

Clear differences in cerebrospinal fluid proteome between women with chronic widespread pain and healthy women – a multivariate explorative cross-sectional study

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Introduction: Frequent chronic local pain can develop into chronic widespread pain (CWP). The spread of pain is correlated with pain intensity, anxiety, and depression, conditions that ultimately lead to a poor quality of life. Knowledge is incomplete about CWP's etiology, although it has been suggested that both central hyperexcitability and/or a combination with peripheral factors may be involved. Cerebrospinal fluid (CSF) could act as a mirror for the central nervous system as proteins are signal substances that activate the formation of analgesics and control nociceptive processes. To this end, this study investigates the CSF protein expression in women with CWP and in female healthy controls.

Materials and methods: This study included 12 female patients with CWP diagnosed according to the American College of Rheumatology criteria with 13 healthy age- and sex-matched pain-free subjects. All subjects went through a clinical examination and answered a health questionnaire that registered sociodemographic and anthropometric data, pain characteristics, psychological status, and quality of life rating. CSF was collected by lumbar puncture from each subject. Two-dimensional gel electrophoresis in combination with mass spectrometry was used to analyze the CSF proteome. This study identifies proteins that significantly discriminate between the two groups using multivariate data analysis (MVDA) (i.e., orthogonal partial least squares discriminant analysis [OPLS-DA]).

Results: There were no clinically significant levels of psychological distress and catastrophization presented in subjects with CWP. MVDA revealed a highly significant OPLS-DA model where 48 proteins from CSF explained 91% (R^2) of the variation and with a prediction of 90% (Q^2). The highest discriminating proteins were metabolic, transport, stress, and inflammatory.

Conclusion: The highest discriminating proteins (11 proteins), according to the literature, are involved in apoptotic regulations, anti-inflammatory and anti-oxidative processes, the immune system, and endogenous repair. The results of this explorative study may indicate the presence of neuro-inflammation in the central nervous system of CWP patients. Future studies should be larger and control for confounders and determine which alterations are unspecific/general and which are specific changes.

Keywords: biomarkers, muscle pain, inflammation

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Introduction

Pain will spread for many patients (9%–25%) with chronic local/regional pain conditions, ultimately leading to chronic widespread pain (CWP). A continuum of chronic pain conditions has been proposed,¹ and CWP – including fibromyalgia syndrome (FMS) – is considered the most negative end point associated with

numerous negative implications. Spreading of pain is associated with increased pain intensity^{2,3} and increased prevalence of psychological symptoms such as anxiety, depression, and catastrophizing.^{3–5} CWP patients often report difficulties with work, increased sick leave, and poor quality of life and health.^{4,6,7} In the west, CWP has a relatively high prevalence (10.6%).⁸

The newly proposed version 11 of the International Classification of Diseases (ICD-11) identifies CWP as the first diagnosis under the category “chronic primary pain”.⁹ Different alterations in the central processing of nociception (e.g., central hyperexcitability), descending facilitation, impairment of descending inhibition, and biochemical and functional alterations in networks (e.g., default mode network) of the brain are present in CWP/FMS.^{10–13} Neuroendocrine and autonomic nervous system alterations have also been reported.^{14,15} Some researchers consider FMS as mainly a central hyperexcitability pain condition.^{12,16,17} However, peripheral tissue alterations,¹⁸ nociceptive C-fiber alterations (e.g., small fiber neuropathy),^{19–23} and nociceptive input^{24–28} in CWP may indicate that peripheral mechanisms maintain the central alterations.

In recent years, research has focused on identifying biomarkers related to various diseases, a development that has seemingly not yet reached its peak. Fluids such as cerebrospinal fluid (CSF), plasma, and saliva have been suggested as interesting areas.^{29–31} CSF may mirror processes in the central nervous system (CNS) with respect to neurons and glial cells, CSF proteins, and peptides.^{30,32} CSF is created by diffusion of interstitial fluid from the brain and passive filtration of blood through the epithelial cells.³³ Intense research using CSF is ongoing in the field of neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis.³² CSF has also attracted the interest of researchers in order to further understand the pathomechanisms of chronic pain conditions (e.g., CWP). Hence, several decades ago, CSF has been investigated with respect to beta-endorphin and substance P in FMS patients.^{34,35} More recently, research has focused on cytokines and neurotrophic factors in CSF of different pain conditions.³⁶ A recent review found evidence for elevated levels of nerve growth factor (NGF), brain-derived neurotrophic factor, interleukin 8, and lowered levels of glial cell-derived neurotrophic factor in FMS.³⁶ In most of these CSF studies of chronic pain conditions, only one or a few substances at a time have been analyzed. However, there is a need to take a more comprehensive approach due to the complexity of the involved nociceptive processes.³⁷ A

panel of multiple biomarkers or bio-clusters will reasonably perform better than a single or few biomarkers with respect to understanding the involved mechanisms.

Proteins act as signal substances, activate the formation of analgesics, and control nociceptive processes. For the last 35 years, high-resolution two-dimensional gel electrophoresis (2-DE) has been the primary tool to impartially analyze the content of proteins in a certain tissue, i.e., the proteome. Since its inception,³² 2-DE has gradually been refined to the technique seen today with immobilized pH gradients (IPG),³³ high-resolution sodium dodecyl sulfate (SDS) protocols, silver staining, and, more recently, the introduction of a new complement – difference in-gel electrophoresis.³⁴ Although modified, the basic premise of 2-DE remains the same: first-dimension separation using isoelectric focusing (IEF) and second-dimension separation by molecular weight (MW), giving a two-dimensional map of up to thousands of proteins. Data sets from the “omics” field (e.g., proteomics) are generally characterized by low subject-to-variables ratios and a large number of intercorrelated molecules/proteins, so these methods often violate central assumptions of traditional statistical methods.³⁸ Thus, it is necessary to use modern multivariate data analysis (MVDA) methods such as advanced principal component analysis (PCA) and different types of partial least square (PLS) regressions.³⁸ Hence, 2-DE,³⁹ mass spectrometry, and subsequent MVDA can be used to separate, quantify, and determine the important pattern of the proteins from a tissue, i.e., proteomics.

Proteomics are frequently applied in clinical research of neurodegenerative disorders,³² but proteomic CSF studies are sparse in the field of chronic pain. Such studies can be important for understanding activated nociceptive mechanisms and hopefully will be used in the long term to clinically guide diagnosis and choice of treatment. A few CSF studies using proteomics have been published – e.g., spinal nerve root injury due to lumbar disk herniation⁴⁰ and neuropathic pain.^{41,42} A few bioinformatics-oriented studies on neuropathic pain also exist.^{43,44} Recently, our group in an explorative study reported that the multivariate protein pattern in CSF of patients with severe peripheral neuropathic pain clearly and significantly differed from that of healthy controls (CON).⁴⁵

In this study, we hypothesized that significant differences in the proteome of CSF exist between CWP and healthy women (CON). Thus, the aim of this explorative and cross-sectional study was to compare the CSF protein expression in CWP and CON using 2-DE and MVDA.

Materials and methods

Subjects

Patients with CWP were recruited among former patients at the Pain and Rehabilitation Centre of the University Hospital, Linköping, Sweden. Inclusion criteria were female sex, age between 20 and 65 years, and CWP according to the American College of Rheumatology (ACR) criteria 46. Exclusion criteria were major psychiatric disorder, addiction, other significant disease (investigator's judgment), and pregnancy. Thirteen women (one excluded; "Results" section) with CWP agreed to participate in the study.

Thirteen healthy women were recruited through advertisements at Linköping University.

A structured interview was conducted to ensure the absence of any significant medical condition. The following areas were specifically assessed in the interview: earlier major trauma; back, joint, muscle, or skeletal disease; heart or vascular disease; lung or bronchial disease; psychiatric symptoms; neurological, ear, or eye disease; digestive tract disease; kidney, urinary, or genital disease; skin disease; tumor or cancer; endocrine disease; hematological disease; birth defects; and other diseases, disabilities, or allergies.

All the subjects in both groups were asked whether they had a bleeding disorder.

All potential subjects were given written and verbal information about the study, and subjects interested in participating signed a consent form that was in accordance with the Declaration of Helsinki. The study was granted ethical clearance by the Linköping University Ethics Committee (Dnr M136-06 and 2012/94-32).

Clinical examination

All subjects went through a brief clinical examination by a physician. The examination included auscultation of heart and lungs, diastolic and systolic blood pressures, routine neurological examination, and tender point examination (number of tender points is reported) according to the ACR criteria.⁴⁶

As a part of the clinical examination, algometry was performed using an electronic pressure algometer (Somedic, Hörby, Sweden) as previously described.⁴⁷ The diameter of the contact area was 10 mm, and the pressure was applied perpendicularly to the skin at a speed of 30 kPa/s. The subjects were instructed to mark the pressure pain threshold (PPT) by pressing a button as the sensation of "pressure" changed to "pain". Before the actual testing of PPT, the subjects were given instructions and allowed to examine the testing procedure. Algometry was performed bilaterally over the medial, middle, and lateral part of the descending

part of the trapezius muscle and over the dominant (CON) or most painful side (CWP) of the tibialis anterior muscle to determine the PPTs. When the button was pressed or when the maximum pressure of 600 kPa was reached, the application of pressure ceased. All PPT measurements were conducted twice in ~5-min intervals. The PPT values of the trapezius muscles were calculated as the mean of these two measurements of lateral, middle, and medial sites on the right and left trapezius muscle.

Health questionnaire

All subjects answered a brief health questionnaire that registered sociodemographic and anthropometric data, pain characteristics, psychological status, and quality-of-life rating.

Sociodemographic and anthropometric data

The following variables were registered: age, self-reported weight (kg), and height (m). Based on these two anthropometric variables, body mass index (BMI; kg/m²) was calculated.

Pain characteristics

All subjects rated pain intensity in nine anatomical regions (neck, shoulders, arms, hands, upper back, lower back, hips, knees, and feet) using a visual analog scale (VAS). The scale was 100 mm long with defined end points ("no pain" and "worst pain imaginable") but without marks in between (results in mm).

Psychological aspects

Hospital Anxiety and Depression Scale (HADS)

The HADS, a short self-assessment questionnaire, measures the level of anxiety and depression. HADS comprises seven items in each of the depression (HADS-D) and anxiety (HADS-A) subscales. Possible subscale scores range from 0 to 21, the lower score indicating the least depression and anxiety. A score of 7 or less indicates a non-case, a score of 8–10 indicates a doubtful case, and a score of 11 or more indicates a definite case. HADS is frequently used and has good psychometric characteristics.^{48,49}

Pain Catastrophizing Scale (PCS)

The PCS is a 13-item self-report measure designed to assess catastrophic thoughts or feelings accompanying the experience of pain.^{50,51} Respondents are asked to reflect on past painful experiences and to indicate the degree to which each of the 13 thoughts or feelings are experienced when in pain. The questionnaire uses a five-point scale ranging from 0 (not at all) to 4 (all the time). Subscales

for rumination, magnification, and helplessness plus a total score are added up. Different cutoff scores that are clinically relevant have been reported.⁵² In this study, the total score was used; a cutoff of 38 is generally used as indicative as catastrophizing.

Quality of Life Scale (QOLS)

The QOLS is a 16-item instrument that measures domains of quality of life: material and physical well-being, relationships with other people, social, community, and civic activities, personal development and fulfillment, recreation, and independence. In this study, the validated Swedish version was used.⁵³ The QOLS is scored by adding up the score on each item to yield a total score ranging between 16 and 112.

Sample handling

For both patients and controls, 10 mL of CSF was retrieved through lumbar puncture with a 27 GA pencil-point Whitacre needle (BD Medical, Franklin Lakes, NJ, USA) and collected into five separate tubes containing 2 mL each of CSF and kept on ice. All samples were centrifuged at 3000× *g* for 10 min and then controlled for blood contamination by a visual erythrocytes check. Samples with visible erythrocytes were pooled and stored separately at −80°C and excluded from the study. Samples without erythrocytes were pooled and stored in aliquots at −80°C pending further quality control and analysis.

Quality control of samples

Samples without obvious erythrocyte contamination were further checked for plasma contamination by determining apolipoprotein B concentration. An apolipoprotein B ratio of plasma (CSF<6000) was considered as non-contaminated.⁵⁴ An ELISA kit (Max Discovery, Bioo Scientific Corporation, Austin, TX, USA) was used to check for plasma contamination by determining apolipoprotein B concentration.

Proteomic analysis

Samples were depleted of the high abundance proteins albumin and immunoglobulin G (IgG) using an Albumin and IgG Depletion SpinTrap kit (GE HealthCare, Buckinghamshire, UK). Aliquots of 200 µL × 5 (final volume 1 mL) CSF were loaded on the SpinTrap and collected through centrifugation according to the user manual. The CSF was then desalted, lyophilized, and solubilized in sample solution as previously described.⁵⁵ Protein concentration was measured before and after the depletion procedure using Bio-Rad protein assay according to Bradford.⁵⁶

An amount of 300 µg protein/sample was diluted in rehydration buffer (Urea 8 M, CHAPS 2%, DTT 0.3%, IPG buffer 0.5%) to a final volume of 350 µL and was applied on nonlinear pH gradient 3–10 IPG strips (0.5 × 3 × 180 mm) (GE HealthCare). IEF was performed on IPGphor as previously described.⁵⁵ The strips were then equilibrated twice (or stored in −80°C until analysis) with SDS equilibration buffer.⁵⁵ The second dimension, 2-DE, was performed vertically on an Ettan™ DALTsix Electrophoresis Unit (Amersham, Pharmacia Biotech, Uppsala, Sweden) per the manufacturer's recommendations. Proteins from the IPG strip were transferred to homogenous gels homemade cast in low fluorescent cassettes (1.0 × 220 × 270 mm, 14%T, 2.6%C) and kept in place with 3 mL agarose solution (0.05%) with a trace of bromophenol blue. The samples were run at 80 V/10 mA* (*per gel) for ~1.5 h, then run at up to 600 V/40 mA* (*per gel) for 4–5 h, i.e., until finished. SDS electrophoresis buffer was used (anodic, 25 mM Tris, 192 mM glycine, 0.1% [w/v] SDS and cathodic, 50 mM Tris, 384 mM glycine, 0.2% [w/v] SDS, approximate pH 8.3). Precision Plus Protein™ All Blue (250–10 kDa) (Bio-Rad) was used as an MW standard. Gels were fixed using 500 mL gel of 10% methanol/7% acetic acid in water overnight and then fluorescently stained with 500 mL SYPRO Ruby (Bio-Rad) and incubated overnight. Fluorescent staining was performed according to the manufacturer's staining protocol (SYPRO Ruby protein gel stain; www.probes.com). Proteins were visualized using a CCD camera VersaDoc™ Imaging system 4000 MP (Bio-Rad).

Finally, protein patterns in the digitized images were analyzed with PDQuest 8.0.1 (Bio-Rad), a computerized imaging 12-bit system designed for evaluations of 2-DE patterns. The amount of protein in a spot was assessed as background-corrected optical density, integrated over all pixels in the spot and expressed as integrated optical density (IOD). If the quantified and matched proteins were present in at least 75% of each group, they were eligible for further multivariate data analysis.

In-gel digestion by trypsin

Protein spots were excised using a homemade spot picker. The picked protein spots were digested with trypsin (Promega/SDS Biosciences, Falkenberg, Sweden). In short, the gel pieces were washed with a mixture of acetonitrile/ammonium bicarbonate, dehydrated with acetonitrile, and incubated with 30 µL of 20 µg/mL trypsin in 25 mM ammonium bicarbonate overnight at 37°C. The supernatant was transferred to a new tube, and the peptides were further extracted from the gel by incubation in 50% acetonitrile/5% trifluoroacetic acid (TFA)

for ~3 h at room temperature while being constantly mixed. The supernatant obtained by the two steps were pooled and dried by the SpeedVac vacuum concentration system (Savant, Farmingdale, NY, USA).

Protein identification by MALDI-TOF/TOF

The dried tryptic samples were dissolved in 3 μ L of 0.1% TFA. The peptides were mixed 1:1 with matrix solutions consisting of dihydroxybenzoic acid (0.04 g/mL) in 70% acetonitrile/0.3% TFA, and 1 μ L was applied on the target plate (stainless steel plate). Analyses of peptide masses were performed using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF)/TOF MS (ultrafleXtreme™ MALDI-TOF; Bruker Daltonik GmbH, Bremen, Germany) operated in reflector mode. Spectra in the mass range of 300–3000 Da were collected, and external mass calibration with a standard peptide mixture was used. The spectra were also internally calibrated using trypsin autolysis peptides as described previously.⁵⁷

Protein identification by Orbitrap mass spectrometry

The dried tryptic samples were dissolved in 6 μ L of 0.1% formic acid. Peptides were analyzed, and data were acquired using Linear Trap Quadrupole (LTQ) Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in conjunction with nanoflow high-performance liquid chromatography system (EASY-Nlc II, Thermo Fisher Scientific). Peptides were separated by reverse-phase chromatography on a 20 mm \times 100 μ m C18 pre-column followed by a 100 mm \times 75 μ m C18 column (particle size 5 μ m; Nanoseparations, Nieuwkoop, the Netherlands) at a flow rate of 300 nL/min for 45 min. The gradient buffers were 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B), and a linear gradient from 0% to 100% of buffer B was used for separation (45 min). Automated online analyses were performed with LTQ Orbitrap Velos Pro hybrid mass spectrometer with nano-electrospray source – 240°C capillary temperature and spray voltage of 2200 V. MS spectra were acquired in profile mode by Fourier transform mass spectrometry at a resolution of 30000 (at m/z 400). The top 20 most intense multiply charged ions were selected with an isolation window of 2.0 and fragmented in the linear ion-trap by collision-induced dissociation with normalized collision energy of 30. Dynamic exclusion of sequenced peptides for 60 s and charge state filtering disqualifying singly charged peptides were activated and predictive automatic gain control was enabled. Centroid

mode was used for collision induced dissociation tandem mass spectrometry.

Database searches

Data processing of the spectra from MALDI-TOF was performed with flexAnalysis v.3.4 (Bruker Daltonik GmbH). The generated peptide mass list (mass + H^+) of the major peaks from MALDI-TOF analysis was submitted to a database search (NCBI or SWISS-PROT) using MS-fit search engines. Parameters were set as species (human), mass tolerance (50 ppm), maximum missed cleavages by trypsin ≤ 1 , fixed modification (carbamidomethylation), dynamic modification (oxidation of methionine), N-terminal glutamine to pyroglutamate, and N-terminus acetylation.

Data from the Orbitrap mass spectrometer were searched with MaxQuant version 1.5 with trypsin as a digestion enzyme against a human taxonomy of the SwissProt database (release February 2016). The following parameters were used: maximum number of missed cleavages 2; fragment ion mass tolerance 0.5 Da; parent ion mass tolerance 6 ppm; fixed modification – carbamidomethylation of cysteine; and the variable modifications – N-terminal acetylation and methionine oxidation. Data were filtered at 1% false discovery rate. Identifications were based on a minimum of two unique peptides.

Statistics

For age, anthropometric data, blood pressures, PPTs, number of tender points, pain intensities, depressive and anxiety symptoms, catastrophizing scale, and quality-of-life data, Student's *t*-test for unpaired data was applied using IBM SPSS v.21.0; $p < 0.05$ was considered significant.

MVDAs were performed using SIMCA v.13.0 (UMETRICS, Umeå, Sweden). When applying MVDA, the recommendations concerning omics data presented by Wheelock and Wheelock³⁸ were followed. Variables were mean centered and scaled for unified variance (UV scaling). An unsupervised PCA was first used to check multivariate outliers among all the observations/proteins.⁵⁸ In the second step, when investigating the multivariate correlations between the proteins and group membership, orthogonal partial least squares discriminant analysis (OPLS-DA) was applied.⁵⁸ In the OPLS-DA, variables (regressors) were considered important if they had regression coefficients with a jackknifed 95% confidence interval not including 0, and the variable of importance (VIP) value was greater than 1. The OPLS-DA analysis was made in two steps. First, all proteins were included, and from this analysis, proteins were selected with $VIP > 1.0$ combined with the jackknifed confidence intervals in the coefficients plot

not including 0 and used in a new regression presented in the results. Coefficients (PLS scaled and centered regression) were used to note the direction of the relationship (positive or negative). The ratio of protein alteration (i.e., the ratio between CWP and CON) was calculated using the mean values of IOD for each protein per group. The $p(\text{corr})$ is the loading of each variable scaled as a correlation coefficient and thus standardizing the range between -1 and $+1$. The $p(\text{corr})$ is stable during iterative variable selection and comparable between models. An absolute $p(\text{corr}) > 0.4$ – 0.5 is generally considered significant.³⁸ R^2 describes the goodness of fit – the fraction of sum of squares of all the variables explained by a principal component. Q^2 describes the goodness of prediction – the fraction of the total variation of the variables that can be predicted by a principal component using cross-validation methods. R^2 should not be considerably higher than Q^2 . A difference > 0.2 – 0.3 implies overfitting, meaning that the robustness of the model is poor.⁵⁸ To validate the model, obtained cross-validated analysis of variance (CV-ANOVA) was used. The multivariate regressions were considered of significant importance if the CV-ANOVA had $p < 0.05$.

Results

Background data

No significant age or height differences were found between the two groups (Table 1). CWP ($n=12$) had significantly higher weight and BMI than CON ($n=13$) (Table 1). Small but significant differences were found in blood pressures. As expected, prominent differences were found in pain intensities and number of tender points (Table 1).

Although significant group differences existed according to the anxiety and depression scales of HADS, none of these values were above the clinical cutoff (i.e., >10), indicating anxiety or depression. A significant difference also existed for PCS, but on the group level, CWP had a level well below the cutoff used for catastrophizing (i.e., >38). QOLS showed a significant group difference with a worse situation for CWP.

Proteome profile

A CSF sample of one patient exceeded the apolipoprotein B ratio, so this patient was not included in the study. The protein concentration of the CWP group (presented as mean ± 1 standard deviation [SD], $n=12$) before removal of the

Table 1 Age, anthropometric data, blood pressures, pressure pain thresholds, number of tender points, pain intensities, depressive and anxiety symptoms, catastrophizing, and quality of life in patients with CWP and in CON

Group Variables	CON, n=13		CWP, n=12		Statistics p-Value
	Mean	SD	Mean	SD	
Age	39.77	15.64	48.25	10.35	0.127
Weight (kg)	63.50	8.93	85.04	19.88	0.002
Height (m)	1.65	0.07	1.68	0.07	0.295
BMI (kg/m ²)	23.4	2.6	30.1	5.9	0.001
BP diastolic (mmHg)	74	7	84	6	0.001
BP systolic (mmHg)	120	9	132	14	0.023
PPT trapezius right (kPa)	291	59	120	46	<0.001
PPT trapezius left (kPa)	268	57	143	123	0.003
PPT tibialis anterior (kPa)	466	131	251	120	<0.001
No. tender points	0.8	1.0	13.6	3.2	<0.001
Pain intensity neck (VAS)	1	2	55	18	<0.001
Pain intensity shoulders (VAS)	2	6	67	18	<0.001
Pain intensity arms (VAS)	2	6	52	22	<0.001
Pain intensity hands (VAS)	0	0	43	25	<0.001
Pain intensity upper back (VAS)	0	0	58	27	<0.001
Pain intensity lower back (VAS)	2	7	65	23	<0.001
Pain intensity hips (VAS)	1	2	63	31	<0.001
Pain intensity knees (VAS)	4	13	41	22	<0.001
Pain intensity feet (VAS)	0	0	41	28	<0.001
HADS-A	2.3	3.0	6.6	4.2	0.007
HADS-D	0.9	1.3	5.9	3.6	<0.001
PCS	3.5	4.5	21.8	13.4	<0.001
QOLS	94.0	10.4	76.8	11.7	0.001

Notes: Mean ± 1 SD is given for each variable. Furthest to the right is the result of the statistical comparisons between the two groups (p-value).

Abbreviations: CWP, chronic widespread pain; CON, healthy controls; SD, standard deviation; BMI, body mass index; PPT, pressure pain threshold; BP, blood pressure; VAS, visual analog scale; HADS-A, Hospital Anxiety and Depression Scale – subscale anxiety; HADS-D, Hospital Anxiety and Depression Scale – subscale depression; PCS, Pain Catastrophizing Scale; QOLS, Quality of Life Scale.

albumin and IgG (i.e., “raw” CSF) was 370 ± 94 and after removal was 90 ± 21 . The protein concentration of the CON group (presented as mean \pm 1 SD, $n=13$) before and after removal of the albumin and IgG was 266 ± 53 and 60 ± 22 , respectively. After the removal of the abundant proteins albumin and IgG, several other proteins could be revealed on the 2-DE, revealing many low abundant proteins or proteins in a higher quantity (Figure 1A and B).

Multivariate data analysis

From the quantification of the 2-DE images, 481 proteins were matched between the gels and eligible for multivariate statistics. As a quality check of outliers, we computed an unsupervised PCA with five principal components ($R^2(\text{cumulative})=0.43$, $Q^2(\text{cumulative})=0.13$), which revealed no moderate (distance to the model in X-space [DModX]) or strong outliers (Hotelling's T^2 statistic test [$T^2\text{Critical } 99\%$]).

To identify the proteins important for group separation of the subjects into CWP and CON, an OPLS-DA regression model (one predictive and two orthogonal latent variables, respectively; Figure 2) was generated. The result showed both excellent fit ($R^2=0.98$) and prediction ($Q^2=0.91$). The CV-ANOVA revealed that the model was highly significant ($p=0.00000118$).

A total of 48 protein spots had a VIP>1, so these were considered important for the group separation (Figure 3; Table S1). The most important protein spots for the discrimination (VIP>1.3, $p(\text{corr})>0.4$; 11 of 48 proteins) between the two groups are marked with a circle in Figure 3 and presented in Table 2.

A final OPLS-DA model (one predictive and one orthogonal latent variable) was computed using the 11 proteins in Table 2 as regressors. This regression also had a very good fit ($R^2=0.89$) and had high predictivity ($Q^2=0.84$).

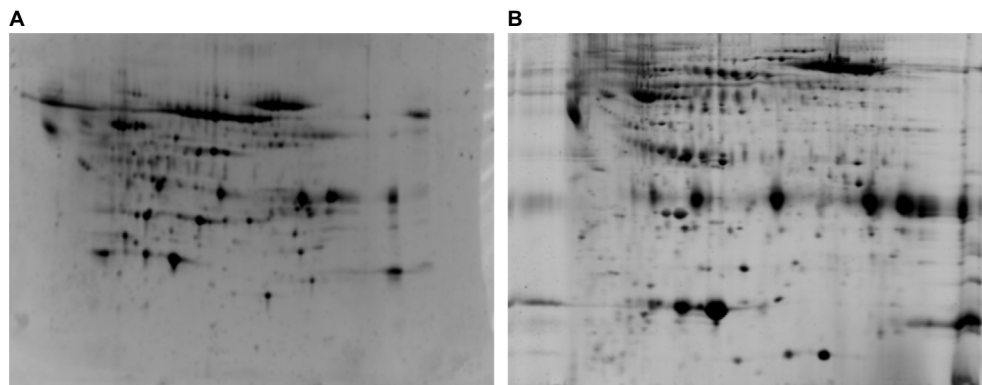


Figure 1 Images of CSF before and after albumin/IgG removal.
Notes: The figure shows the “raw” CSF (A) compared to the fractionated CSF (B).
Abbreviations: CSF, cerebrospinal fluid; IgG, immunoglobulin G.

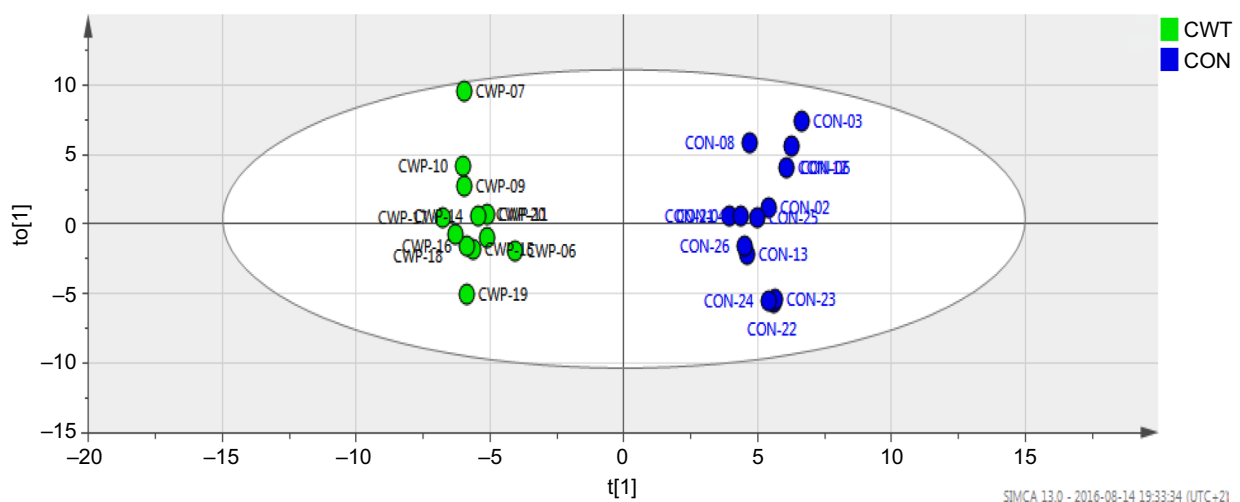


Figure 2 An OPLS-DA (the first principal component $t[1]$ vs. the first orthogonal component $to[1]$) model showing the discriminant separation between the CWP patients (green filled circles) and the CON (blue filled circles).

Notes: The longitudinal dimension (Y-axis) shows the interclass discrimination, and the latitudinal dimension (X-axis) shows the intraclass discrimination between CWP and CON.

Abbreviations: OPLS-DA, orthogonal partial least square discriminant analysis; CWP, chronic widespread pain; CON, healthy controls.

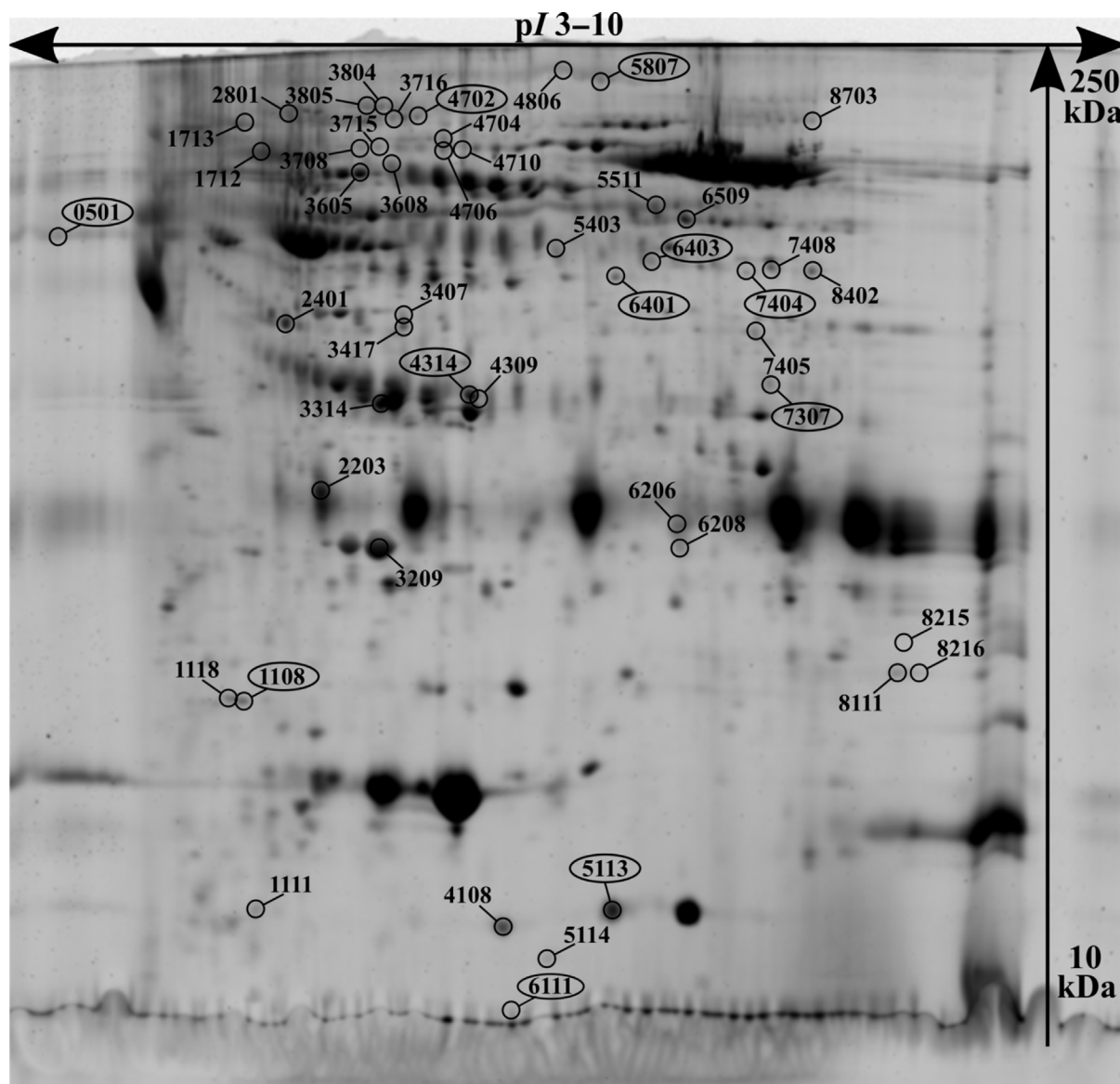


Figure 3 The 2-DE CSF proteins.

Notes: The image shows the proteins important for the group separation between CWP and CON. Protein numbers that are circled had a VIP>1.3 and were highly significant for the model.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; CSF, cerebrospinal fluid; CWP, chronic widespread pain; CON, healthy controls; VIP, variable of importance.

This model was also highly significant according to CV-ANOVA ($p=0.0000000756$). Hence, clear group separation was achieved when only including these 11 proteins as regressors.

Protein identification

The 48 protein spots that were important for the separation between CWP and CON (VIP>1) were excised from the gel and in-gel digested by trypsin for identification by mass spectrometry. The protein spots that were highly stained were identified by MALDI-TOF, and the remaining protein spots of low abundance were identified by Orbitrap mass spectrometer. For most identified proteins, the apparent

molecular mass and pI determined in the 2-DE were in agreement with the theoretical values. When identifying fragments of proteins (spot no. 1111), the position of the matched peptides within the theoretical sequence of the protein were controlled in UniProt database using “Compute MW/pI” to be in agreement with the apparent MW/pI on the gel. The identified proteins were grouped based on biological processes according to UniProt database (Tables 2 and S1); 19% of the significant proteins were expressed in more than one form, i.e., isoforms (alpha-2-macroglobulin [$\alpha 2M$], apolipoprotein E, beta-2-glycoprotein 1, alpha-1B-glycoprotein, prostaglandin-H2 D-isomerase, and beta-2-microglobulin form pI 5.3, Contactin-1) (Table S1).

Table 2 The most important proteins (VIP>1.3) discriminating between CWP and CON according to the OPLS-DA

Spot number	Protein	VIP	p(corr)	CoeffCS	IOD ratio (CWP/CON)	Type
6111	Insulin-like growth factor II	2.00	-0.95	+	24.40	M
5807*	Alpha-2-macroglobulin	1.66	0.64	-	0.26	S&I
7404	Alpha-enolase	1.56	0.61	-	0.28	M
5113	Beta-2-microglobulin	1.54	-0.75	+	2.23	S&I
4314	Transthyretin	1.47	0.72	-	0.19	T
1108*	Beta-2-microglobulin form pI 5.3	1.45	0.67	-	0.33	S&I
4702	Neuronal cell adhesion molecule	1.37	0.44	-	0.36	T
6401	Pigment epithelium-derived factor	1.36	0.56	-	0.68	R
6403	Beta-1,4-glucuronyltransferase I	1.34	0.58	-	0.56	M
7307	Malate dehydrogenase, cytoplasmic	1.34	0.53	-	0.59	M
0501	Dickkopf-related protein 3	1.32	0.68	-	0.42	R

Notes: For the CoeffCS, "+" indicates upregulation and "-" indicates downregulation of the protein in the CWP group compared to the CON. Spot numbers marked with * were present as an isoform on the 2-DE. The proteins were divided based on UniProt database (<http://web.expasy.org>) definition on biological process in different groups (labeled Type): M, metabolic; S&I, stress and inflammatory; T, transport; R, regulation proteins.

Abbreviations: VIP, variable of importance; CWP, chronic widespread pain; CON, healthy controls; OPLS-DA, orthogonal partial least squares discriminant analysis; IOD, integrated optical density; CoeffCS, scaled and centered coefficients.

Discussion

Using 2-DE in combination with mass spectrometry, we separated and identified proteins in CSF from women with CWP and healthy female subjects. MVDA was used to determine the most important proteins for the separation of the two groups, and 48 of 481 proteins contributed significantly to the separation between CWP and CON; this highly significant OPLS-DA model had nearly perfect fit and predictivity ($R^2=0.98$ and $Q^2=0.91$). The following discussion highlights the most significant proteins (i.e., those with a VIP>1.3 and a $p(\text{corr})>0.4$) (Table 2); the significant OPLS-DA model only using these 11 proteins as regressors was also characterized by a very good fit and predictivity ($R^2=0.89$ and $Q^2=0.84$).

The protein with the highest discriminating power (VIP=2.0) was the insulin-like growth factor II (IGF2). It was upregulated in the CWP group. IGF2 is a protein that controls growth activity and in cultured cells triggers mitosis.⁵⁹ It also has anti-apoptotic properties and inhibits apoptotic cell death (e.g., via antagonizing activation of pro-inflammatory cytokine signaling).^{60,61} Furthermore, pulsed radiofrequency right after nerve injury inhibited the development of neuropathic pain. This was found to be due to downregulation of IGF2 and the inhibition of ERK1/2 activity in microglial cells.⁶²

The $\alpha 2\text{M}$ is a protease inhibitor and belongs to the I39 family of macroglobulins. It is synthesized by numerous cell lineages including astrocytes.⁶³ It inhibits by trapping the protease after a conformational change has been triggered in the protein when the protease cleaves $\alpha 2\text{M}$ s bait region.⁶⁴ Thus, a strong covalent binding is made through the hydrolyzation of a thioester bond. Although the protease almost remains totally inhibited to the higher MW substrates, it is still able to cleave the substrates with lower MWs. The $\alpha 2\text{M}$ binds several cytokines (e.g., IL-6 and IL-1 β), growth

factors (e.g., NGF and platelet-derived growth factor), and hormones.^{63,64} It is considered as an acute-phase protein,⁶⁵ and it has been suggested that $\alpha 2\text{M}$ has antioxidant effects and anti-inflammatory ability.⁶³ With respect to the latter suggestion, the formation of the $\alpha 2\text{M}$ -pro-inflammatory cytokine complex has been suggested to protect from the immediate toxic effect of cytokines. Increased levels of $\alpha 2\text{M}$ have been associated with dysfunctional blood-CSF barrier.⁶⁶ This protein was downregulated in the present CWP cohort. Significantly lower levels of $\alpha 2\text{M}$ were also found in CSF from patients with schizophrenia, in subjects with risk of psychosis, and in depressed patients.^{67,68}

Alpha-enolase is a multifunctional protein found in almost all human tissues. It is a metabolic enzyme as well as an important protein involved in growth control and allergic responses and stimulates immunoglobulin production. Alpha-enolase has also been described as a hypoxic stress protein, a heat shock protein, and a neurotrophic factor.⁶⁹ It has been characterized as a marker of pathological stress⁷⁰ and as a biomarker of damaged glial cells.⁷⁰ During ontogenesis, there is a transition from the alpha/alpha homodimer to the alpha/beta heterodimer in striated muscle cells and to the alpha/gamma heterodimer in nerve cells. Alpha-enolase was downregulated in the present CWP group. Previous studies found increased CSF levels in patients with multiple sclerosis, cerebral infarction, and peripheral neuropathy.⁷⁰

β -2-Microglobuline was upregulated in the present CWP cohort. It is a small membrane protein of ~11 kDa expressed widely in all body fluids.⁷¹ Increased levels of CSF have been associated with an activation of the immune system, and it has been proposed as a reliable CSF biomarker of different inflammatory disorders or neoplastic CNS disorders (e.g., purulent meningitis, leptomeningeal metastasis, viral

meningitis/encephalitis, and neuroborreliosis in CSF).⁷² After acute nociception (i.e., non-neurological surgery), significant increases in CSF β -2-microglobuline, but not in serum, were noted, which was ascribed to increased cellular turnover and immunological activation in CNS but not to peripheral inflammatory activity or changes in blood–brain barrier permeability.⁷³ A significant increase in CSF β -2-microglobuline has also been found in chronic fatigue, chronic fatigue syndrome, and depression.^{68,74} In a relatively small study, an age dependence (i.e., higher CSF levels) was reported for subjects >50 years.⁷⁵ β -2-microglobuline has also been investigated in several studies of Alzheimer's disease and multiple sclerosis, but yet no consistent patterns have been established.^{76–79} Partially, in contrast to the results of this study, no differences were found in CSF levels between patients with polyneuritis/polyneuropathies and CON, which the authors suggested could have been due to heterogeneity aspects.⁷²

Transthyretin (TTR; originally labeled prealbumin) has an MW of 55 kDa and binds thyroxine (T4) and is secreted into the CSF by the choroid plexus. Most likely, it stands for the transportation of T4 to the CSF and the brain.^{80–82} It has also been suggested that TTR acts as an endogenous anti-inflammatory mediator.⁸³ It was downregulated in the present CWP group. Low level of TTR in CSF has been reported to correlate with depression and low serotonin function.^{68,84} Decreased CSF levels have been found in patients with first-onset schizophrenia.⁸⁵ This protein was also of significant importance but upregulated in a proteomic study of CSF in patients with neuropathic pain.⁴⁵ Increased CSF levels of TTR in response to oxidative stress have been reported.⁸⁶ TTR has also been investigated in CSF from Alzheimer's disease, and both significant up- and downregulations have been reported.⁷⁶

Neuronal cell adhesion molecule (NCAM, also called CD56) is part of the immunoglobulin superfamily mediating calcium-independent intercellular adhesion. This glycoprotein plays a part in the development of the nervous system and is involved in the increase of T cells and dendritic cells, which are of importance in the surveillance of the immune system.⁸⁷ NCAM participates in contact-mediated interactions among neurons, astrocytes, oligodendrocytes, and myotubes⁸⁸ and in plasticity of synaptic connection in memory formation, learning, and CNS injury repair.^{88,89} NCAM was downregulated in CWP. CSF NCAM was also significantly lower in polyneuropathy compared to CON;⁹⁰ low NCAM levels were suggested to represent extensive neuronal damage or lowered potential for endogenous repair due to chronicity or neurodegenerative etiology. Significant reductions have

also been reported for multiple sclerosis, Alzheimer's disease, and meningitis.⁹⁰ Both increased and decreased levels of this protein in CSF have been reported for subjects with depression.^{68,91} Animal experiments have shown changes in the expression patterns of NCAM isoforms that occur in the spinal cord as a consequence of peripheral injury and regeneration,^{92,93} which may indicate that NCAM participates in the reorganization of neural circuitry in the spinal cord.⁸⁹

Pigment epithelium-derived factor (PEDF) is found in many tissues and fluids in the body (e.g., CNS and CSF).⁹⁴ PEDF has neurotrophic, neuroprotective, and anti-oxidative properties.^{95–98} Although a member of the serpin (serine antiprotease) protein family, PEDF does not possess this activity.⁹⁷ The protein has been described as having a neuroprotective effect from degeneration due to overexcitation by glutamate.⁹⁶ Glutamate, through the *N*-methyl-D-aspartate receptor activation, can cause cell damage, resulting in an increase of nitric oxide (NO). This in turn will accumulate toxic peroxynitriles that form pro-apoptotic complexes with reactive oxygen species following apoptosis.⁹⁶ Whether PEDF is anti-angiogenic or neurotrophic depends on the site and number of phosphorylations.⁹⁴ PEDF was downregulated in CWP. Downregulations of this protein have been reported in CSF studies of neuropathic pain conditions.^{41,45} Whether it is up- or downregulated in depression is unclear.⁶⁸

Beta-1,4-glucuronyltransferase was downregulated in CWP. It has its optimum pH at 7.0 and is involved in protein glycosylation, and in adults, it is highly expressed in brain, heart, and skeletal muscles.⁹⁹ Mutations of the beta-1,3-*N*-acetylglucosaminyltransferase 1 have been reported to cause congenital muscular dystrophies.¹⁰⁰

Malate dehydrogenase is a cellular enzyme and has an oxidoreductase activity and is involved in the production of NO through nicotinamide adenine dinucleotide phosphate in the mitochondria. NO is a highly diffusible messenger that is involved in many biological functions including modulation of nociception, immune function, and neurotransmission.^{101,102} Malate dehydrogenase is stimulated by dehydroepiandrosterone and thyroid-free T3. Furthermore, it has also been suggested to be associated with the pain modulation in FMS.¹⁰³ It was downregulated in CWP. Decrease of malate dehydrogenases leads to lower levels of malate, and it has been reported that a supplemental use of malic acid can improve pain conditions in FMS.¹⁰⁴ A mouse study of global cerebral ischemia has reported decreased levels of this protein in the spinal cord.¹⁰⁵ Significant elevations in CSF have been reported in Alzheimer's disease and in Lewy body dementia.¹⁰⁶

The dickkopf-related protein 3 (dkk-3) is a 38-kDa protein and counteracts Wnt signaling by inhibiting low-density lipoprotein receptor-related protein 5 and 6 interaction with Wnt.¹⁰⁷ The dickkopf-related proteins play a significant role in vertebrate development by inhibiting the Wnt-regulated processes such as limb development, somitogenesis, and eye formation.¹⁰⁷ There are also some evidence suggesting that dkk-3 has a role in the immune system.¹⁰⁸ The dkk-3 was downregulated in the present CWP cohort. A recent study of patients with multiple sclerosis also reported a CSF down-regulation of dkk-3.¹⁰⁹ Recently, it was suggested that dkk-3 may be an A β -associated protein in Alzheimer's disease.¹¹⁰

In conclusion, the most important and significant proteins are involved in apoptotic regulations, anti-inflammatory and anti-oxidative processes, activity of the immune system, and endogenous repair. Taken together, these findings indicate the presence of neuroinflammation in chronic pain and especially CWP, a possibility suggested by other authors.^{110,111} Although a pattern of proteins clearly discriminating CWP and CON (Table 2) was found, our study cannot determine if these alterations are primarily due to CNS processes or secondary consequences of peripheral factors. In fact, our group reported prominent proteomic alterations in the interstitium of the trapezius muscle and in biopsies of the same muscle in another cohort of CWP patients and controls.^{112,113} Interestingly, when comparing the results of this study (Table 2) with a recently published similar study from our group on neuropathic pain patients,⁴⁵ the top protein lists of the two studies partly overlap, TTR and PEDF being significant proteins in both studies. In addition, among the other significant proteins were partial overlaps between the two studies identified – i.e., prostaglandin-H2 d-isomerase (downregulated in both conditions), gelsolin (downregulated in both conditions), apolipoprotein-A-I (generally upregulated in neuropathic pain but downregulated in the CWP), and haptoglobin (upregulated in both conditions) (Table S1). This might reflect, on the one hand, that different chronic pain conditions share common mechanisms but, on the other hand, that there are pain condition-specific mechanisms at work (in this case, in CWP vs. posttraumatic peripheral neuropathic pain). The most important proteins in this study have also been investigated and/or been identified in proteomic studies of neurodegenerative and psychiatric conditions. Hence, some of the alterations reported in pain and neurodegenerative and psychiatric conditions may be general and unspecific and not disease/condition specific.

This explorative study has several limitations. The number of subjects in each group was relatively low, although

most proteomic studies of CSF in humans are relatively limited with respect to the number of subjects. The use of MVDA has several advantages in proteomic studies as mentioned in the “Introduction” section – e.g., the ability to handle the fact that the ratio between subjects (12+13=25 subjects) and variables (481 proteins) was low. Moreover, selection mechanisms may have been present in the group of patients such as overrepresentation of patients with severe CWP. Although we have investigated patients with CWP, they had low levels of psychological strain. This explorative study must be confirmed in larger studies that also include different groups of chronic pain conditions (e.g., with respect to degree of pain spreading on the body). Future studies should be larger and include analyses of possible confounders and to what extent pain and depression, e.g., may share inflammation as a common mediator.¹¹⁰ Determination of unique and common regressors may also be applicable for factors such as BMI, concomitant medication, and blood pressures.¹¹⁴ The comparisons with proteomic studies concerning neurodegenerative and psychiatric conditions made earlier may be biased since several of these studies have not determined the most important proteins using MVDA or similar techniques. Thus, the discriminating and predictive ability of potentially interesting proteins was not considered.

Conclusion

In this explorative study, we used proteomics in combination with MVDA and found 48 proteins in CSF that were important in discriminating women with CWP from CON. The highest discriminating proteins (11 proteins) are, according to the literature, involved in apoptotic regulations, anti-inflammatory and anti-oxidative processes, activity of the immune system, and endogenous repair. Our results suggest the presence of neuroinflammation in the CNS of CWP patients. Future studies should include more participants, control for confounders, and determine which alterations are unspecific/general and which are specific changes.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Altered proteins in cerebrospinal fluid identified by 2-DE and nLC-MS/MS.

Spot number	Protein name	Accession number	MW (kDa)/pI	Matched unique peptides	Sequence coverage (%)	IOD ratio (CWP/CON)
6111	Insulin-like growth factor II	P01344	20.1/6.4	2	13.9	24.4
5807	Alpha-2-macroglobulin	P01023	163.3/6.0	20	16.9	0.26
7404	Alpha-enolase	P06733	47.2/7.0	18	46.1	0.28
5113	Beta-2-microglobulin	P61769	13.7/6.1	5	31.1	2.23
4314	Transthyretin	P02766	15.9/5.3	8	72.8	0.19
1108	Beta-2-microglobulin form pI 5.3	P61769	13.7/5.3	2	16.8	0.33
4702	Neuronal cell adhesion molecule	Q92823	143.9/5.3	20	16.9	0.36
6401	Pigment epithelium-derived factor	P36955	46.3/5.9	6	16.3	0.68
6403	Beta-1,4-glucuronyltransferase I	O43505	47.1/6.8	5	11.8	0.56
7307	Malate dehydrogenase, cytoplasmic	P40925	36.4/6.9	12	35	0.59
0501	Dickkopf-related protein 3	Q9UBP4	38.4/4.5	6	21.4	0.42
2801	Voltage-dependent calcium channel subunit alpha-2/delta-1	P54289	124.6/5.1	27	25.6	0.15
8111	Prostaglandin-H2 d-isomerase	P41222	21.0/8.4	4	31.1	0.44
3605	Alpha-1B-glycoprotein	P04217	54.2/75.6	14	48.1	1.75
6509	Beta-2-glycoprotein I	P02749	38.3/8.4	8	27.8	3.13
3804	Contactin-I	Q12860	113.3/5.6	27	35.2	0.43
3716	Neuronal cell adhesion molecule	Q92823	143.9/5.3	24	23.8	0.35
7408	Procollagen C-endopeptidase enhancer I	Q15113	48.0/7.5	11	30.5	0.65
3715	Vitamin D-binding protein	P02774	53.0/5.2	20	45.1	0.32
4710	Gelsolin	P06396	85.7/5.7	22	23	0.09
3209	Apolipoprotein A-I	P02647	30.8/5.4	19	61.8	0.48
3407	Apolipoprotein A-IV	P06727	45.4/5.2	19	55.3	2.81
3805	Contactin-I	Q12860	113.3/5.6	31	38.1	0.39
1712	Amyloid-like protein I	P51693	72.2/5.4	20	34.9	0.19
8215	Complement component C8 gamma chain	P07360	22.3/8.5	5	33.2	0.73
5511	Beta-2-glycoprotein I	P02749	38.3/8.4	9	34.2	1.91
3417	Haptoglobin	P00738	45.2/6.1	12	27.6	2.45
3314	Apolipoprotein E	P02649	36.1/5.5	14	46.1	0.43
1713	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	Q13822	99.0/6.6	21	25.7	0.34
4704	Neural cell adhesion molecule 2	O15394	93.0/5.4	16	20.9	0.32
6208	Metalloproteinase inhibitor 2	P16035	24.4/6.5	6	20.9	0.55
8216	Prostaglandin-H2 D-isomerase	P41222	21.0/8.4	4	31.1	0.30
3708	Alpha-1B-glycoprotein	P04217	54.2/5.6	8	20.4	0.17
4706	Contactin-I	Q12860	113.3/5.6	19	23.8	0.56
4108	Beta-2-microglobulin form pI 5.3	P61769	13.7/5.3	2	16.8	2.13
3608	Hemopexin	P02790	51.7/6.4	9	30.3	1.90
1111	Prosaposin; Saposin-A; Saposin-B-Val; Saposin-B; Saposin-C; Saposin-D fragment	P07602	58.0/5.0	5	7.1	1.13
2401	Clusterin precursor	P10909	52.4/5.9	8	21.4	1.89
5114	Acyl-CoA-binding protein	P07108	10.0/6.1	3	49.4	1.41
5403	Neuronal pentraxin-I precursor	Q15818	47.1/5.8	15	30.6	2.32
4309	Apolipoprotein E	P02649	36.1/5.5	20	60.3	0.26
6206	Prostaglandin-H2 D-isomerase	P41222	21.0/8.4	4	24.7	0.22
4806	Alpha-2-macroglobulin precursor	P01023	163.3/6.0	47	37.9	0.27
7405	Fructose-bisphosphate aldolase C	P09972	39.4/6.5	14	41.2	0.68
1118	Proprotein convertase I inhibitor	Q9UHG2	27.4/5.6	6	29.6	0.35
8703	Plasminogen precursor	P00747	90.6/7.1	21	33	0.06
2203	Prostaglandin-H2 D-isomerase	P41222	21.0/8.4	5	24.7	1.48
8402	Procollagen C-endopeptidase enhancer I	Q15113	48.0/7.5	15	43.9	0.26

Note: The spot numbers refer to the marked spots in Figure 3.

Abbreviations: MW, molecular weight; IOD, integrated optical density; CWP, chronic widespread pain; CON, healthy controls; 2-DE, two dimensional gel electrophoresis; nLC-MS/MS, nano liquid chromatography tandem mass spectrometry.

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