were expressed as mean ± standard deviation (SD) and the dichotomous variables as percentages. The comparison between means was done by one-way ANOVA, while Fisher's exact test was used for binary variables. Differences were considered significant with a P value <0.05. Regarding the fold-changes of spot abundance, values > 1.5 were considered as significant.

3. Results and discussion

The evaluation of the protein profiles of human blood, using proteomic methodologies, provides a window on the state of illness or health of an individual. Accordingly, the results of our study showed that the serum proteome of migraine patients (MM and PM groups), during the attack-free interval, was different from that of non-headache women (C group). Based on proteins resolution by 2-DE (Fig. 1) and PDQuest 2-D image analysis software, 13 protein spots were differentially expressed in MM and PM groups compared to C group. These proteins were identified by LC-ESI-QToF-MS/MS analyses as 12 unique proteins, since two spots resulted different isoforms of the same protein (Table 2). Specifically, the first and second columns of Table 2 reported the protein entry name and the primary accession number, respectively, both derived from the UniProt knowledge database, while the third column showed the protein full names. MS data are illustrated in the last 4 columns: the highest scores obtained with MASCOT search engine, the number of total peptide and the significant peptides matching the identified proteins, the number of total and significant sequences, and the percentage of sequenced amino acids for each identified protein.

In particular, prothrombin (THRB), serum amyloid P-component (SAMP), Ig kappa chain C region (IGKC), apolipoprotein A-I (APOA1), serum amyloid A-4 protein (SAA4) resulted differentially expressed in both MM and PM groups compared to C group; 2 protein fragments, inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and complement C4-A (CO4A) were differentially expressed only in MM group *vs* C group; other 5 proteins, transthyretin (TTHY), tetranectin (2 spots, $TETN^1$ and $TETN^2$), alpha-1-antitrypsin (A1AT), haptoglobin (HPT) and apolipoprotein A-IV (APOA4) were differentially expressed only in PM group *vs* C group. Moreover, a second set of replicated protein spots was further analyzed by LC-ESI-QO-MS/MS System, confirming all proteins previously identified by LC-ESI-QToF-MS/MS analysis (results and method are presented in Supplementary Material).

By means of the research carried out by UniProt and The Human Protein Atlas databases, most (58 %) of the identified proteins were inflammatory and binding/transport proteins, while the oth-

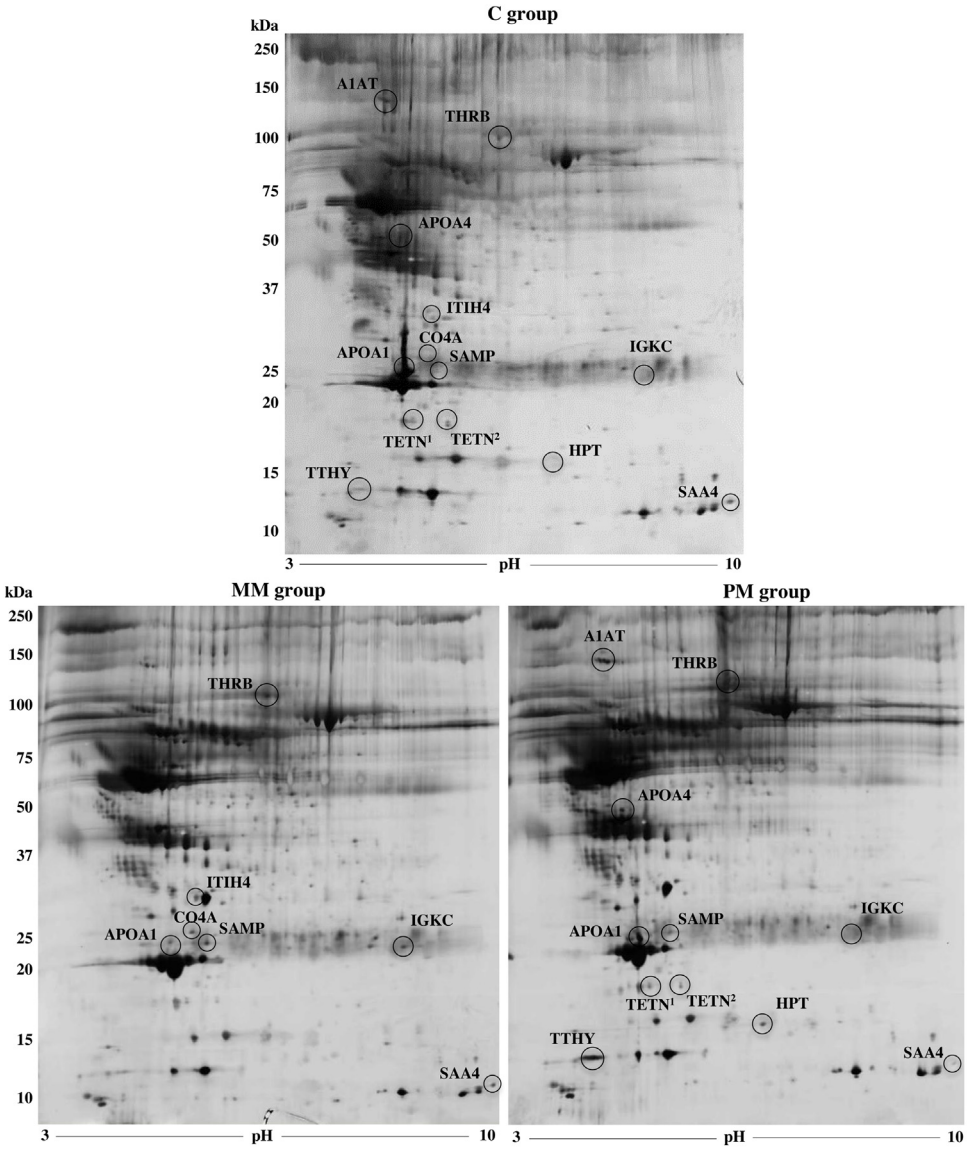

Fig. 1. Representative 2-DE gels obtained from serum of non-headache control females (C group), menstrually-related migraine (MM group) and post-menopausal migraine patients (PM group). The differentially expressed protein spots detected in migraineurs *vs* controls are highlighted by a circle; abbreviations of protein names derive from the UniProt database (protein entry name) and correspond to those reported in Table 2.
2-DE: 17 cm IPG strip, pH 3–10, in first-dimension separation (IEF) and 8–16 % polyacrylamide gradient gel for second-dimension separation (SDS-PAGE). Protein spots evidenced by Silver-staining. Molecular weight marker (kDa): Precision Plus Protein™ Standards All Blue.

ers were involved in lipid metabolism, immunity and inhibition functions (Fig. 2).

3.1. *Proteins differentially expressed in both MM and PM groups vs C group*

The fold-changes of protein expression are graphically shown in Fig. 3 (the raw data are provided as Supplementary Material). Five proteins, deregulated in both MM and PM groups *vs* controls suggested a particular link between these proteins and migraine without aura. Specifically, 3 proteins were up-regulated in migraine *vs* non-headache women: THRB (MM +2.19; PM +4.06), SAMP (MM +3.88; PM +2.19) and IGKC (MM +1.86; PM +1.76), while 2 proteins, APOA1 (MM −16.8; PM −2.01) and SAA4 (MM −2.05; PM −4.36), were found down-regulated. THRB, a serine protease, is fundamental, as coagulation factor II, in blood homeostasis regulating inflammatory response, cell proliferation, apoptosis and maintaining vascular integrity [6]. THRB would be also involved in neuroinflammation [7]. Its blood levels increase in migraine patients and this tendency to hypercoagulability was associated with the risk of stroke in migraine with aura. Therefore, our results supported the hypothesis that THRB could represent a specific marker of migraine [8]. Another up-regulated inflammatory protein was SAMP. It is a constituent of amyloid deposit, which belongs to the pentraxine superfamily, acute phase proteins, induced by IL-6 that mediates inflammation and innate humoral immunity [9]. SAMP levels were higher in MM than in PM group. Probably, inflammatory processes change with disease progression because migraine patients with a headache history lasting more than five years present lower serum levels of pentraxin-3 than patients with a relatively recent diagnosis [10]. The third up-regulated protein was IGKC, a promising biomarker in the diagnosis and monitoring of the therapeutic response of several solid tumours [11]. Thus, as in tumours, an inflammatory and immune response could be also activated in migraine. Among the under-expressed proteins there was APOA1, the main polypeptide of human plasma high-

Table 2
Differentially expressed serum proteins identified by LC-ESI-QToF-MS/MS analysis.

Entry name[a]	Acc. number[b]	Protein name	Score[c]	Pep/pep. sign.[d]	Seq/seq. sign.[e]	Cov.[f](%)
Proteins differentially expressed in both MM and PM groups vs C group						
THRB	P00734	Prothrombin	154	12/8	7/4	10
SAMP	P02743	Serum amyloid P-component	87	7/6	5/5	21
IGKC	P01834	Ig kappa chain C region	655	40/33	7/6	86
APOA1	P02647	Apolipoprotein A-I	493	78/35	24/16	63
SAA4	P01834	Serum amyloid A-4 protein	53	37/7	7/4	47
Proteins differentially expressed only in MM group vs C group						
ITIH4	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4 (fragment)	61	9/5	5/3	6
CO4A	P0C0L4	Complement C4-A (fragment)	177	20/13	7/5	4
Proteins differentially expressed only in PM group vs C group						
TTHY	P02766	Transthyretin	105	25/8	7/5	42
TETN[1]	P05452	Tetranectin	78	5/2	4/1	21
TETN[2]	P05452	Tetranectin	43	4/3	2/1	11
A1AT	P01009	Alpha-1-antitrypsin	171	25/10	11/6	31
HPT	P00738	Haptoglobin	170	40/11	9/6	20
APOA4	P06727	Apolipoprotein A-IV	1083	139/72	39/28	79

[a] Entry name from UniProt knowledge database (www.uniprot.org).
[b] Primary accession number from UniProt database.
[c] The highest score with MASCOT search engine.
[d] Number of total peptides/significant peptides matching the identified protein.
[e] Number of total sequences/significant sequences.
[f] Sequence coverage: percentage of sequenced amino acids for each identified protein.

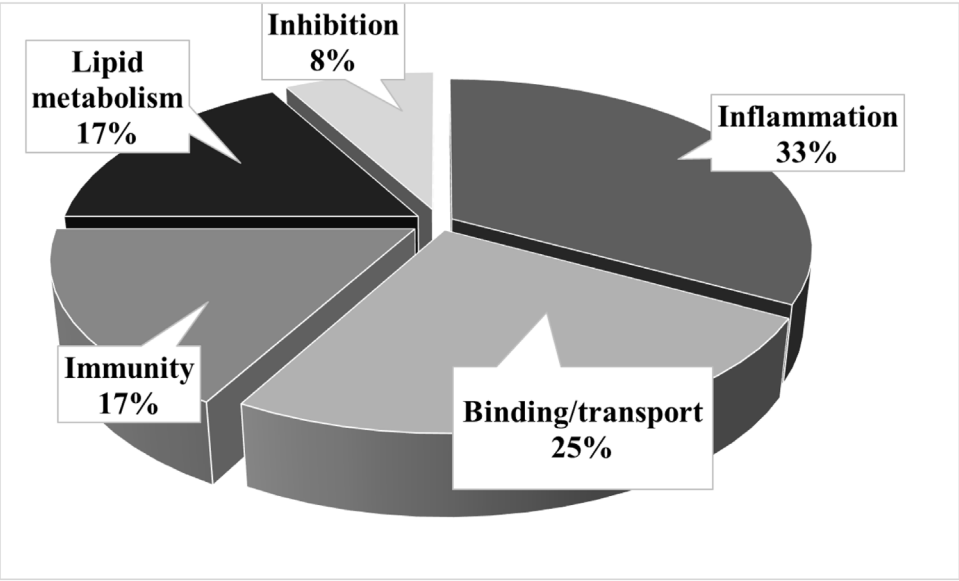

Fig. 2. Main functions of the identified proteins. Inflammation: Haptoglobin (HPT), Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), Prothrombin (THRB), Serum amyloid A-4 protein (SAA4); Binding/transport: Serum amyloid P-component (SAMP), Transthyretin (TTHY), Tetranectin (TETN); Immunity: Ig kappa chain C region (IGKC), Complement C4-A (CO4A); Lipid metabolism: Apolipoprotein A-I (APOA1), Apolipoprotein A-IV (APOA4); Inhibition: Alpha-1-antitrypsin (A1AT). Protein functions were retrieved by the UniProt database.

density lipoprotein (HDL). It is a negative acute phase protein, which expression is reduced more than 25 % during the acute phase response. It exhibits also anti-apoptotic, anti-inflammatory and antioxidant functions [12]. The other under-expressed protein was SAA4. It belongs to the serum amyloid family and is a minor acute-phase reactant in humans [13]. *In vitro* studies suggest a chemo-attractive, protective function in the brain. The decline in its plasma concentrations has been hypothesized as a marker in the early diagnosis of Alzheimer's disease [14].

Essentially, our results showed that a subclinical inflammatory state was present in migraine women, supporting the role of inflammation in the pathogenesis of migraine. In particular, it is assumed that the activation of the trigeminal sensory pathways leads to an inflammatory response involving immune, vascular and neuron cells with release of neuropeptides and pro-inflammatory neurotransmitters, capable of inducing a state of sterile inflammation in the intracranial meninges and, consequently, sensitization of trigeminal meningeal nociceptors. Neuroinflammation in the trigeminovascular system, although it does not completely explain the onset of the attack, could be crucial in promoting the progression of episodic migraine into a chronic form [15].

3.2. Proteins differentially expressed only in MM group vs C group

Always taking the fold-change as a parameter (Fig. 3), 2 protein fragments resulted up-regulated exclusively in MM group compared to controls, namely ITIH4 (+1.89) and CO4A (+2.04). ITIH4 belongs to inter-alpha trypsin inhibitors and is a type II acute-phase protein involved in inflammatory responses; its blood levels rise from 1.4 to 3 times in acute-phase processes [16]. Moreover, it

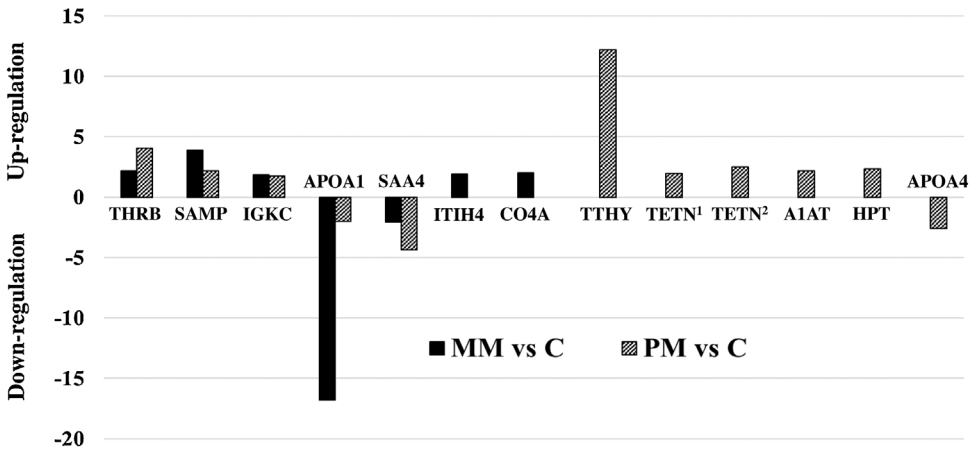

Fig. 3. Representation of the fold-change data showing the up-regulated and down-regulated proteins in the menstrually-related migraine (MM) and post-menopausal migraine (PM) groups compared to control (C) group. The fold-changes of spot abundance were calculated as ratio between the values of spot volume detected by the PDQuest 2-D image analysis software.

has been associated to neuroinflammation in depression [17]. CO4A is a mediator of local inflammation with antimicrobial properties that contributes to propagating the inflammatory response *via* the classical complement pathway [18]. Menstrual-related migraine is included, in the last ICHD-3 classification [1], only in the appendix, pending further scientific evidence to characterize it [19]. It can be assumed that the high inflammatory state found in MM women could be at the basis of the severity of the menstrual attacks. Interestingly, the protein fragments are very promising biomarkers. In fact, their generation from the specific processes of a disease could reduce the overlap between diagnostic groups [20].

3.3. Proteins differentially expressed only in PM group vs C group

Lastly, 4 proteins were found up-regulated only in PM group *vs* C group (Fig. 3): TTHY (+12.2), TETN (+1.94, spot 1; +2.52, spot 2), A1AT (+2.16) and HPT (+2.33), while APOA4 was down-regulated (−2.61). The most relevant aspect was the up-regulation of TTHY, a carrier protein with the primary function of retinol and thyroid hormones transport. TTHY promotes the inflammatory response and in the central nervous system (CNS) is associated with cognitive functions, memory, and emotion control through the regeneration of damaged neurons [21]. In animal models of oligoemia, a typical condition of shock and migraine, TTHY increases in cerebrospinal fluid (CSF), as an indicator of oxidative stress. Overexpression of this protein is reported in different conditions of chronic pain with oxidative stress, where TTHY could ensure neuroprotection [22]. Two TETN isoforms were more expressed in PM group. This protein belongs to type C lectins. It is an indicator of proteolysis, fibrinolysis and tissue remodelling. In fact, TETN concentration is high in the CSF after an epileptic attack [23]. Furthermore, TETN is a potential biomarker for Parkinson's disease and coronary artery disease [24,25]. A1AT and HPT, both positive acute phase proteins, were also upregulated. Serum A1AT concentration increases in response to inflammation or tissue trauma. By inhibiting the production of proinflammatory cytokines, A1AT limits the damage in inflamed tissues [26]. HPT concentration substantially increases during acute inflammation in response to interleukin-6. It inhibits prostaglandin synthesis, angiogenesis and exerts anti-inflammatory, antioxidant, protective for endothelium and weak antibacterial activity [27]. Increased plasma HPT concentration has been found in patients with Parkinson's disease, Guillain-Barré syndrome, multiple sclerosis and idiopathic intracranial hypertension [28]. Finally, APOA4 was downregulated in PM group. This protein plays a crucial role in lipid absorption; moreover, it is involved in mitigating the excessive inflammatory activation at the endothelial level and shows anti-atherosclerotic properties [29]. Thus, in the PM group, proteins with anti-inflammatory functions, involved in protective and reparative processes were also abundant. Therefore, it can be hypothesized that the long history of migraine in PM group had triggered an opposite response, defensive against inflammation. This phenomenon, in addition to the cessation of hormonal fluctuations, could contribute to the reduction of the severity and prevalence of migraine after menopause [19].

Notably, many of the differentially expressed proteins in the MM and PM groups (Table 2) had a dual role, as regulators of vessel integrity and inflammation. Migraine, especially with aura, has been associated with an unfavourable risk profile for ischemic vascular events. The exact biological mechanism of this increased risk is unknown [30]. From our proteomic analysis, the deregulation of proteins with a double involvement, in the inflammatory response and in the endothelial activation, could precisely underlie the increased risk of ischemic events and support the link between migraine and vascular diseases.

3.4. Limitations and strengths

The main limitations of our pilot study were the low sample size and the cross-sectional design that did not allow establishing whether the deregulated protein pattern was the cause or consequence of migraine. Inflammation could be a normal process in menstrual cycle and menopause (more details in Supplementary Material). The serum proteomic profile found in the PM group could be affected by the higher age of these patients (Table 1). However, there were no significant differences between the PM and MM groups due to comorbidities, migraine days and analgesic consumption. PM women only suffered from a longer migraine history. Moreover, we had no elements to define whether the serum proteome could reflect the environment in CNS; nevertheless, the same proteins have been found in other neurological diseases, characterized, like migraine, by neuroinflammation such as Parkinson's disease, multiple sclerosis, Alzheimer's disease and chronic pain. Our study was conducted rigorously: the differentially expressed proteins were first identified and subsequently verified and validated by two different mass spectrometers, the LC-ESI-QTof-MS/MS and LC-ESI-QO-MS/MS systems, respectively. All proteins identified in the first analysis session were confirmed by the second analysis. To the best of our knowledge, this is the first study providing insight into the differential expression of proteins in women suffering from menstrual-related and postmenopausal

migraine with respect to non-headache women; so, to date, we have no possibility of comparison. Therefore, our results need to be confirmed regarding their diagnostic value on larger samples, also using more sensitive analytical methods in next investigations.

4. Conclusions

In summary, the results of our pilot study showed that: 1. the proteomic approach was able to distinguish between migraine and non-headache status; 2. migraine women, in both fertile and post menopause age, presented a great abundance of inflammatory and regulatory of vascular integrity proteins; 3. MM group showed a great down-regulation of APOA1 (−16.8), a negative acute phase protein, intimately linked with inflammation and over expression of inflammatory protein fragments (ITIH4 and CO4A) compared to the C group; this abundant inflammatory proteome might explain the severity of the menstrual attacks and poor response to acute treatments; 4. PM group, older than MM, displayed a noteworthy up-regulation of TTHY (+12.2) and a number of proteins differentially expressed, higher than C group; these proteins played also an important role in anti-inflammatory and reparative processes, suggesting that the long history of migraine could induce a protective response. Finally, the relevant inflammatory status highlighted by proteomic analysis, if confirmed in larger series, could represent a target for menstrual-related migraine treatment.

Credit author statement

All authors participated in study design, performed research, data analysis, and drafting of manuscript content. All authors reviewed and approved the manuscript.

Funding

This study was carried out by research funds from the University of Modena and Reggio Emilia (Italy) attributed to Dr. Anna Ferrari (FAR 2016).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the “Centro Interdipartimentale Grandi Strumenti” (CIGS) of the University of Modena and Reggio Emilia for technical assistance. LC–MS/MS instruments at CIGS were granted by the Foundation “Fondazione Cassa di Risparmio di Modena – FCRM.”

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113165.

References

[1] Headache Classification Committee of the International Headache Society (IHS), The international classification of headache disorders Cephalalgia, 38, 3rd edition, 2018, pp. 1–211, http://dx.doi.org/10.1177/0333102417738202.
[2] B.W. Somerville, Estrogen-withdrawal migraine: I. Duration of exposure required and attempted prophylaxis by premenstrual estrogen administration, Neurology 25 (1975) 239–244, http://dx.doi.org/10.1212/WNL.25.3.239.
[3] E.A. MacGregor, T.W. Victor, X. Hu, Q. Xiang, R.A. Puenpatom, W. Chen, J.C. Campbell, Characteristics of menstrual vs nonmenstrual migraine: a post hoc, within-woman analysis of the usual-care phase of a nonrandomized menstrual migraine clinical trial, Headache 50 (2010) 528–538, http://dx.doi.org/10.1111/j.1526-4610.2010.01625.x.
[4] M. Kowalska, M. Prendecki, W. Kozubski, M. Lianeri, J. Dorszewska, Molecular factors in migraine, Oncotarget 7 (2016) 50708–50718, http://dx.doi.org/10.18632/oncotarget.9367.
[5] C. Vogel, E.M. Marcotte, Insights into the regulation of protein abundance from proteomic and transcriptomic analyses, Nat. Rev. Genet. 13 (2012) 227–232, http://dx.doi.org/10.1038/nrg3185.
[6] H. Krenzlin, V. Lorenz, B. Alessandri, The involvement of thrombin in the pathogenesis of glioblastoma, J. Neurosci. Res. 95 (2017) 2080–2085, http://dx.doi.org/10.1002/jnr.24049.
[7] K. Göbel, P. Kraft, S. Pankratz, C.C. Gross, C. Korsukewitz, R. Kwiecien, R. Mesters, B.E. Kehrel, H. Wiendl, C. Kleinschnitz, S.G. Meuth, Prothrombin and factor X are elevated in multiple sclerosis patients, Ann. Neurol. 80 (2016) 946–951, http://dx.doi.org/10.1002/ana.24807.
[8] G.E. Tietjen, S.A. Collins, Hypercoagulability and migraine, Headache 58 (2018) 173–183, http://dx.doi.org/10.1111/head.13044.
[9] B. Bottazzi, A. Inforzato, M. Messa, M. Barbagallo, E. Magrini, C. Garlanda, A. Mantovani, The pentraxins PTX3 and SAP in innate immunity, regulation of inflammation and tissue remodelling, J. Hepatol. 64 (2016) 1416–1427, http://dx.doi.org/10.1016/j.jhep.2016.02.029.
[10] M. Ceylan, O.F. Bayraktutan, S. Becel, Ö. Atis, A. Yalcin, D. Kotan, Serum levels of pentraxin-3 and other inflammatory biomarkers in migraine: association with migraine characteristics, Cephalalgia 36 (2016) 518–525, http://dx.doi.org/10.1177/0333102415598757.
[11] J.P. Pandey, E. Kistner-Griffin, L. Black, A.M. Namboodiri, M. Iwasaki, Y. Kasuga, G.S. Hamada, S. Tsugane, IGKC and FcγR genotypes and humoral immunity to HER2 in breast cancer, Immunobiology 219 (2014) 113–117, http://dx.doi.org/10.1016/j.imbio.2013.08.005.
[12] P. Sirniö, J.P. Väyrynen, K. Klintrup, J. Mäkelä, M.J. Mäkinen, T.J. Karttunen, A. Tuomisto, Decreased serum apolipoprotein A1 levels are associated with poor survival and systemic inflammatory response in colorectal cancer, Sci. Rep. 7 (2017) 5374, http://dx.doi.org/10.1038/s41598-017-05415-9.
[13] C.L. Chen, T.S. Lin, C.H. Tsai, C.C. Wu, T. Chung, K.Y. Chien, M. Wu, Y.S. Chang, J.-S. Yu, Y.-T. Chen, Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics, J. Proteomics 85 (2013) 28–43, http://dx.doi.org/10.1016/j.jprot.2013.04.024.
[14] L. Ijsselstijn, J.M. Papma, L.J.M. Dekker, W. Calame, C. Stingl, P.J. Koudstaal, N.D. Prins, P.A.E. Sillevis Smitt, T.M. Luider, Serum proteomics in amnestic mild cognitive impairment, Proteomics 13 (2013) 2526–2533, http://dx.doi.org/10.1002/pmic.201200190.
[15] L. Edvinsson, K.A. Haanes, K. Warfvinge, Does inflammation have a role in migraine? Nat. Rev. Neurol. 15 (2019) 483–490, http://dx.doi.org/10.1038/s41582-019-0216-y.
[16] L. Zhuo, V.C. Hascall, K. Kimata, Inter-α-trypsin inhibitor, a covalent protein-glycosaminoglycan-protein complex, J. Biol. Chem. 279 (2004) 38079–38082, http://dx.doi.org/10.1074/jbc.R300039200.
[17] Q. Wang, X. Su, X. Jiang, X. Dong, Y. Fan, J. Zhang, C. Yu, W. Gao, S. Shi, J. Jiang, W. Jiang, T. Wei, iTRAQ technology-based identification of human peripheral serum proteins associated with depression, Neuroscience 330 (2016) 291–325, http://dx.doi.org/10.1016/j.neuroscience.2016.05.055.
[18] T.E. Hugli, Biochemistry and biology of anaphylatoxins, Complement 3 (1986) 111–127.
[19] K.G. Vetvik, E.A. MacGregor, Sex differences in the epidemiology, clinical features, and pathophysiology of migraine, Lancet Neurol. 16 (2017) 76–87, http://dx.doi.org/10.1016/S1474-4422(16)30293-9.
[20] Y.R. Yang, S.L. Liu, Z.Y. Qin, F.J. Liu, Y.J. Qin, S.M. Bai, Z.Y. Chen, Comparative proteomics analysis of cerebrospinal fluid of patients with Guillain–Barré syndrome, Cell. Mol. Neurobiol. 28 (2008) 737–744, http://dx.doi.org/10.1007/s10571-007-9257-7.
[21] J. Brouillette, R. Quirion, Transthyretin: a key gene involved in the maintenance of memory capacities during aging, Neurobiol. Aging 29 (2008) 1721–1732, http://dx.doi.org/10.1016/j.neurobiolaging.2007.04.007.
[22] M. Sharma, S. Khan, S. Rahman, L.R. Singh, The extracellular protein, transthyretin is an oxidative stress biomarker, Front. Physiol. 10 (2019) 5, http://dx.doi.org/10.3389/fphys.2019.00005.
[23] L. Wang, Y. Pan, D. Chen, Z. Xiao, Z. Xi, F. Xiao, X. Wang, Tetranectin is a potential biomarker in cerebrospinal fluid and serum of patients with epilepsy, Clin. Chim. Acta 411 (2010) 581–583, http://dx.doi.org/10.1016/j.cca.2010.01.022.
[24] E.S. Wang, H.B. Yao, Y.H. Chen, G. Wang, W.W. Gao, Y.R. Sun, J.G. Guo, J.W. Hu, C.C. Jiang, J. Hu, Proteomic analysis of the cerebrospinal fluid of Parkinson's disease patients pre- and post-deep brain stimulation, Cell. Physiol. Biochem. 31 (2013) 625–637, http://dx.doi.org/10.1159/000350082.
[25] Y. Chen, H. Han, X. Yan, F. Ding, X. Su, H. Wang, Q. Chen, L. Lu, R. Zhang, W. Jin, Tetranectin as a potential biomarker for stable coronary artery disease, Sci. Rep. 5 (2015) 17632, http://dx.doi.org/10.1038/srep17632.
[26] O. Repetto, L. Mussolin, C. Elia, L. Martina, M. Bianchi, S. Buffardi, A. Sala, R. Burnelli, M. Mascarin, V. De Re, Proteomic identification of plasma biomarkers in children and adolescents with recurrent Hodgkin lymphoma, J. Cancer 9 (2018) 4650–4658, http://dx.doi.org/10.7150/jca.27560.

[27] C.B.F. Andersen, K. Stødkilde, K.L. Sæderup, A. Kuhlee, S. Raunser, J.H. Graversen, S.K. Moestrup, Haptoglobin, Antioxid. Redox Signal. 26 (2017) 814–831, http://dx.doi.org/10.1089/ars.2016.6793.
[28] J. Hannerz, K. Ericson, D. Greitz, P.H.B. Skejo, G. Edman, Orbital phlebography in idiopathic intracranial hypertension and chronic tension-type headache, Acta Radiol. Short Rep. 2 (2013), 204798161349886, http://dx.doi.org/10.1177/2047981613498861.
[29] J. Peng, X. Li, Apolipoprotein A-IV may inhibit inflammation via regulating NLRP3/IL-1 path to anti-atherosclerosis, Int. J. Cardiol. 278 (2019) 223, http://dx.doi.org/10.1016/j.ijcard.2018.11.103.
[30] T. Kurth, P. Ridker, J. Buring, Migraine and biomarkers of cardiovascular disease in women, Cephalalgia 28 (2008) 49–56, http://dx.doi.org/10.1111/j.1468-2982.2007.01467.x.