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Plasma proteins from several components of the immune system differentiate chronic widespread pain patients from healthy controls – an exploratory case-control study combining targeted and non-targeted protein identification

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Abstract

Chronic widespread pain (CWP), including fibromyalgia (FM), is characterized by generalized musculoskeletal pain and hyperalgesia. Plasma proteins from proteomics (non-targeted) and from targeted inflammatory panels (cytokines/chemokines) differentiate CWP/FM from controls. The importance of proteins obtained from these two sources, the protein-protein association network, and the biological processes involved were investigated. Plasma proteins from women with CWP (n = 15) and CON (n = 23) were analyzed using two-dimensional gel electrophoresis analysis and a multiplex proximity extension assay for analysis of cytokines/chemokines. Associations between the proteins and group were multivarietly analyzed. The protein-protein association network and the biological processes according to the Gene Ontology were investigated. Proteins from both sources were important for group differentiation; the majority from the two-dimensional gel electrophoresis analysis. 58 proteins significantly differentiated the two groups (R^2 = 0.83). A significantly enriched network was found; biological processes were acute phase response, complement activation, and innate immune response. As with other studies, this study shows that plasma proteins can differentiate CWP from healthy subjects. Focusing on cytokines/chemokines is not sufficient to grasp the peripheral biological processes that maintain CWP/FM since our results show that other components of the immune and inflammation systems are also highly significant.

Abbreviations: 2-DE = two-dimensional gel electrophoresis, ACR = American College of Rheumatology, AHSG = alpha-2-HS-glycoprotein, BDNF = brain-derived neurotrophic factor, BMI = body mass index, CON = control, CV-ANOVA = cross validated analysis of variance, CWP = chronic widespread pain, FM = fibromyalgia, GO = gene ontology, IgG = immunoglobulin G, IL = interleukin, JAK/STAT = Janus kinase-signal transducer and activator of transcription pathway, LIFR = leukaemia inhibitory factor receptor, MAPK = mitogen-activated protein kinase signaling pathway, MVDA = multivariate data analysis, NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells pathway, NGF = nerve growth factor, OPLS-DA = Orthogonal Partial Least Squares discriminant analysis, PANTHER = Protein ANalysis THrough Evolutionary Relationships, PEA = multiplex proximity extension assay, PROM = Patient Reported Outcome Measures, Q^2 = goodness of prediction, R^2 = goodness of fit, SIRT2 = NAD-dependent protein deacetylase sirtuin-2, SRs = systematic reviews, STRING = Search Tool for Retrieval of Interacting Genes/Proteins, UV = unit variance, VIPpred = variable influence on projection (predictive) value.

Keywords: 2-DE, biomarker, chemokine, cytokine, fibromyalgia, immune, inflammation, proteomics, widespread pain

1. Introduction

Chronic widespread pain (CWP), including fibromyalgia (FM), has a high population prevalence (5%–10%) with a female predominance.[1–3] CWP is often associated with depressive and anxiety symptoms (e.g., insomnia) as well as their consequences such as personal suffering and high socio-economic costs.[4–6] Nociplastic pain is a new pain mechanistic descriptor (IASP definition: "Pain that arises from altered nociception despite no clear evidence of actual or threatened

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tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain." Source: https://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698#Nociplasticpain), which classifies FM (a subgroup of CWP) as a nociplastic pain condition.[7]

Clinical management, including interventions, are hampered by lack of valid biomarkers.[8,9] Omic methods are increasingly applied in pain research as emerging insights indicate that pain involves complex patterns of molecular changes.[10] The proteome of a tissue regulates biological processes and integrates the effects of genes with, for example, age, behaviors, comorbidities, drugs, and environmental factors.[10–12] In 2015, we reported significant differences in the trapezius muscle proteome between CWP (mainly FM) and controls.[13] Later, we and others have found significant differences in plasma/serum proteome between CWP/FM and healthy controls.[14–17] Proteins obtained from proteomic analysis are generally at nano and micro molar levels, whereas inflammation-related substances (e.g., cytokines and chemokines) are typically found at pico molar levels. Also, targeted omics-like methods using panels of 70 to 90 inflammation-related proteins [mainly cytokines and chemokines (Hereafter labeled *Inflammatory panel*.)] have been applied when investigating CWP/FM; proteins from such panels can significantly differentiate CWP/FM patients from controls.[18,19]

We recently published two studies of the same subjects aimed at differentiating CWP from control (CON) using plasma biomarkers.[14,18] In the first study, proteomic analyses (two-dimensional gel electrophoresis [2-DE]: number of proteins: 325) were applied and a highly significant differentiation was obtained with 22 significant proteins (e.g., acute phase proteins, complement factors, and coagulation factors) (R^2 = 0.84, Q^2 = 0.60).[14] In the second study, an inflammation panel of 92 proteins was used, and 11 proteins (e.g., 3 chemokines, 1 cytokine, and 2 growth factors) significantly differentiated the two groups (R^2 = 0.58, Q^2 = 0.37). Thus, proteins identified using proteomic analysis were somewhat more important than the proteins from the inflammatory panel for group differentiation. As the important proteins identified in the two studies are mainly part of the host defence/immunity system, it is reasonable to assume tight interactions between the different components of this system. However, the relative importance of the proteins from these two protein sources for group differentiation is not known. Understanding this may be important for the design of larger biomarker studies of CWP/FM and in the long perspective for management and treatment of CWP/FM.

Based on the two previous studies,[14,18] we assumed that it will be possible to obtain a significant regression differentiating the two groups of subjects using a broad pattern of plasma proteins from pico to micro molar levels. Hence, this exploratory study investigates the relative importance of proteins obtained from the two sources—i.e., a targeted inflammatory panel and proteins obtained from proteomics (untargeted analysis)–for group differentiation (CWP and CON) and whether a significant protein-protein association network exists among the important proteins. In addition, this exploratory study maps the biological processes involved according to the gene ontology (GO).

2. Subjects and Methods

2.1. Subjects

The two groups of subjects have been described in detail elsewhere.[20] All subjects received a standardized clinical examination to confirm the individual eligibility. As this project was initiated in 2009, the American College of Rheumatology (ACR) criteria from 1990 was used to classify FM/CWP.[6] None of the subjects used any anticoagulatory, opioid, or steroidal medication. Exclusion criteria were medical history record of bursitis, tendonitis, capsulitis, postoperative conditions in the neck/shoulder area, previous neck trauma, disorder of the spine, neurological disease, rheumatoid arthritis or any other systemic diseases, metabolic disease, malignancy, severe psychiatric illness, pregnancy, and difficulties understanding Swedish. A total of 19 CWP and 24 CON were initially recruited, and, as previously reported, 15 CWP patients and 23 CON subjects were included in the final cohorts[18,21]; for a flow chart see.[22]

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 in the Declaration of Helsinki. All participants signed a written consent before the start of the study and after receiving verbal and written information about the objectives and procedures of the study.

2.2. Clinical variables

2.2.1. Age and body mass index. Age (years), weight (kg), and height (m) were registered at the clinical examination. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). All subjects answered a brief health questionnaire and these data have been presented elsewhere[21,23] and here some of these variables (Patient Reported Outcome Measures [PROM]) are used as brief clinical descriptions of the two groups (Table 1). Descriptions of these PROM measures are given below; for details, see.[21,23]

2.2.2. Pain intensity. The subjects rated their pain intensity on the whole body using an 11-grade (0–10) Numeric Rating Scale with two endpoints: zero indicating no pain at all and 10 indicating worst possible pain.[24]

2.2.3. Psychological distress. The Hospital Anxiety and Depression Scale measures symptoms of anxiety and depression using 14 items.[25] A total score of Hospital Anxiety and

Table 1

Background and PROM variables (Mean and SD together with Median and Range) for CON and CWP; data have been published elsewhere.[21,23]

Group	CON	n = 23			CWP	n = 15			Statistics
Variables	Mean	SD	Median	Range	Mean	SD	Median	Range	*P* value
Age (yr)	41.0	10.2	42.0	29.0	49.2	8.9	50.0	31.0	.014
BMI (kg/m^2)	24.0	2.8	23.2	12.4	26.0	5.0	23.8	15.6	.185
NRS-whole body	0.0	0.0	0.0	0.0	4.9	2.0	6.0	7.0	<.001
HADS	3.3	2.8	2.0	9.0	14.0	5.3	13.0	17.0	<.001
QOLS	93.1	9.7	94.0	37.0	82.5	13.1	85.0	46.0	.013

BMI = body mass index, CON = healthy control group, CWP = chronic widespread pain group, HADS = Hospital Anxiety and Depression Scale, NRS = Numeric Rating Scale, PROM = Patient Reported Outcome Measures, QOLS = Quality-of-Life Scale.

Depression Scale (score range: 0–42) was used to indicate psychological distress.[26]

2.2.4. Quality-of-Life. The Quality-of-Life Scale is composed by 16 items measured on seven-point satisfaction scales (total score: 16–112).[27] A higher total score reflects higher satisfaction.

2.3. Protein analyses

Venous blood samples were collected in a EDTA vacutainer and centrifuged (1000 × g, 15 minutes) and plasma was collected. 2-DE in combination with mass spectrometry (MALDI-TOF MS and nLC-MS/MS) was used for the proteomic analysis as described previously.[13,14,28] Briefly, depleted plasma proteins were separated first according to *pI* (isoelectric focusing) in the first dimension then according to the molecular weight in the second dimension. The separated proteins were stained fluorescently by SYPRO Ruby® (Bio-Rad Laboratories, Hercules, CA) and visualized/analyzed as digitizing images using a charge-coupled device camera (VersaDoc™ Imaging system 4000 MP; Bio-Rad) in combination with a software system designed for evaluation of 2-DE and quantification of protein spots (PDQuest Advanced v. 8.0.1, Bio-Rad). The protein spots of interest were excised from the gels. After enzymatic digestion by trypsin, the samples were subjected to mass spectrometer. A database search of proteins was used to identify the obtained peptide peak list from mass spectrometer as previously described.[14,29]

A multiplex proximity extension assay (PEA) was used to analyze a panel (n = 92) of inflammation related proteins (mainly cytokines, chemokines, and growth factors; see Supplementary Digital Content text file #1, http://links.lww.com/MD/H551). This targeted PEA was conducted using Proseek® Multiplex Inflammation I (Olink Bioscience, Uppsala, Sweden); for details, see previous publication.[18]

2.4. Statistics

For comparison, background and PROM variables are reported mean ± SD together with median and range. For group comparisons of values of background and PROM variables, Student's *t* test was applied using IBM SPSS (version 27.0; IBM Corporation, Route 100 Somers, New York, NY). $P < .05$ was considered significant in all analyses. Analysing omics data and a large number of variables in relation to number of subjects (i.e., broad, and short data tables) requires advanced statistical techniques such as Multivariate Data Analysis (MVDA).[30,31] Here, we have use the recommendations for MVDA of omics data.[30] We have investigated which proteins can be used to differentiate CWP from CON (i.e., group belonging) with Orthogonal Partial Least Squares discriminant analysis (OPLS-DA) using SIMCA-P + (version 17.0; Sartorius Stedim Biotech, Umeå, Sweden).[32] As this procedure has been described in detail elsewhere,[13,14,23,29] we only give a summary. All variables were mean centered, scaled to unit variance (UV-scaling), and log-transformed if necessary. No multivariate outliers were identified according to a principal component analysis. Variables with variable influence on projection (predictive) value (VIPpred) > 1.0 (combined with jack-knifed 95% confidence intervals in the regression coefficients plot not including zero) and with absolute p(corr) ≥ 0.40 were considered significant; p(corr) is the loading of each variable scaled as a correlation coefficient and therefore results in a standardized range from −1 to +1.[30] Number of predictive and orthogonal components are also reported. The OPLS-DA was made in two steps. In the first step, all identified proteins from 2-DE and inflammatory panel analysis (several hundred) were included in the analysis. In the second step, the proteins with VIPpred > 1.5 were used in a new OPLS-DA provided that the first analysis resulted in a significant component according to the internal rules used in SIMCA-P+.[32] When investigating the possible influences of age and BMI, we added the two variables to the final OPLS-DA regression of group membership and analyzed whether they were significant. Furthermore, an OPLS regression was made using the *t* score of the predictive component of the second OPLS-DA as a dependent variable and with age and BMI as independent variables. R^2 describes the goodness of fit and Q^2 describes goodness of prediction.[32] Cross validated analysis of variance (CV-ANOVA) with a $P \leq .05$ was used to validate the obtained models.

2.5. Network analysis

The protein-protein association network of the important proteins for group differentiation was analyzed using the online database Search Tool for Retrieval of Interacting Genes/Proteins (STRING; version 11).[33] Protein accession numbers (UniProt) for the important significant proteins (i.e., proteins with VIPpred ≥ 1.5) were entered in STRING's search engine (multiple proteins) with the following parameters: species set to *Homo sapiens*; maximum number of interactions for query proteins only; and interaction score set to minimum required interaction score of medium confidence (0.400). For the obtained network, PPI enrichment *P* value and average local clustering coefficient were reported. In the network figure, each protein is represented by a coloured node, and protein-protein interaction and association are represented by an edge visualized as a line. Higher combined confidence scores are represented by thicker lines/edges. Using Protein ANalysis THrough Evolutionary Relationships (PANTHER; version 16.0), the Cellular Component, Molecular Function, and Biological Processes according to the Gene Ontology (GO; http://geneontology.org/docs/ontology-documentation/) were listed.[34] The following settings were used in PANTHER: Organism: Homo sapiens; Analysis: complete; Test type: Fisher's Exact; and Correction: Bonferroni correction for multiple testing.

3. Results

3.1. Clinical variables

The two groups have been presented earlier and some clinical measures are reported in Table 1.[21,23] Hence, the CON group was younger, had a higher quality of life, and had significantly lower levels of psychological distress. The CWP group had considerable pain intensities, whereas the CON group was pain free.

3.2. Regression analysis of group belonging

Using all proteins (proteomic analysis: n = 325; measurable proteins from the inflammation panel: n = 72), we obtained a significant OPLS-DA with the following characteristics: R^2 = 0.996; Q^2 = 0.529; and CV-ANOVA *P* value = .0044 (one predictive and three orthogonal components). When only the most important proteins were included (i.e., VIPpred > 1.5) (Table 2), the significant regression had the following characteristics: R^2 = 0.829; Q^2 = 0.644; CV-ANOVA *P* value = 7.0e-07 (one predictive and one orthogonal component). In total, 58 proteins (including proteoforms) had a VIPpred > 1.5.

Both proteins determined from the 2-DE analysis and from the inflammation panel were important for the group differentiation although the majority belonged to the proteins from the proteome (Table 2). The four most important proteins for the group differentiation (i.e., highest absolute p(corr) and VIPpred value) were two complement factors (Complement C3c alpha chain fragment 2 and Complement factor I), alpha-2-HS-glycoprotein, and AXIN1.

Several of the important proteins had proteoforms; most proteoforms were noted for ceruloplasmin (n = 10), complement C1r subcomponent (n = 7), and plasminogen (n = 5).

Table 2

OPLS-DA regression of group membership (CWP or CON). The most important plasma proteins (i.e., VIPpred > 1.5) are presented in alphabetical order. Numbers of proteoforms are reported together with mean VIPpred and mean p(corr). A positive p(corr) indicates higher levels in CWP. *Characterisation* is in a pain-immune-nociception context. Proteins in bold are from the panel of 92 inflammation-related proteins.

Accession number	Protein	Abbreviation	No. of proteoforms	Mean VIPpred	Mean p(corr)	*Characterisation*
Q13541	**Eukaryotic translation initiation factor 4E-binding protein 1**	EIF4EB-P1/4E-BP1	1	1.59	0.46	Control of protein translation & sensitivity of neurons
P01009	Alpha-1-antitrypsin	SERPINA1	2	1.62	0.43	Acute phase protein
P08697	Alpha-2-antiplasmin	SERPINF2	2	1.60	0.43	Acute phase protein
P02765	Alpha-2-HS-glycoprotein	AHSG	1	2.33	0.62	Immune response
P01023	Alpha-2-macroglobulin	A2M	2	1.55	0.41	Acute phase protein
P02647	Apolipoprotein A-I	APOA1	2	1.69	–0.45	Antiinflammation
O15169	**AXIN1**	AXIN1	1	2.05	0.58	Downregulates Wnt signaling pathway
Q14790	**Caspase-8**	CASP8	1	1.51	0.43	Modulate IL-1b & inflammation; Regulation of NF-κB
P78556	**C-C motif chemokine 20**	CCL20	1	1.94	0.53	Chemokine
P00450	Ceruloplasmin	CP	10	1.85	0.49	Acute phase protein
P05160	Coagulation factor XIII B chain	F13B	1	1.77	0.47	Coagulation factor
P00736	Complement C1r subcomponent	C1R	7	1.90	0.51	Complement factors
P01024	Complement C3c alpha chain fragment 2	C3	1	2.42	0.64	Complement factors
P00751	Complement factor B	CFB	1	1.66	0.44	Complement factors
P05156	Complement factor I	CFI	1	2.42	0.64	Complement factors
P02679	Fibrinogen gamma chain	FGG	3	1.93	0.51	Acute phase protein
O75636	Ficolin-3	FCN3	1	1.75	0.47	Pattern recognition molecule—activate complement
P06396	Gelsolin	GSN	2	1.75	0.47	Anti-inflammatory
P00738	Haptoglobin	HP	1	1.73	0.46	Acute phase protein
P02790	Hemopexin	HPX	1	1.50	0.40	Acute phase protein
P01877	Ig alpha-2 chain C region	IGHA2	1	1.73	–0.46	Immunoglobulin
P42702	**Leukaemia inhibitory factor receptor**	LIFR	1	1.56	–0.39	Activates JAK/STAT and MAPK signaling pathways
P00747	Plasminogen	PLG	5	1.87	0.50	Acute phase protein
P02787	Serotransferrin	TF	1	1.77	–0.47	Acute phase protein
Q8IXJ6	**NAD-dependent protein deacetylase sirtuin-2**	SIRT2	1	1.74	0.50	Anti-inflammatory; Regulation of NF-κB
O95630	**STAM-binding protein**	STAMPB	1	1.62	0.47	regulation of endosomal-lysosomal degradation pathway
P01135	**Protransforming growth factor alpha**	TGFA	1	1.59	0.46	Restoring function in neurodegenerative disorders
P02766	Transthyretin	TTR	1	1.79	–0.48	Acute phase protein
O43508	**Tumour necrosis factor ligand superfamily member 12**	TNFSF12	1	1.65	0.44	Cytokine
P02774	Vitamin D-binding protein	GC	3	1.78	0.47	Immunity

AXIN1 = axin-1, CON = healthy control group, CWP = chronic widespread pain group, GC = vitamin d-binding protein, IL = interleukin, JAK/STAT = Janus kinase-signal transducer and activator of transcription pathway, MAPK = mitogen-activated protein kinase signaling pathway, NF-κB = nuclear factor kappa-light-chain-enhancer of activated b cells pathway, OPLS-DA = Orthogonal Partial Least Squares discriminant analysis, VIPpred = variable influence on projection (predictive) value, TF = serotransferrin, TGFA = protransforming growth factor alpha, TNFSF12 = tumour necrosis factor ligand superfamily member 12.

Most proteins were increased in CWP (i.e., a positive p(corr)) but apoliprotein A-I, Ig alpha-2 chain C region, leukaemia inhibitory factor receptor [LIFR], serotransferrin, and transthyretin were higher in the CON (i.e., negative p(corr)) (Table 2).

3.3. Possible influences from age and BMI

When age and BMI were added as regressors to the second OPLS-DA regression of group membership, none of these two variables were significant regressors (i.e., VIPpred < 1.0). We also regressed the *t* score of the predictive component obtained from the second OPLS-DA using age and BMI as regressors, but the model was not significant. Hence, age and BMI did not significantly influence the important proteins differentiating the CWP from the CON.

3.4. The network and enrichment analysis together with GO analysis

The network and enrichment analysis of the important proteins differentiating CWP from CON (n = 58) (Table 2) identified a significantly enriched protein-protein interaction network (note that proteoforms cannot be included in STRING) (Fig. 1). This means that the proteins included in the analysis have more interactions among themselves than what would be expected for a random set of proteins of similar size drawn from the genome. Thus, the proteins are at least partially biologically connected as a group. When scrutinizing Figure 1, it was found that five of nine proteins from the inflammation panel did not show interactions: SIRT2, AXIN1, LIFR, EIF4EBP1 (4E-BP1), and STAMPB.

Biological Processes according to the GO analysis (PANTHER) identified in the enriched network included acute phase response, regulation of complement activation, complement activation,

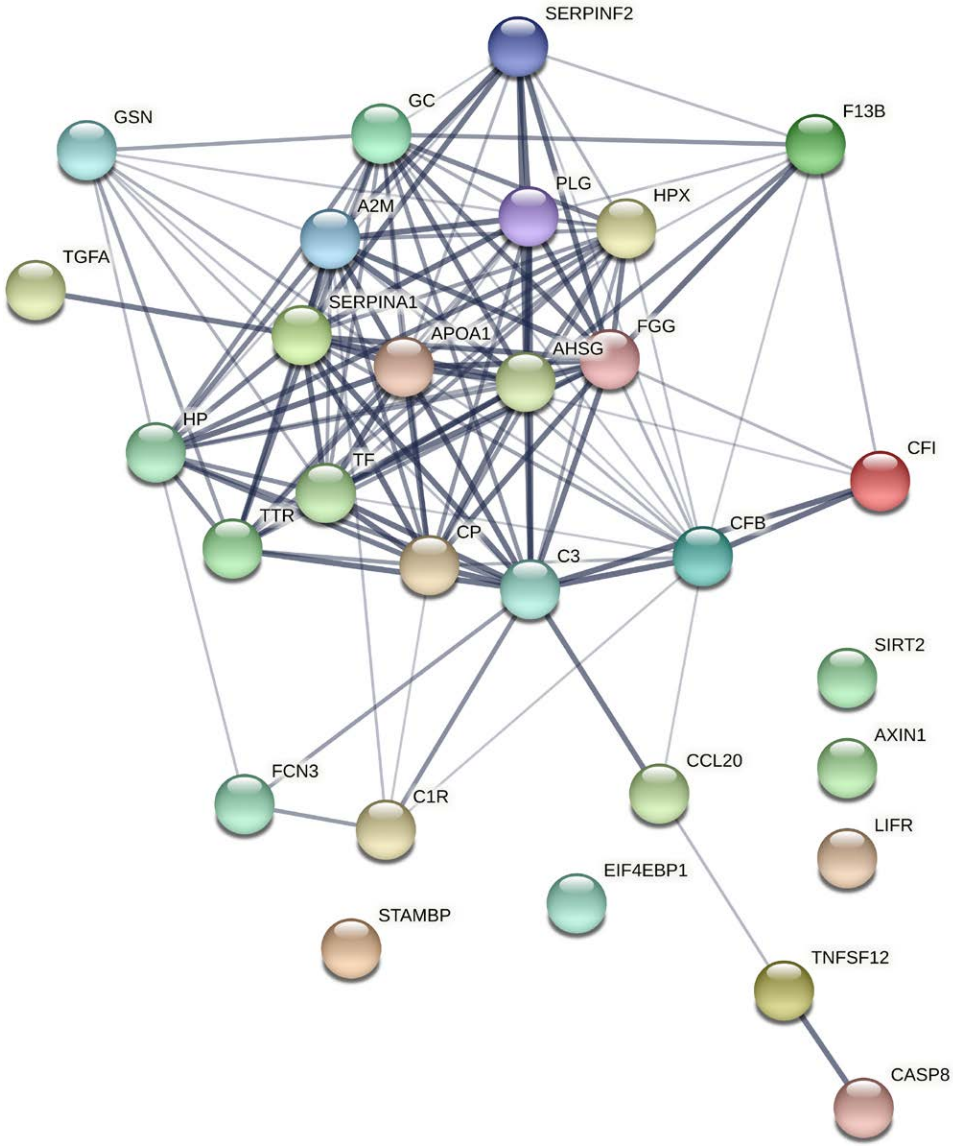

Figure 1. Network analyses of important proteins differentiating CWP from CON. The network had the following characteristics: number of nodes: 29; number of edges: 129; average node degree: 8.9; average local clustering coefficient: 0.62; expected number of edges: 10; PPI enrichment *P* value: < 1.0e-16. Note that Ig alpha-2 chain C region is not included in STRING. A2M = alpha-2-macroglobulin, AHSG = alpha-2-HS-glycoprotein, APOA1 = apolipoprotein A-I, AXIN1 = axin-1, C1R = complement C1r subcomponent, C3 = complement C3, CASP8 = caspase-8, CCL20 = C-C motif chemokine 20, CFB = complement factor B, CFI = complement factor I, CP = ceruloplasmin, EIF4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), F13B = coagulation factor XIII B chain, FCN3 = ficolin-3, FGG = fibrinogen gamma chain, GC = vitamin D-binding protein, GSN = gelsolin, HP = haptoglobin, HPX = hemopexin, LIFR = leukaemia inhibitory factor receptor, PLG = plasminogen, SERPINA1 = alpha-1-antitrypsin, SERPINF2 = alpha-2-antiplasmin, SIRT2 = NAD-dependent protein deacetylase sirtuin-2, STAMBP = STAM-binding protein, TF = serotransferrin, TGFA = protransforming growth factor alpha, TNFSF12 = tumour necrosis factor ligand superfamily member 12, TTR = transthyretin.

regulation of endopeptidase activity, fibrinolysis, and innate immune response (see Supplementary Digital Content Table 1, http://links.lww.com/MD/H552). Detailed results from the GO analyses are presented in the Supplementary Digital Content Tables 1–3, http://links.lww.com/MD/H552: http://links.lww.com/MD/H553: http://links.lww.com/MD/H554; for molecular function and cellular component see Supplementary Digital Content Tables 2–3, http://links.lww.com/MD/H553: http://links.lww.com/MD/H554.

4. Discussion

As expected, a broad pattern of mainly interacting immune- and inflammation-related plasma proteins from pico to micro molar levels differentiated CWP from CON. Proteins from the plasma proteomics were somewhat more important than proteins from the inflammation panel. The pattern of interacting proteins was neither age- nor BMI-dependent. The protein-protein interaction network was significantly enriched, and the identified biological processes included acute phase response, aspects of complement activation, and innate immune response. The broad involvement of the immune system peripherally supports suggestions that systemic low-grade inflammation is present in CWP.

Both proteins identified from the proteome and from the inflammatory panel contribute to group differentiation (CWP vs. CON). Most of the important proteins were obtained from the plasma proteome although proteins from the inflammation panel also contributed (Table 2). Our results broaden the perspective and clearly suggest that proteins from pico to micro molar levels must be considered to obtain a more complete understanding of the plasma alterations in CWP/FM. Due to

the intimate relationships between the immune system and nociception, the present protein alterations differentiating CWP from CON might indicate a more continuous input (immune and nociceptive) to the CNS maintaining central alterations and clinical presentations.

Currently, there are four plasma/serum proteome studies of CWP/FM versus controls.[14–17] These studies indicate that peripheral immune proteins are involved in differentiating CWP/FM from controls. Several studies have investigated peripheral (blood) cytokines and chemokines and their roles in FM, but recent systematic reviews (SRs) have not found clear evidence (except for IL-6) as they disagree about which cytokines were altered in FM.[35,36] One SR reported significantly higher levels of IL-6, IL-17A, and IL-4 in FM.[35] Another SR reported higher IL-6, IL-8, TNF-α, and chemokine eotaxin levels, and decreased IL-10 in FM.[36] The present study did not identify any of the cytokines/chemokines from the SRs as important among proteins with VIPpred > 1.5 (Table 2) or among those with VIPpred > 1.0 (data not shown). Most of the studies included in the SRs investigated relatively few proteins. Using broad plasma inflammatory panels (mainly cytokines and chemokines) in combination with MVDA, two studies report that CWP/FM can be differentiated from controls[18,19]; several of the significantly differentiating proteins were similar in the two studies (i.e., STAMPB, SIRT2, AXIN1, and 4e-BP1). However, this does not exclude that the cytokines/chemokines identified in the SRs are important in the initiation of chronic pain or for certain pain variables. Moreover, recent studies of CWP/FM have reported altered plasma levels of other substances (e.g., glutamate, lactate, NGF, BDNF, and lipid mediators),[20,37–39] findings that also advocate for a broad approach to understanding the peripheral contribution in CWP/FM.[40]

The highly significant enriched protein-protein interaction network (Fig. 1) indicated biologically meaningful interactions. The identified biological processes according to the GO analysis agreed with earlier proteomic studies (e.g., acute phase response, regulation of complement activation, complement activation, and innate immune response).[14–17] These are parts of the host defence/immunity system and support interpretations of low-grade inflammation in CWP/FM. Scrutinising the important proteins in Table 2 in more detail in an immune-nociception-pain context revealed that several of the proteins were acute phase proteins (n = 10), complement related factors (n = 5), chemokine/cytokine (n = 2), other inflammation-related proteins (n = 3), coagulation factor (n = 1), and immunoglobulin factor (n = 1). Furthermore, three proteins that affect pathways—e.g., the Wingless-related integration site pathway (AXIN1), Janus kinase-signal transducer and activator of transcription pathway (JAK/STAT) (LIFR), mitogen-activated protein kinase signaling pathway (MAPK) (LIFR), and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (SIRT2)–were also present. These pathways are involved in the induction of cytokines and the inflammatory effects of cytokines.[41–45] JAK-STAT regulates durations and magnitude of pro- and anti-inflammatory cytokines and is involved in homeostasis and inflammation.[46,47]

Several proteins identified by 2-DE had proteoforms—i.e., the acute phase proteins ceruloplasmin (n = 10) and plasminogen (n = 5), and one complement factor (Complement C1r subcomponent; n = 7). Thus, future large studies should characterize in more detail the types, functions, and patterns of the proteoforms and how they are altered in CWP. Because the network analysis in STRING does not handle proteoforms, other methods are required to consider these alterations.

The aetiopathogenesis including pathophysiological alterations, diagnostic, and classification criteria of CWP/FM is still a matter of debate.[48] In the literature, CWP/FM has been associated with molecular, morphological, and functional (e.g., central sensitization) alterations both in the central nervous system (CNS) and in the periphery (e.g., systemic low-grade inflammation, autoreactive IgG, nociceptor impairment, and muscle mitochondrial disturbance).[40,49–62] The circulatory system is vital for the immune-system-based host defence mechanisms and tissue homeostasis.[63,64] Nociceptors have neuro-immune and neural-vascular interactions,[65] and bidirectional signaling pathways exist between immune and nervous systems at peripheral and central levels.[66–71] Furthermore, the CNS exerts top-down effects on peripheral inflammatory activity via neuroendocrine and autonomic mechanisms.[67,72] Information on peripheral inflammatory activity is transmitted via peripheral nociceptors and humoral and neuronal pathways to the CNS, leading to, for example, sickness behavior, decreased endogenous pain inhibition, and neuroinflammation.[66,71,73–75] Altered blood-brain barrier permeability due to influences from cytokines and complement factors facilitates this communication to the CNS,[76–78] and in FM and CWP signs of CNS neuroinflammation have been found.[19,29] These and the present findings indicate complicated interactions between peripheral and central alterations and processes in CWP/FM.

Generalised hyperalgesia for pressure is a criteria for FM according to the 1990 ACR criteria and are, as in the present patient cohort, generally found in CWP.[23] The generalized hyperalgesia is often attributed to central sensitization and is associated with activation of certain parts of the CNS.[79] However, it has also been proposed that neuroinflammation in the peripheral and central nervous system and/or dys-regulation in the immune-nociception signaling might be associated with or drives of central sensitization, which drives CWP/FM.[77,80] Other authors suggest that different peripheral stimuli/stressors including pro-inflammatory cytokines can lead to long-term potentiation, "wind up", and/or central sensitization.[81,82]

4.1. Strength and limitations

Limitations of this study were the small sample size, the cross-sectional design, and including only women. Larger studies of both sexes with repeated measures are important for validation. Traditional statistical analyses are challenging with few subjects and many probably highly intercorrelated proteins. In these situations, MVDA is the backbone as pointed out elsewhere and applied in this study.[31] The necessary removal procedure of large abundant proteins (i.e., albumin and IgG) could have removed other low abundant proteins that could be of interest. Research using peripheral proteomics in CWP/FM is in an initial exploratory stage and larger studies are required to understand what changes are specific to the pain condition. Immunological and inflammatory biological processes have also been reported for depression, obesity, and aging.[83–85]

5. Conclusions

This study contributes to the emerging knowledge that plasma proteins can differentiate CWP, including FM from healthy controls. Proteins involved in the host defence/immune system are generally identified and give support to the interpretation that low-grade inflammation is present in CWP/FM. Focusing on traditional cytokines/chemokines might not be sufficient to grasp the peripheral biological processes that maintain CWP/FM. Our results indicate that other parts of the immune and inflammation systems are also important and that a combination of different methods need to be used to identify proteins involved in these processes. Unconditional exploratory studies with a wide range of proteins and other substances investigating interactions and biological processes are necessary. Within such efforts it will be important to determine the primary biological and molecular processes in CWP/FM. When such an understanding has been achieved, it will be fruitful to focus on key proteins for identification of clinical plasma biomarkers.

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Author contributions

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