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ORIGINAL RESEARCH

Systemic alterations in plasma proteins from women with chronic widespread pain compared to healthy controls: a proteomic study

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Abstract: Chronic widespread pain (CWP) is a complex pain condition that is difficult to treat. The prevalence of CWP approximates ~10% of the general population, with higher prevalence in women. Lack of understanding of molecular mechanisms has been a challenge for diagnosis and treatment of chronic pain. The aim of this study was to explore the systemic protein changes in CWP compared to those in healthy controls (CON). By applying 2-dimensional gel electrophoresis, we analyzed the protein pattern of plasma samples from women with CWP (n=16) and healthy women (n=23). The proteomic data were analyzed using multivariate statistical models, and altered proteins were identified using mass spectrometry. The proteome analysis was further validated by gel-free Western blot. Multivariate statistical data analysis of quantified proteins revealed 22 altered proteins in women with CWP, compared to CON group. Many of the identified proteins are previously known to be involved in different parts of the complement system and metabolic and inflammatory processes, e.g., complement factor B, vitamin D-binding protein, ceruloplasmin, transthyretin and alpha-2-HS-glycoprotein. These results indicate that important systemic protein differences exist between women with CWP and healthy women. Further, this study illustrates the potential use of proteomics to detect biomarkers that may provide new insights into the molecular mechanism(s) of chronic pain. However, further larger investigations are required in order to confirm these findings before it will be possible to identify proteins as potential pain biomarkers for clinical use.

Keywords: inflammation, biomarker, painomics, complement system, GC protein

Introduction

Chronic widespread pain (CWP) is a complex pain condition that is difficult to treat. The prevalence of CWP in the European population is ~10%,[1–3] whereof 2–4% is further diagnosed with fibromyalgia syndrome (FMS).[4–6] According to the American College of Rheumatology (ACR) criteria from 1990, CWP is defined as pain present in at least 3 of 4 body quadrants, including axial skeleton pain, and persists for at least 3 months.[7] Individuals diagnosed with CWP/FMS often experience tiredness, sleeping disorders and other psychological and somatic disorders, e.g., irritable bowel syndrome and stiffness in muscle and joints.[1,8–11] There is evidence that CWP/FMS is more prominent among women with increased age, obesity, depression and low physical activity.[4,12]

Central alterations in the pain matrix in the brain and central hyperexcitability are known to be part of the underlying mechanism in CWP/FMS.[13–15] There are several studies suggesting that peripheral factors and alterations could have a prominent role and therefore contribute to the maintenance of pain and increased pain sensitivity in

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CWP/FMS, e.g., alterations in muscle[16–19] and peripheral nociceptors.[20–22] However, the molecular mechanisms behind CWP are not fully elucidated.

Applying proteomics as an approach to study new biomarkers in different body tissues, from individuals with different diseases, has been used in several studies. The proteome of serum from rheumatoid arthritis (RA) patients,[23] cerebrospinal fluid (CSF) from patients with neuropathic pain[24] and the interstitial fluid of the trapezius muscle from patients with CWP/FMS[25] have been investigated. A preliminary proteomic study of human serum from FMS patients found upregulated levels of 3 proteins, α1-antitrypsin, transthyretin and retinol-binding protein 4, compared to healthy controls (CON).[26] Further, the metabolic profile in serum from women with localized and widespread pain has recently been investigated, suggesting systemic changes in processes related to lipid metabolism and energy consumption.[27]

We have previously performed proteomics on muscle biopsies from patients with CWP/FMS and found protein alterations involved in metabolic pathways, inflammation and muscle recovery.[28] Further, the pain intensity and pressure pain threshold correlated significantly with specific proteomic patterns.[29] In this study, from the same cohort, we aimed to analyze the protein pattern, and potential new biomarkers, in plasma sample from female CWP/FMS patients compared to healthy CON.

Materials and methods
Subjects
The recruitment process, including the inclusion and exclusion criteria for the patients with CWP and healthy CON, has been described in detail previously.[30] Briefly, according to the exclusion criteria, none of the included participants in either group used any type of anticoagulatory, opioid or steroidal medication. Further, if a medical history record of bursitis, tendonitis, capsulitis, postoperative conditions in the neck/shoulder area, previous neck trauma, disorder of the spine, neurological disease, RA or any other systemic diseases, metabolic disease, malignancy, severe psychiatric illness or pregnancy or difficulties understanding the Swedish languages were present, participants were excluded from the study. The healthy CON group was recruited through local newspaper advertisement and consisted of 23 women aged between 20 and 65 years. Female patients with CWP were recruited to this project by either being among former patients with CWP at the Pain and Rehabilitation Centre of the University Hospital, Linköping, Sweden, or from an organization for FMS patients. The ACR criteria from 1990

were used for classification of FMS.[7] The recruitment process resulted in 19 CWP patients. As it has been reported previously[30] based on the concentration of lactate using power and sample size calculation (a=0.05, power=0.8), it was found that 17 subjects in each group were needed.

To confirm the individual eligibility, all participants (CON and CWP) received a standardized clinical examination. The examination was followed by a health questionnaire concerning chronic pain,[30] which included questions about pain intensity in the neck–shoulder and low back regions using numeric rating scale (NRS, 0=no pain and 10=worst possible pain), anxiety and depressive symptoms (Hospital Anxiety and Depression Scale [HADS; cutoff values for both subscales >10]), catastrophizing (Pain Catastrophizing Scale [PCS]; cutoff >37) and quality of life (QoL).

All participants signed a written consent before the start of the study, after receiving verbal and written information about the objectives and procedures of the study. The study was approved by the Regional Ethical Review Board in Linköping, Sweden (Dnr: M10–08, M233–09, Dnr: 2010/164–32) and followed the guidelines according to the Declaration of Helsinki. All methods were carried out in accordance with the approved ethical application.

Sample collection
The participants were asked not to take nonsteroidal anti-inflammatory drugs for 7 days and/or paracetamol medication for 12 hours prior to the sampling. Venous blood samples were collected in EDTA vacutainer and centrifuged at 1000× g for 15 minutes, plasma was collected, aliquoted and stored at −70°C until use. All samples were blinded before analysis. There were some difficulties with blood sampling for 2 subjects, and the sample from 1 subject was not sufficient for further proteomic analysis. Therefore, plasma samples from 16 women with CWP were included in this study.

Proteomics
The procedure for 2-dimensional gel electrophoresis (2-DE) has been described in previous studies.[24,28] Briefly, 40 μL plasma sample from each subject was depleted of albumin and immunoglobulin G (IgG) using ProteoPrep (Sigma-Aldrich Co, St Louis, MO, USA), followed by protein concentration measurement using 2D-Quant Kit (GE Healthcare, Little Chalfont, UK). Samples were further desalted with PD-10 columns (GE Healthcare) and lyophilized prior to use in the first dimension. Lyophilized proteins were resolved in 2-DE urea sample buffer according to Gorg et al,[31] and 100 μg of total protein from each subject was applied in the

first dimension and further run in second dimension using Ettan™ DALTsix Electrophoresis unit (Amersham, Pharmacia Biotech, Uppsala, Sweden).[24,28]

Separated proteins were fluorescently stained with SYPRO Ruby® (Bio-Rad Laboratories, Hercules, CA, USA), and gels were visualized using a charged coupled device (CCD) camera (VersaDoc™ Imaging system 4000 MP; Bio-Rad Laboratories). 2-DE protein patterns were analyzed and quantified using software PDQuest Advanced version 8.0.1 (Bio-Rad Laboratories). Protein spots of interest were excised from the gel and, after tryptic digestion, were analyzed by mass spectrometry using ultrafleXtreme™ matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF; Bruker Daltronik GmbH, Bremen, Germany). Database search was performed in ProteinProspector MS-Fit version 5.14.4 including Swiss-Prot database version 2015.3.5 as described in previous studies.[28,32]

Immunological analysis of vitamin D-binding protein (VDBP)

Plasma samples were analyzed using Peggy Simple Western size assay (ProteinSimple, Santa Clara, CA, USA) according to the user manual and as described previously.[33] Briefly, plasma samples were mixed with master mix containing 80 mM dithiothreitol and fluorescent molecular markers and heated at 95°C for 5 minutes. The samples, blocking reagents, primary antibody, secondary antibody and chemiluminescence substrate were loaded onto a 384-well plate according to the user manual. The primary antibody (VDBP, monoclonal mouse anti-human, LS-B3318; Nordic BioSite, Täby, Sweden) was diluted in antibody diluent in a 1:50 dilution. The chemiluminescence signals were digitized using a CCD camera. The digital images were analyzed with Compass software version 2.7.1 (ProteinSimple). To evaluate the concentration of VDBP in plasma, a 6-point standard curve, consisting of recombinant VDBP (Nordic BioSite), was run simultaneously with the plasma samples. The concentration of VDBP was calculated from the standard curve (R^2=0.99), and the data are presented as median ± min–max µg/µL.

Vitamin D (25-hydroxyvitamin D) concentration in plasma

Analysis of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 was performed with high-performance liquid chromatography (HPLC) according to a method previously described.[34] The HPLC system consisted of a JASCO PU-2089 pump and a JASCO UV-975 detector set on the wavelength 265 nm (both from Japan Spectroscopic Company, Tokyo, Japan). The

column was a Grace Smart RP 18 (100 × 2.1 mm, 3 µm), and the mobile phase consisted of methanol:water (80:20, v/v) and the flow rate was 0.4 mL/min. Twenty microliters of the samples were injected, and the concentration was determined based on a standard curve that was prepared by an in-house reference. Standards of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Together with standards with known concentration, a reference was analyzed together with the samples. The inter-assay and intra-assay coefficient of variation was 3.9% and 5.7%, respectively. As a quality control, we used plasma references from Vitamin D External Quality Control Scheme (DEQAS; http://www.deqas.org). Because sampling was performed on different occasions over the year, the concentrations of vitamin D were adjusted for what time of the year it was drawn against a standard curve, obtained from results of a British study of 7437 individuals.[35]

Statistics

All statistics regarding the anthropometric, vitamin D and VDBP data were calculated in IBM SPSS version 23 (IBM Corporation, Armonk, NY, USA). For normally distributed data, a Student t-test was applied, and for non-normally distributed data, a Mann–Whitney U test was applied. $p \leq 0.05$ was considered significant. Data are presented as mean values ± 1 standard deviation (SD) if not stated otherwise.

Multivariate correlation between membership of groups and quantified proteins were analyzed with orthogonal partial least square discriminant analysis (OPLS-DA) using SIMCA-P+ version 13.0 (UMETRICS, Umeå, Sweden). The procedure to compute multivariate correlation models has been described earlier[24,28] and is in accordance with Wheelock and Wheelock.[36] Briefly, prior to OPLS-DA analysis, a principle component analysis (PCA) was created to control for multiple outliers. No detectable multivariate outliers were identified in this study. The OPLS-DA model contains data variables (quantified proteomic data) that were mean centered and scaled for unified variance (UV-scaled). The variable influence of projection (VIP value) is a value that describes the importance and relevance of each X variable (quantified proteins), pooled over all dimensions, and Y variables (CWP or CON) that include the group of variables that best explain Y. In this study, a VIP>1.0 with a jacked-knifed 95% confidence interval was considered significant as reported in previous study.[28] The OPLS-DA model was performed in a 2-step analysis. First, a pre-model was built including all proteins. From this model, proteins with a VIP value>1.0 including the

jacked-knifed 95% confidence interval, not including zero, were further used in a new regression model. 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. To validate the model, we used cross-validated analysis of variance (CV-ANOVA), which was considered of significant importance when $p<0.05$.

Results

Clinical and anthropometric data

In total, plasma samples were retrieved from 16 CWP patients (14 of 16 were diagnosed with FMS) and 23 healthy women (CON). No significant differences were found between the groups regarding age, height, weight, body mass index (BMI) or pain duration. As expected, CWP had higher pain intensities than CON for neck, shoulder and low back (Table 1). The CWP group reported significant higher HADS scores for the anxiety and depression subscales compared to CON, but on group level, these values were below the cutoffs indicating clinically depressive or anxiety states. No significant differences existed in PCS or QoL (Table 1).

Multivariate regression analysis to assess group belonging

To identify systemic differences in plasma between CWP and CON, a proteomic approach was used in combination with PCA and OPLS-DA models. In total, 414 ± 21 (CWP: 423 ± 20, CON: 408 ± 20) protein spots from each gel were detected in the 2-DE analysis. Out of these proteins, 325 protein spots in each plasma gel were further quantified, matched and initially analyzed with an unsupervised PCA model. No detectable strong, or moderate, outliers were identified in either group in this study. Further, an OPLS-DA model was applied consisting of 1 predictive and 1 orthogonal component. The model showed a clear separation between groups with a good fit (R^2=0.84) and predictivity (Q^2=0.60) and presented a significant CV-ANOVA, p=2.31e^{-06} (Figure 1).

Altered proteins

Twenty-two proteins, including several different isoforms, had a VIP value >1.0 and were considered to be important for group belongings and were significantly altered in the CWP group compared to CON. The identified proteins were divided into 4 classes according to their involvement

Table 1 Anthropometric data and data from the health questionnaire

Variables	CON (n=23)	CWP (n=16)	Statistics (*p*-value)
Age (years)	41.0 ± 10.2	47.4 ± 11.3	0.915[a]
Height (cm)	168.7 ± 7.3	167.3 ± 4.9	0.060[a]
Weight (kg)	68.7 ± 11.2	72.4 ± 15.4	0.898[b]
BMI (kg/m²)	24.0 ± 2.8	25.8 ± 4.9	0.361[b]
Pain duration (years)	NA	12.5 ± 8.3	NA
Pain intensity the last 7 days			
Neck (NRS)	0.1 ± 0.4	5.8 ± 2.4	<0.001*,[a]
Shoulders (NRS)	0.0 ± 0.0	5.9 ± 1.9	<0.001*,[a]
Low back (NRS)	0.0 ± 0.0	5.6 ± 2.2	<0.001*,[b]
HADS-Anxiety	2.3 ± 2.2	7.5 ± 3.4	<0.001*,[b]
HADS-Depression	1.2 ± 1.6	5.8 ± 3.6	<0.001*,[b]
PCS	6.5 ± 6.6	12.5 ± 6.9	0.982[a]
QoL	93.0 ± 9.9	86.6 ± 13.6	0.209[a]
Also diagnosed with FMS (n)	0	14	NA

Notes: Age and anthropometric data of included subjects with CWP (n=16) and CON (n=23). The health questionnaire included questions concerning pain duration, pain intensity the last 7 days, depression, anxiety, catastrophizing and QoL. For the variables are reported mean ± 1 SD. The results from the statistical analysis between the 2 groups are presented in the column furthest to the right. [a]Statistical test used: Student's *t*-test. [b]Statistical test used: Mann–Whitney U test. *Denotes statistical significance.
Abbreviations: CON, controls; CWP, chronic widespread pain; BMI, body mass index; NRS, numeric rating scale; HADS-Anxiety, Hospital Anxiety and Depression Scale – Subscale Anxiety; HADS-Depression, Hospital Anxiety and Depression Scale – Subscale Depression; PCS, Pain Catastrophizing Scale – Total Score; QoL, quality of life; NA, not applicable; FMS, fibromyalgia syndrome.

in different biological processes found in Uniprot Database (uniprot.org), as follows: iron ion hemostasis and immunity, metabolic and inflammatory processes (Figure 2; Table 2).

Two proteins belonged to cellular iron ion hemostasis (ceruloplasmin [CERU], 9 upregulated isoforms; serotransferrin, 1 downregulated isoform). Eight proteins belonged to immunity process/complement activation (complement C1r subcomponent, 6 upregulated isoforms; complement factor B, 1 upregulated isoform; complement C1s subcomponent, 1 upregulated isoform; Ig alpha-2 chain C region, 1 downregulated isoform; complement C3 alpha chain fragment 2, 1 upregulated isoform; complement factor I light chain, 1 upregulated isoform; ficolin-3, 1 upregulated isoform, Ig kappa chain C region, 1 upregulated isoform and 1 downregulated). Eight proteins belonged to metabolic processes (gelsolin, 2 upregulated isoforms; plasminogen, 6 upregulated isoforms; coagulation factor XIII B chain, 1 upregulated isoform; prothrombin, 1 upregulated isoform; alpha-2-antiplasmin, 2 upregulated isoforms; fibrinogen gamma chain, 3 upregulated isoforms; apolipoprotein A-I, 1 downregulated isoform; transthyretin, 1 downregulated isoform). Three proteins belonged to inflammatory response (VDBP, 3 upregulated isoforms; alpha-2-HS-glycoprotein, 1 upregulated isoform; haptoglobin beta chain, 1 upregulated
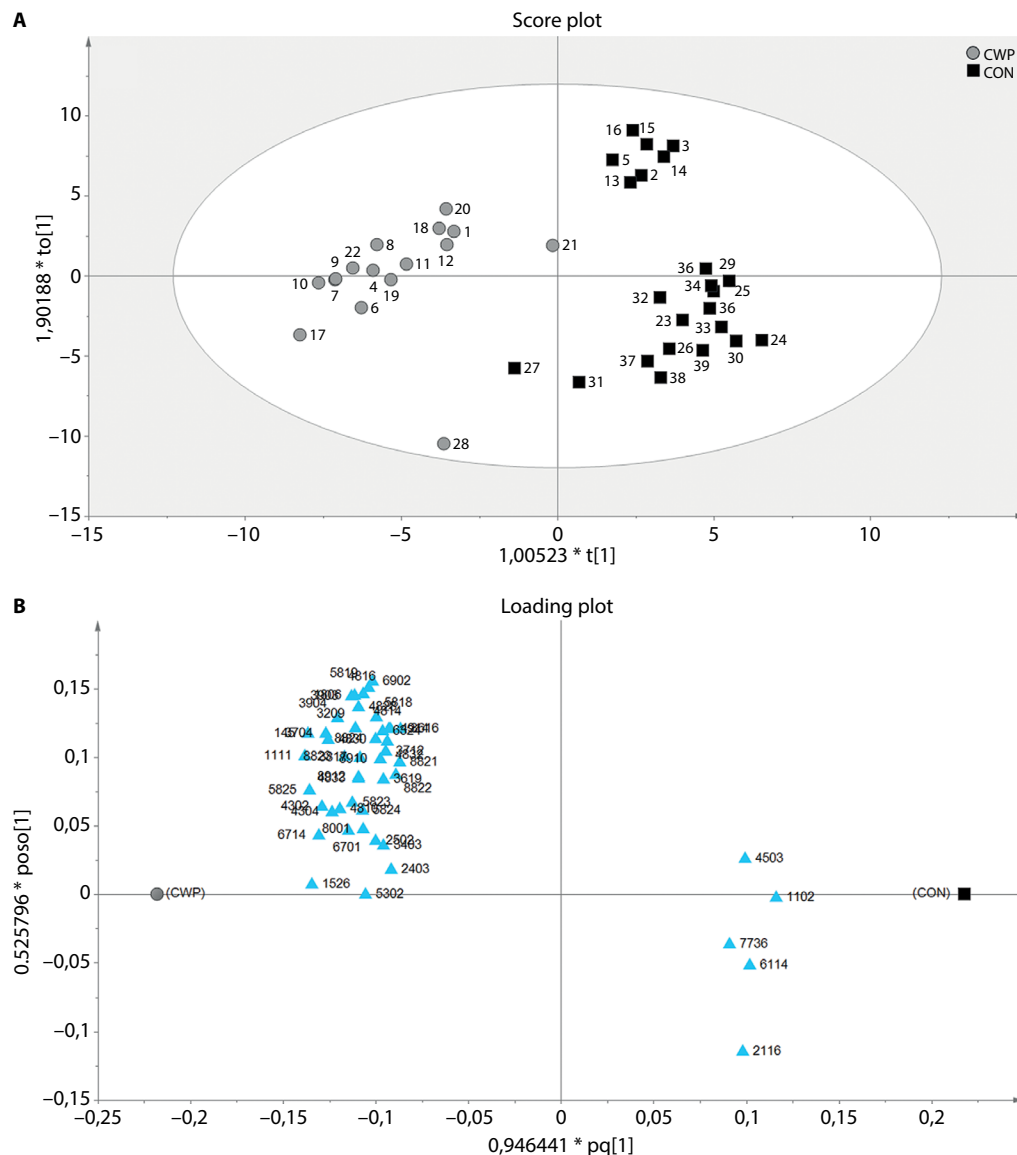
Figure 1 OPLS-DA model of quantified proteins from plasma.
Notes: (**A**) A score plot of CWP compared to CON, visualizing clear separation between the 2 included groups. (**B**) Loading plot corresponding to significant proteins, with a VIP value >1 (triangles), between CWP and CON. The parameters for the models were R^2=0.84, Q^2=0.60, CV-ANOVA, *p*-value=2.31e^{-06}
Abbreviations: OPLS-DA, orthogonal partial least square discriminant analysis; CWP, chronic widespread pain; CON, controls; VIP, variable influence of projection; CV-ANOVA, cross-validated analysis of variance.

isoform) and 1 unidentified protein was upregulated. A more detailed table over the mass spectrometry result is shown in Table S1.

VDBP and vitamin D concentration

The VDBP result from 2-DE was further verified using gel-free Western blot. The VDBP concentration in plasma samples from CWP (n=14) and CON (n=22) was analyzed, and significant elevated concentrations of VDBP were found in CWP group compared to CON (CWP=162.4 ± 98.2–309.8 ng/μL; CON=138.8 ± 89.3–214.3 ng/μL, *p*=0.034; Figure 3). Additionally, significant higher concentration of vitamin D (25-hydroxyvitamin D) in plasma was found in

CWP group compared to CON (CWP=67.7 ± 18.8 nmol/L; CON=48.5 ± 16.8 nmol/L, *p*=0.002).

Discussion

In this novel study, significant differences were found between CWP and CON in several plasma proteins belonging to inflammatory, immunity, and metabolic processes using proteomics in combination with multivariate data analysis.

There are limited studies that have been conducted exploring systemic changes of plasma proteins in CWP. Balfoussia et al[37] investigated the plasma proteome of male elite runners before and after performing a running race of 246 km within 36 hours. They found several proteins that
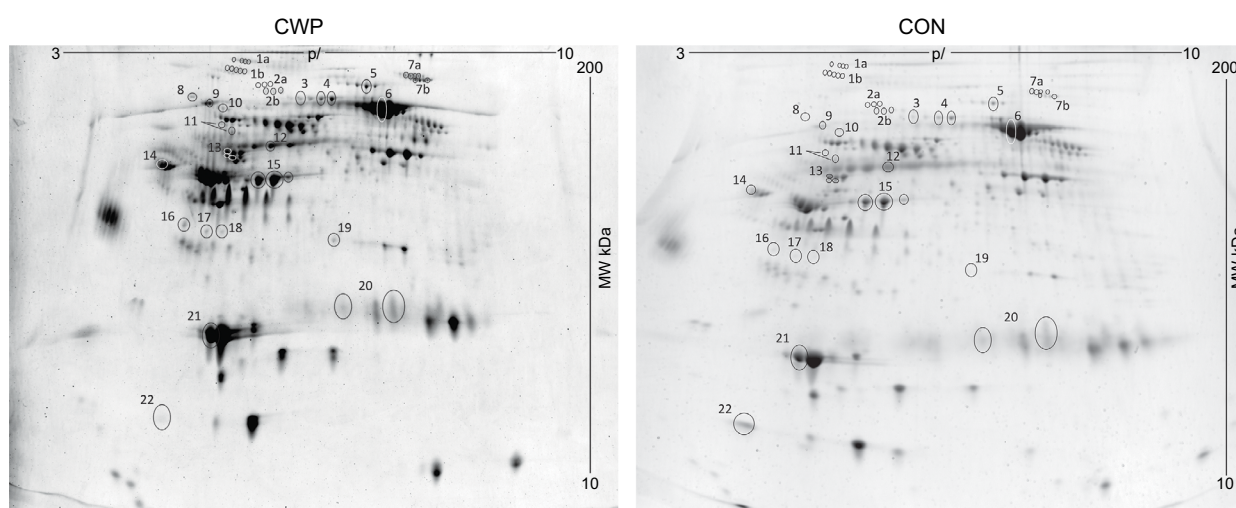
Figure 2 A representative SYPRO Ruby® stained 2-DE gel of the plasma proteome from one woman with CWP (right) and one healthy CON (left).
Notes: A total of 22 proteins (marked, numbered spots), including different isoforms, had a VIP value >1, were highly significant for the model, and were further identified.
Abbreviations: 2-DE, 2-dimensional gel electrophoresis; CWP, chronic widespread pain; CON, controls; VIP, variable influence of projection; MW, molecular weight; p*I*, isoelectric point.

are similar in significance and alteration as in our study, e.g., upregulated levels of complement factor B and VDBP and downregulated levels of transthyretin, Ig kappa chain C region and apolipoprotein A-I.[37] In a recent plasma proteomic study of male farmers with musculoskeletal disorders from our group,[38] increased levels of complement factor B, haptoglobin and serotransferrin and decreased levels of alpha-2-HS-glycoprotein, VDBP and apolipoprotein A-1 were found, suggesting that these farmers may be subjected to systemic inflammation. In this study, the same proteins were found significant, however, differentially altered, e.g., increased levels of alpha-2-HS-glycoprotein and VDBP and decreased levels of serotransferrin. It is interesting to reflect upon that both studies share the same type of altered proteins even though they are differently expressed. This could be due to different pain disorders, gender or type of environmental exposures. In contrast to our results, significantly decreased levels of VDBP and fibrinogen gamma chain among others have been reported in serum from patients with carpal tunnel syndrome.[39] All the abovementioned studies have one thing in common, ie, the altered proteins are mainly involved in inflammation, either by themselves or through activation of different pathways of the complement system or metabolic processes.[37–39]

Regarding our study, reflecting plasma protein changes in women with CWP, a vast amount of the proteins belong to different parts of the complement system. The complement system is characterized by a cascade of proteolytic cleavages after recognition of different molecular patterns

from mainly microorganisms, impaired host cells or other molecules, leading to production of different components mainly regarded as pro-inflammatory proteins, through the recruitment of different immune cells.[40,41] Six proteins were significantly upregulated and belong to the complement system activation, through either of the 3 pathways; the classical pathway (complement C1r subcomponent, C1s subcomponent, complement C3c alpha chain fragment 2, complement factor I light chain), alternative pathway (complement factor B) or the lectin pathway (ficolin-3). Complement C3c alpha chain fragment 2 (CO3), a component of C3, is one of the central proteins of both the classical and alternative pathways of the complement system.[40] After cleavage of CO3, pro-inflammatory fragments of C3a are produced leading to activation of several different cell types, mainly leukocytes, as well as increased influx of Ca^{2+} in these cells that induces an inflammatory response.[42] Furthermore, complement factor B has been shown to be involved in muscle inflammation.[41] Since a majority of the proteins involved in the complement and coagulation cascade in this study were significantly upregulated and were among the ones most important for group separation with highest VIP values, it is tempting to speculate that an increased activity of these proteins leads to a larger production and activation of pro-inflammatory substances that contribute to an overall increased inflammation in the muscles of chronic pain patients.

Several isoforms of CERU were found upregulated in CWP (2 isoforms were among the ones with highest VIP

Table 2 Identified proteins in plasma

Protein number	MVDA number	Protein name	Accession number	Biological process	VIP	OD CWP (mean ± SD)	OD CON (mean ± SD)	OD ratio (CWP/ CON)	Alteration CWP vs. CON
1a	3817	Ceruloplasmin	P00450	Iron ion hemostasis	1.12	178 ± 157	62 ± 103	2.89	↑
	4830				1.34	355 ± 243	98 ± 197	3.61	↑
	4832				1.18	268 ± 164	121 ± 181	2.21	↑
	4833				1.33	233 ± 164	81 ± 109	2.86	↑
1b	4816	Ceruloplasmin	P00450	Iron ion hemostasis	1.25	204 ± 123	67 ± 87	3.07	↑
	4828				1.18	301 ± 236	97 ± 109	3.11	↑
	3903				1.26	382 ± 266	119 ± 162	3.21	↑
	3904				1.24	315 ± 151	132 ± 153	2.38	↑
	4814				1.07	335 ± 270	145 ± 169	2.31	↑
2a	4914	Complement C1r subcomponent	P00736	Immunity	1.21	111 ± 99	28 ± 45	4.03	↑
	5818				1.11	213 ± 181	59 ± 94	3.61	↑
	5819				1.24	233 ± 153	81 ± 110	2.89	↑
2b	4810	Complement C1r subcomponent	P00736	Immunity	1.23	563 ± 232	247 ± 174	2.28	↑
	5825				1.44	488 ± 186	246 ± 144	1.98	↑
	5824				1.07	277 ± 125	162 ± 94	1.70	↑
3	5823	Coagulation factor XIII B chain	P05160	Metabolic	1.19	699 ± 353	384 ± 199	1.82	↑
4	6701	Gelsolin	P06396	Metabolic	1.06	1506 ± 463	1065 ± 442	1.41	↑
	6714				1.28	2192 ± 582	1569 ± 426	1.40	↑
5	6902	Complement factor B	P00751	Immunity	1.08	2226 ± 507	1605 ± 690	1.39	↑
6	7736	Serotransferrin	P02787	Iron ion hemostasis	1.06	42780 ± 10036	54298 ± 12906	0.79	↓
7a	8910	Plasminogen	P00747	Metabolic	1.16	773 ± 340	463 ± 237	1.67	↑
	8912				1.35	718 ± 286	392 ± 205	1.83	↑
	8823				1.32	683 ± 331	319 ± 211	2.14	↑
	8824				1.23	525 ± 255	239 ± 150	2.20	↑
7b	8822	Plasminogen	P00747	Metabolic	1.08	739 ± 327	430 ± 292	1.72	↑
	8821				1.00	773 ± 326	460 ± 352	1.68	↑
8	1806	Complement C1s subcomponent	P09871	Immunity	1.21	628 ± 347	259 ± 355	2.42	↑
9	3704	Prothrombin	P00734	Metabolic	1.36	3602 ± 1269	1752 ± 1410	2.06	↑
10	3712	Unidentified	–	–	1.07	369 ± 161	225 ± 132	1.64	↑
11	3616	Alpha-2-antiplasmin	P08697	Metabolic	1.01	220 ± 156	94 ± 100	2.35	↑
	3619				1.07	822 ± 760	414 ± 620	1.99	↑
12	4503	Ig alpha-2 chain C region	P01877	Immunity	1.20	6583 ± 3797	10969 ± 4475	0.60	↓
13	2502	Vitamin D-binding protein	P02774	Inflammatory	1.03	3906 ± 2507	1946 ± 1240	2.01	↑
	2403				1.17	535 ± 249	248 ± 170	2.16	↑
	3403				1.18	738 ± 272	453 ± 242	1.63	↑
14	1526	Alpha-2-HS-glycoprotein	P02765	Inflammatory	1.52	1324 ± 332	862 ± 247	1.54	↑
15	4302	Fibrinogen gamma chain	P02679	Metabolic	1.28	17320 ± 5749	11371 ± 3908	1.52	↑
	4304				1.28	25712 ± 8442	16721 ± 6137	1.54	↑
	5302				1.09	4382 ± 2210	2441 ± 1696	1.80	↑
16	145	Complement C3c alpha chain fragment 2	P01024	Immunity	1.56	871 ± 301	360 ± 299	2.42	↑
17	1111	Complement factor I light chain	P05156	Immunity	1.51	770 ± 328	293 ± 273	2.63	↑
18	3209	Haptoglobin beta chain	P00738	Inflammatory	1.10	390 ± 181	210 ± 171	1.86	↑
19	6324	Ficolin-3	O75636	Immunity	1.06	604 ± 330	313 ± 271	1.93	↑
20	6114	Ig kappa chain C region	P01834	Immunity	1.01	602 ± 867	1733 ± 2089	0.35	↓
	8001				1.02	18099 ± 12404	8460 ± 8529	2.14	↑
21	1102	Apolipoprotein A-I	P02647	Metabolic	1.07	15848 ± 5685	22160 ± 6806	0.72	↓
22	2116	Transthyretin	P02766	Metabolic	1.13	1218 ± 1348	3497 ± 2539	0.35	↓

Notes: For each protein, protein name, accession number (Uniprot), biological process (Uniprot), VIP values for each protein isoform, quantified OD for each group and OD ratio between CWP and CON are displayed. The MVDA number refers to specific protein isoforms, and ranges from acidic to basic (p*I* 3–10). ↑=upregulated protein, ↓=downregulated protein.

Abbreviations: MVDA, multivariate data analysis; VIP, variable influence of projection; OD, optical density; CWP, chronic widespread pain; SD, standard deviation; CON, controls; p*I*, isoelectric point.
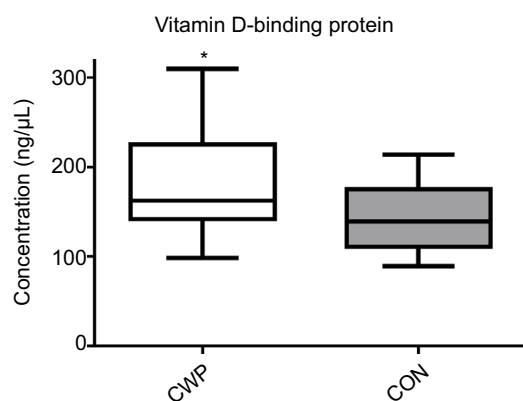
Figure 3 Concentration of VDBP in plasma for the 2 groups of subjects.
Note: Significantly elevated concentration of VDBP was found in CWP compared to CON (*p=0.0343).
Abbreviations: VDBP, vitamin D-binding protein; CWP, chronic widespread pain; CON, controls.

value). CERU is primarily produced in the liver and secreted out in the circulation and is known to be one of the larger copper-carrier proteins in the body. Arner et al[43] found elevated levels of CERU secreted from adipose cells and circulating levels in obese females compared to non-obese subjects. The detected isoforms of CERU in our study were found elevated. Although, there were no significant differences in weight or BMI in this cohort, it is generally known that one of the risk factors is obesity in females with CWP/FMS.[44,45] Several studies have also found a correlation between higher levels of CERU, haptoglobin and complement proteins in plasma and serum to be associated with major depressive disorders.[46–50] In this study, the CWP group reported statistically significant higher HADS scores (anxiety and depression) compared to CON. However, their HADS scores were <10, and they showed no clinical signs of depression or anxiety. Hence, the findings in this study are not primarily related to depression or anxiety in females with CWP and could therefore not explain the increased levels of these proteins in the CWP group compared to CON.

VDBP main function is as a carrier and transporter of vitamin D in the circulation. It also acts as an actin scavenger, forming complexes with G-actin and gelsolin, primarily by depolarization of free actin that is released upon injury to prevent damage to the microcirculation.[51] VDBP further works as a macrophage-activating factor (MAF), initiating macrophage activity.[52] In this study, both VDBP and gelsolin were upregulated in the CWP group. We also verified the 2-DE findings of VDBP with an additional method. So far, to our knowledge, no one has investigated the VDBP levels in plasma from CWP patients. However, it is more common to analyze vitamin D metabolites, and several studies have

found decreased levels in chronic low back pain[53] and FMS/CWP patients.[54,55] This is in conflict with our result, showing increased levels of both vitamin D and VDBP. The cause of elevated levels of VDBP in the CWP group is unknown; one theory could be physiological, since the levels of vitamin D is increased, this could follow with increased levels of VDBP, due to elevated transportation of the protein is required. Another theory could be VDBP's function as a cofactor, together with one of the by-products from the alternative and classical pathway of complement, c5a, which essentially needs VDBP to be able to work as chemoattractant for neutrophils.[56] One could speculate that since several proteins from the complement system are upregulated in the CWP group, it could possibly lead to elevated levels of complement by-products. Therefore, excessive levels of VDPB together with complement by-product, e.g., c5a, can lead to more recruitment of neutrophils and in turn activate and/or prolong an immune response. Additionally, studies have suggested that vitamin D supplement could be an alternative for decreased pain among women with CWP/FMS.[54,57,58] Hence, in our study, the higher levels of both vitamin D and VDBP cannot explain the reported pain in the CWP patients.

Finally, the coagulation system interacts with the complement system via thrombin activation of C5.[59] Coagulation factor XIII B chain, fibrinogen gamma chain and prothrombin are proteins involved in the coagulation/fibrinolytic system. In this study, these proteins were all upregulated in the CWP group compared to CON. It has been shown in several studies that patients suffering from chronic pain states have impaired microcirculation.[16,60,61] We have earlier in the same cohort also found significantly increased concentration of lactate and glutamate, in the interstitial muscle and plasma, in the CWP group compared to CON.[30] Impaired microcirculation in the peripheral tissue might affect the presence of circulating factors and cells in the muscle tissue in patients with chronic pain, which could be an explanation to the altered levels of proteins seen in the CWP group compared to CON.

This study demonstrates that there are systemic changes in women with chronic pain that might contribute to central nervous system alterations. However, one should keep in mind that this proteomic study only reflects the plasma proteome at one explicit time point, where precautions of its significance should be reflected upon. Further, the number of participants might be considered as low and should in future studies be increased although the number of subjects in this study compared to other proteomic studies reporting biomarker candidates for different pain conditions is relatively high.[39,62]

Conclusion

This study illustrates the potential use of proteomics for identifying systemic protein changes associated with CWP. We have found significantly altered levels of inflammatory, immunity, and metabolic proteins in plasma samples from women with CWP compared to healthy CON, suggesting that patients with CWP are subjected to a low-grade systemic inflammation. The identified differences in the plasma proteome in this study provide novel information about the biological mechanisms in CWP, which, together with earlier proteome studies in biopsies from the same cohort, might contribute to better understanding of the involved nociceptive and pain mechanisms in CWP.

Acknowledgments

Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Mass spectrometry and MVDA data from identified proteins with a VIP>1

Protein number	MVDA number	Protein name	Biological process	Accession number	MW/pI	MOWSE score	Number of peptides	% Cov.	VIP	OD CWP (mean ± SD)	OD CON (mean ± SD)	OD ratio (CWP/CON)	Alteration CWP Vs. CON
1a	3817	Ceruloplasmin	Iron ion hemostasis	P00450	122206/5.4	–	–	–	1.12	178 ± 157	62 ± 103	2.89	↑
	4830					92120	14	15.6	1.34	355 ± 243	98 ± 197	3.61	↑
	4832					–	–	–	1.18	268 ± 164	121 ± 181	2.21	↑
	4833					1.05e+6	10	12.5	1.33	233 ± 164	81 ± 109	2.86	↑
1b	4816	Ceruloplasmin	Iron ion hemostasis	P00450	122206/5.4	–	–	–	1.25	204 ± 123	67 ± 87	3.07	↑
	4828					–	–	–	1.18	301 ± 236	97 ± 109	3.11	↑
	3903					4.09e+10	17	20.2	1.26	382 ± 266	119 ± 162	3.21	↑
	3904					–	–	–	1.24	315 ± 151	132 ± 153	2.38	↑
	4814					1.81e+8	17	18.7	1.07	335 ± 270	145 ± 169	2.31	↑
2a	4914	Complement C1r subcomponent	Immunity	P00736	80119/5.8	–	–	–	1.21	111 ± 99	28 ± 45	4.03	↑
	5818					4.38	4	8.4	1.11	213 ± 181	59 ± 94	3.61	↑
	5819					822015	12	15.4	1.24	233 ± 153	81 ± 110	2.89	↑
2b	4810	Complement C1r subcomponent	Immunity	P00736	80119/5.8	2.98E+09	10	20.6	1.23	563 ± 232	247 ± 174	2.28	↑
	5825					1.13e+11	22	33.0	1.44	488 ± 186	246 ± 144	1.98	↑
	5824					1.71e+12	19	30.6	1.07	277 ± 125	162 ± 94	1.70	↑
3	5823	Coagulation factor XIII B chain	Metabolic	P05160	75511/6.0	5.13e+7	13	23.9	1.19	699 ± 353	384 ± 199	1.82	↑
4	6701	Gelsolin	Metabolic	P06396	85698/5.9	3.01e+11	21	37.5	1.06	1506 ± 463	1065 ± 442	1.41	↑
	6714					1.64e+12	23	40.4	1.28	2192 ± 582	1569 ± 426	1.40	↑
5	6902	Complement factor B	Immunity	P00751	85534/6.7	1.20e+16	30	49.7	1.08	2226 ± 507	1605 ± 690	1.39	↑
6	7736	Serotransferrin	Iron ion hemostasis	P02787	77065/6.8	7.39e+15	29	49.1	1.06	42780 ± 10036	54298 ± 12906	0.79	↓
7a	8910	Plasminogen	Metabolic	P00747	90570/7.0	9.09e+17	29	46.5	1.16	773 ± 340	463 ± 237	1.67	↑
	8912					–	–	–	1.35	718 ± 286	392 ± 205	1.83	↑
	8823					5.94e+12	22	30.6	1.32	683 ± 331	319 ± 211	2.14	↑
	8824					–	–	–	1.23	525 ± 255	239 ± 150	2.20	↑
7b	8822	Plasminogen	Metabolic	P00747	90570/7.0	6.00e+16	30	50.4	1.08	739 ± 327	430 ± 292	1.72	↑
	8821					–	–	–	1.00	773 ± 326	460 ± 352	1.68	↑
8	1806	Complement C1s subcomponent	Immunity	P09871	76685/4.9	169203	14	21.9	1.21	628 ± 347	259 ± 355	2.42	↑
9	3704	Prothrombin	Metabolic	P00734	70038/5.6	7.65e+12	26	58.2	1.36	3602 ± 1269	1752 ± 1410	2.06	↑
10	3712	Unidentified	–	–	–	–	–	–	1.07	369 ± 161	225 ± 132	1.64	↑
11	3616	Alpha-2-antiplasmin	Metabolic	P08697	54566/5.9	239508	11	30.8	1.01	220 ± 156	94 ± 100	2.35	↑
	3619					12596	12	28.9	1.07	822 ± 760	414 ± 620	1.99	↑
12	4503	Ig alpha-2 chain C region	Immunity	P01877	36527/5.7	481	5	18.8	1.20	6583 ± 3797	10969 ± 4475	0.60	↓

(Continued)

Table S1 (Continued)

Protein number	MVDA number	Protein name	Accession number	Biological process	MW/pI	MOWSE score	Number of peptides	% Cov.	VIP	OD CWP (mean ± SD)	OD CON (mean ± SD)	OD ratio (CWP/CON)	Alteration CWP Vs. CON
13	2502	Vitamin D-binding protein	P02774	Inflammatory	52964/5.4	1.22e+9	15	33.5	1.03	3906 ± 2507	1946 ± 1240	2.01	↑
	2403					3.03e+6	12	29.1	1.17	535 ± 249	248 ± 170	2.16	↑
	3403					1.21e+8	15	41.6	1.18	738 ± 272	453 ± 242	1.63	↑
14	1526	Alpha-2-HS-glycoprotein	P02765	Inflammatory	39325/5.4	7.78e+6	9	27.5	1.52	1324 ± 332	862 ± 247	1.54	↑
15	4302	Fibrinogen gamma chain	P02679	Metabolic	51512/5.4	3.84e+13	19	53.4	1.28	17320 ± 5749	11371 ± 3908	1.52	↑
	4304					1.14e+16	18	59.2	1.28	25712 ± 8442	16721 ± 6137	1.54	↑
	5302					6563	8	22.5	1.09	4382 ± 2210	2441 ± 1696	1.80	↑
16	145	Complement C3c alpha chain fragment 2	P01024	Immunity	39488/4.79	6.34e+7	15	56.0	1.56	871 ± 301	360 ± 299	2.42	↑
17	1111	Complement factor I light chain	P05156	Immunity	27592/6.24	1580	6	32.0	1.51	770 ± 328	293 ± 273	2.63	↑
18	3209	Haptoglobin beta chain	P00738	Inflammatory	27265/6.32	35609	8	34.7	1.10	390 ± 181	210 ± 171	1.86	↑
19	6324	Ficolin-3	O75636	Immunity	32903/6.2	31230	6	20.1	1.06	604 ± 330	313 ± 271	1.93	↑
20	6114	Ig kappa chain C region	P01834	Immunity	11609/5.6	1139	2	30.2	1.01	602 ± 867	1733 ± 2089	0.35	↓
	8001					1.69e+6	5	80.2	1.02	18099 ± 12404	8460 ± 8529	2.14	↑
21	1102	Apolipoprotein A-I	P02647	Metabolic	30778/5.6	2.39e+13	19	68.9	1.07	15848 ± 5685	22160 ± 6806	0.72	↓
22	2116	Transthyretin	P02766	Metabolic	15887/5.5	97.5	4	30.0	1.13	1218 ± 1348	3497 ± 2539	0.35	↓

Notes: ↑=upregulated protein. ↓=downregulated protein.
Abbreviations: MVDA, multivariate data analysis; MW, molecular weight; pI, isoelectric point; MOWSE, molecular weight search; Cov., coverage; VIP, variable influence of projection; OD, optical density; CWP, chronic widespread pain; SD, standard deviation; CON, controls.