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REVIEW

Proteomic studies of common chronic pain conditions - a systematic review and associated network analyses

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ABSTRACT

Introduction: The lack of biomarkers indicating involved nociceptive and/or pain mechanisms makes diagnostic procedures problematic. Clinical pain research has begun to use proteomics.

Areas covered: This systematic review covers proteomic studies of chronic pain cohorts and in relation to clinical variables. Searches in three databases identified 96 studies from PubMed, 161 from Scopus and 155 from Web of Science database. Finally, 27 relevant articles were included. Network analyses based on the identified proteins were performed.

Expert opinion: Small pain cohorts were investigated and the number of studies per diagnosis and tissue is small. The use of proteomics in chronic pain research is exploratory and larger proteomic studies are needed. It will be necessary to standardize the descriptions of the pain cohorts investigated. There is a need to identify the mechanisms underlying the whole clinical presentation of specific chronic pain conditions. Multivariate methods capable of handling and identifying intercorrelated protein patterns must be applied. Rather than focusing on a few proteins, future studies should use network analyses to investigate interactions and biological processes. Proteomics in combination with bioinformatics have a huge potential to identify previously unknown panels of proteins involved in chronic pain and relevant when devising new pain control strategies.

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1. Introduction

1.1. Chronic pain – clinical presentations and prevalence

Acute pain is part of the body's alarm system. The inability to experience pain due to rare recessive gene mutations is associated with tissue damage, tissue mutilation, and reduced life expectancy [1]. In contrast, most chronic pain conditions (i.e. pain for at least three months) are considered maladaptive and mechanistically different from acute protective pain [2]. Chronic pain is sometimes labeled as pathological pain.

One-fifth of the European population has chronic pain of at least moderate intensity [3]. In addition to significant pain intensity, these conditions are associated with sick leave, poor health, psychological distress, and high socioeconomic costs [4]. Chronic pain conditions are in complex ways associated with increased risk for lowered physical activity and increased body mass index (BMI). Typically, modern clinical practice applies a bio-psycho-social framework as chronic pain is influenced by and interacts with psychological, neurobiological, and social factors in complex and partially unknown ways [5].

The prevalence of local chronic pain conditions is high – e.g. 8% chronic neck shoulder pain (CNSP) and 19.5% chronic low back pain (CLBP) [6–8]. Based on population surveys, 3–8% of the population has neuropathic pain [9]. Local pain conditions such as CNSP and CLBP can gradually become more easily triggered and spread to most of the body (i.e. chronic widespread pain (CWP) with a 5–10% prevalence) [10]. Fibromyalgia (FM) (community prevalence: 2–4%) is a subgroup of CWP with generalized hyperalgesia according to the 1990 American College of Rheumatology (ACR) criteria [11–13]. Although CWP/FM is considered the most negative extreme of chronic pain, the etiologies of these conditions as well as the risk factors are insufficiently understood [14,15].

1.2. Chronic pain definition

The International Association for the Study of Pain (IASP) defines pain as '[a]*n unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*.' [16]. Although this definition is clinically accepted, it has attracted some criticism over the years [17]. For example, the definition does not capture the fact that pain may be both protective and pathological and does not consider the needs of non-verbal individuals as it requires the experience to be described verbally [2]. Pain that persists for more than three months is labeled chronic pain. Typically, chronic pain diagnoses are based on the duration and anatomical location[s] such as chronic low back pain. Chronic pain patients are largely managed using trial-and-error [18]. Different activated neurobiological mechanisms such as the extent of peripheral biochemical alterations may

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Supplemental data for this article can be accessed here.

Article highlights

- The use of proteomics in chronic pain research is in its infancy.
- Peripheral and central mechanisms have been investigated.
- The identified studies reported proteins that significantly differed in expression between patients and controls.
- Our network analyses showed interactions among most proteins.
- The overlap at the level of single proteins is limited and necessitates a focus on identifying the biological processes.
- Larger proteomic studies with standardised descriptions of the pain cohorts are needed.

explain the small to moderate effect sizes of common non-pharmacological interventions [19–22]. Hence, a certain clinical diagnosis may mechanistically combine different phenotypes [23]. In clinical practice, efforts are made to implement mechanism-based classification of pain conditions. Few mechanism-based diagnoses exist for chronic pain. One exception is neuropathic pain: pain that arises as a direct consequence of a lesion or diseases that affect the somatosensory system. In addition to neuropathic pain and nociceptive pain, a new mechanism has recently been identified by IASP – nociplastic pain. The IASP includes FM and nonspecific CLBP as examples of pain conditions associated with nociplastic pain mechanisms.

1.3. Peripheral or central mechanisms

The lack of easily obtained markers – e.g. blood biomarkers – indicating involved nociceptive and/or pain mechanisms make diagnostic procedures problematic [24]. Current diagnostic tools lack specificity for identifying pain drivers [18]. Major drug developments have failed mainly because the underlying mechanisms are not understood and therefore are not targeted [25]. Because the processes driving the pain are difficult to identify and target for treatment, the effective management of chronic pain is difficult [2].

Several decades ago, chronic pain conditions such as CNSP, non-neuropathic CBLP, and FM/CWP were perceived as being of peripheral origin, a conclusion supported by acute animal experiments. When potential or actual tissue pain is experienced, nociceptors respond to and can be sensitized by single or combinations of noxious mechanical stimuli, temperature, and chemicals [18]. However, the peripheral origin theory was challenged by evidence gathered from imaging techniques such as functional Magnetic Resonance Imaging (fMRI); these techniques found evidence for altered central (CNS) nociceptive/pain processing and morphology in CNSP, CLBP, and CWP/FM [26–32]. Therefore, some researchers have characterized these diseases as central pain conditions [26,33].

Understanding of the relative roles of peripheral and central factors is fundamental for developing treatments. That is, a more complex picture has emerged of the interaction between peripheral and central factors as well as of pain systems overall. It has been suggested by us and others that central nervous system (CNS) alterations can be driven by peripheral nociception generators that produce the clinical presentations [34,35]. For example, CNS alterations in CLBP or chronic hip osteoarthritis are normalized after effective peripheral treatment (facet joint injections or surgery) [36–38]. Moreover, studies have uncovered support for a peripheral muscle involvement such as increased muscle levels of serotonin, glutamate, pyruvate, and lactate in CNSP and CWP/FM and decreased concentrations of adenosine triphosphate (ATP) and phosphocreatine (PCr) in FM [39–42]. However, only a few molecules have been investigated and it is unclear whether important pathophysiological mechanisms have been targeted in hypothesis-driven studies that focus on a few molecules. Hence, to achieve a true mechanistic understanding of the biological factors maintaining pain conditions, it is necessary to understand the activated molecular mechanisms from a broader system biology perspective.

1.4. What are biomarkers, and can they be used for chronic pain conditions?

Objective biomarkers (e.g. proteins from different tissues) are considered essential for facilitating and improving diagnosis of chronic pain conditions [43]. Several clinical areas would benefit from the use of pain biomarkers – e.g. routine patient diagnosis and management, anaesthetized and comatose patients, non-verbal persons including neonates, clinical trials, and analgesic drug discovery and development [2].

Several, mainly overlapping, definitions of a biomarker have been used. The National Institutes of Health Biomarkers Definitions Working Group defines a biomarker as '*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*' [44]. Hence, biomarkers are by definition objective, quantifiable characteristics of biological processes, and they may but do not necessarily correlate with a patient's experience and perceived health [45]. Preferably, a biomarker should be non-invasively accessible, inexpensive, highly specific, sensitive, and easy to interpret. In the search for reliable biomarkers, it is important to find an accurate method that it is applicable in a clinical setting. High clinical (diagnostic, progression, and monitoring) accuracy should be maintained regardless of, for example, differences in sample handling protocols.

Clinical endpoints are variables that reflect an individual's health and wellbeing. Clinical endpoints are primary and to some extent the only relevant endpoints of all clinical research and ultimately of all biomedical research [45]. Biomarkers generally must be viewed as surrogate endpoints – i.e. substitutes for clinically meaningful endpoints [45] – although not all biomarkers can be surrogate endpoints. A surrogate endpoint (i.e. biomarker) is characterized by solid scientific evidence that the biomarker consistently and accurately predicts a clinical outcome as either a benefit or a harm [45].

In a note to the above IASP definition of pain, the authors emphasize that '[p]*ain is always subjective*'. If pain is always subjective, research attempting to identify objective biomarkers may appear strange. Can an objective biomarker be identified for something, i.e., pain that in the clinic and in research setting is considered subjective? Moreover, clinicians seem to use the word 'subjective' inconsistently. For example, some seem to think that 'subjectivity' means that no objective

measure is available, some seem to think that 'subjectivity' means that it is impossible to gauge whether a patient has pain, and some seem to think 'subjectivity' reflects that pain is very complicated. Furthermore, some individuals use the word 'subjective' to be dismissive, patronizing, or express distrust. Some have argued that we can never capture the experience of pain with biomarkers but can possibly identify biomarkers that reflect nociception or the consequences of pain. To complicate the matter further, philosophers use the notion of 'subjective' to discuss consciousness [46], an historically slippery concept in itself. Moreover, the philosophical conception of subjective appears to be distinct from its everyday use [47]. Similarly, the philosophical concept of' objectivity' has historically been met with controversy.

Based on our current knowledge, when we die, we no longer experience pain – i.e. the experience of pain depends on various biological and physiological processes. If this is so, then there should be an opportunity to describe these processes and how pain is created and maintained, factors that can be objectively measured. However, these chemical and physiological processes can be so complex and dynamic that we will never be able to capture and describe them with high precision and when, how, and if they result in pain perception. However, a biomarker may be associated with a certain risk that a certain mechanistic process or pain is present.

1.5. Single molecules in blood and CSF – drawbacks

Chronic pain conditions are associated with increased prevalence of different co-morbidities. Furthermore, available data suggest that chronic pain is a complex process involving interactions of an array of biochemical, transmitters, and receptors both in the central and peripheral nervous systems. It is highly unlikely that conditions such as chronic pain and cancer can be captured in their entirety by one biomarker as these conditions are heterogenous and the result of interacting complex cellular networks [48,49]. To date, no unidimensional reliable biomarker for pain has been identified. Panels of multiple molecules (i.e. molecular signatures) should perform better than a single molecule when it comes to understanding the role activated nociceptive and pain mechanisms have in chronic pain conditions. Hence, composite biomarker signatures (e.g. obtained from advanced analytical and statistical tools such as machine learning, neural networks, and artificial intelligence) are more likely to be fruitful for understanding nociceptive processes and pain and for developing new treatments for patients with chronic pain conditions [2,50]. Proteins, for example, are directly responsible for maintaining cellular function, signaling substances of pain, regulating pain modulation, and activating the production of other pain mediators [51].

1.6. What is omics and proteomics?

Omics methods characterize and quantify pools of many molecules (up to several 1000). Since a large number of substances can be analyzed simultaneously, omics is a potentially valuable tool in examining the relationship between multiple substances in conjunction with their cellular functions and in the context of various chronic pain conditions such as CNSP, CLBP, CWP/FM, and neuropathic pain.

The human genome, which has three billion bases with an estimated 20–40,000 genes [52]. The proteome is much larger than the genome, because of such factors as alternatively spliced RNA, posttranslational modifications of proteins, temporal regulation of protein synthesis, and varying protein-protein interactions. The proteome represents the composite readout of gene expression, translation, and post-translational modulation [53]. Investigating the proteome will in comparison from studying the genome and the transcriptome means a huge increase in the complexity [54].

The process of identifying the proteome is called proteomics. Since proteins are molecules directly responsible for maintaining correct cellular function, they are also directly involved in both normal and disease-associated biochemical processes. A more complete understanding of diseases may be gained by looking directly at the proteins present within diseased cells, tissues, or compartments. Such investigations can be achieved through proteomics. Proteomics, frequently used in psychiatric and neurodegenerative disease research, has recently been identified as an unbiased method that can be used to explore pain pathophysiology [55,56].

1.7. Methods used for proteomics in the field of pain

Proteomic pain research tries to understand the expression, function, and regulation of the entire set of proteins involved in nociception (and associated with pain) in a certain tissue. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in combination with mass spectrometry are key technologies used to study how proteins are expressed, regulated, and modified throughout the living system. Although 2-DE was first described in 1975, it fits very well into the new concept of proteomics: *'old, old-fashioned, but it still climbs up the mountains'* [57,58].

The technique resolves the complex protein mixture in the first dimension by isoelectric focusing, during which proteins are migrated in a pH gradient until they reach their isoelectric point pI (the pH where the protein has zero net charge). In the second dimension, proteins are separated according to their relative mass (Mr) using sodium dodecyl sulfate (SDS). Several 2-DE databases have been established for human tissues/body fluids and different cell lines in health and disease. The World 2D-PAGE index (https://world-2dpage.expasy.org/portal) provides access to the most relevant databases such as Heart-2DPAGE, plasma 2D database, serume-2DPAGE, and the 2-DE map of CSF.

Since up to 1000 proteins can be visualized on a single gel [59], high throughput techniques are needed to analyze and identify all the proteins. Using mass spectrometry, protein identification has become much easier and faster and the technique allows a sensitive and precise detection of the total peptide contents of complex mixtures [60]. Peptide mass fingerprinting (PMF) is a process by which proteins are identified from their peptides. Protein spots of interest are excised from the gel and are subjected to a digestion procedure resulting in signature peptide fragments that can be compared with peptide fragments in databases. The mass

spectrometry (MS) technologies that can be used for PMF are matrix-assisted laser desorption ionization time of flight (MALDI-TOF) and electrospray ionization (ESI) source-equipped mass spectrometry. For routine fast analysis of unseparated protein digests, MALDI-TOF- MS is the mass spectrometer of choice. ESI in combination with high-performance liquid chromatography (HPLC) is the method of choice for shotgun proteomics. This method, which is an MS-based proteomic, provides direct analysis of complex mixtures of digested peptides in the entire batch of proteins. The complex peptide mixtures are separated based on its hydrophobicity on a C-18 column in a gradient of organic solvent. Eluting peptides are ionized by ESI and transferred to the on-line coupled high-resolution MS where selected peptides are fragmented by tandem mass spectrometry (MS/MS) [61]. Automated computational tools such as MaxQuant can extract quantitative data from the large amount of the generated MS/MS spectra and can be used to identify proteins [62]. The generated data are entered into databases containing original protein sequences and open reading frames or putative predicted sequences from mRNA or genomic DNA sequences. The National Center for Biotechnology Information (NCBI) and UNIProt databases are the most used databases for interpretation of the experimental data obtained from mass spectrometry analysis [63,64]. The search algorithms identify the MS/MS spectra using the theoretically predicted peptide sequence from the protein databases that fit the experimental data with a certain false discovery rate (FDR). Some more search criteria are applied such as parent ion mass tolerance, enzyme digestion, and post-translational or chemical modifications [65,66]. These technologies have been used to identify and quantify all the proteins found in muscle tissues and body fluids from patients with chronic pain.

The proteomic approach provides enormous amounts of raw data that can be handled with the help of bioinformatic tools such as STRING (Search Tool for Retrieval of Interacting Genes/Proteins), which is available on the World Wide Web [67]. The output of proteomic studies is often a panel of multiple proteins instead of single proteins. The majority of the identified proteins do not function independently, because they regulate activity and induce/reduce expression levels of other proteins, it is reasonable to study protein-protein interactions to better understand the physiology and the biological processes proteins affect. Gomez-Varela et al. suggest using the term protein disease signatures (PDS) rather than biomarkers – PDS is loosely defined as proteins that differ between disease conditions and controls [68]. A network construction is needed that can organize the large amount of proteomics data, a prerequisite for the identification of the underlying mechanisms of chronic pain conditions [69,70]. Once the interesting pathways and functions are identified, a hypothesis can be created that considers the specific proteins involved in chronic pain.

1.8. Aim

This systematic review was motivated by the increasing recognition that the biological basis and maintenance of chronic pain are unlikely to be related to single molecules but to biological processes and complex cellular networks. This view means proteins should be of special interest since they are directly involved in maintaining cellular function, nociceptive signaling, and modulation and in interactions with other pain mediators. Moreover, the proteome is the composite and dynamic readout of gene expression, translation, and post-translational modulation. It has been noted that proteomics has been increasingly applied to the field of pain conditions [55,56]. To avoid focussing on single proteins, it is necessary to apply a systems biology approach that starts by mapping the involved networks associated with nociception and chronic pain, including their broad clinical presentations. Hence, this systematic review has the following aims:

(1) Systematically review the literature (primary studies) concerning proteomics applied to different tissues (muscle, saliva, blood and cerebrospinal fluid (CSF)) in humans with chronic pain conditions (neck-shoulder pain including trapezius myalgia, low back pain, widespread pain including FM and neuropathic pain) regarding ability to differentiate versus healthy controls and in relation to clinical variables (e.g. pain intensity, psychological distress, disability, etc.) for those with pain.
(2) Based on the identified proteins from the systematic review, comprehensively perform network analyses using the online database tool Search Tool for Retrieval of Interacting Genes/Proteins (STRING) and identify the important biological processes involved in chronic pain.

2. Methods

We performed (a) a systematic review of the literature and b) for the important proteins reported in the identified studies for a certain diagnosis – tissue combination protein-protein association network analysis was made.

2.1. Electronic search strategy

After consulting university librarians, we searched three databases: PubMed; Scopus; and Web of Sciences (Supplementary Figure 1). The search was done on 18 February 2020. The search strings for each database are shown in Supplementary Text File 1.

2.2. Selection criteria and population

We included primary studies of humans (no cadaveric studies) with the following chronic (≥3 months duration) pain conditions: chronic neck-shoulder pain (CNSP) including trapezius myalgia; chronic widespread pain (CWP) including fibromyalgia (FM); chronic low back pain (CLBP), and chronic neuropathic pain. At least 75% of the patients in a pain cohort had to experience chronic pain (≥3 months duration). We included studies of these pain conditions that analyzed the following tissues: muscle; blood (i.e. plasma and serum); saliva; and cerebrospinal fluid (CSF). The following types of studies were included: methodological proteomic studies (e.g. developing

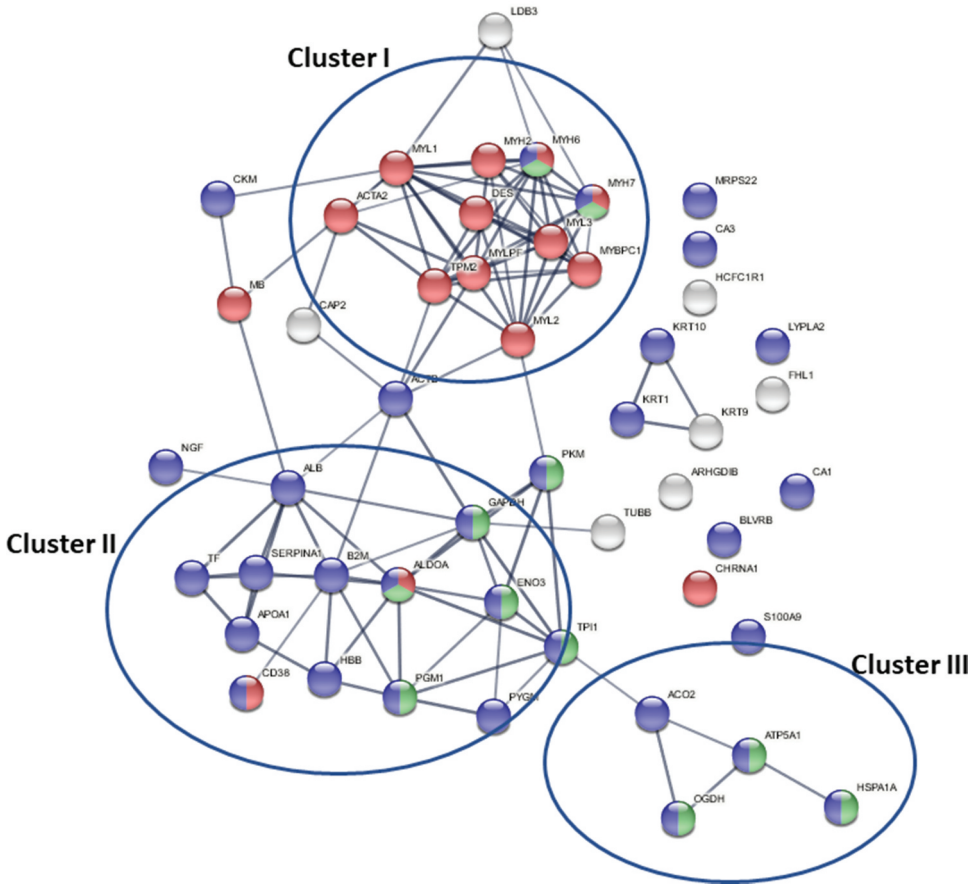

Figure 1. Protein network interaction of altered proteins in muscle from patients with trapezius myalgia compared to healthy controls identified in three studies [81–83]. Nodes denote genes/peptides. The protein-protein Interaction (PPI) enrichment analysis ($P < 1.0e-16$) separated the identified proteins in 3 clusters. Cluster I is represented by proteins involved in muscle contraction (red – 11 proteins: ACTA2, DES, MYBPC1, MYH2, MYH6, MYH7, MYL1, MYL2, MYL3, MYLPF, and TPM2). Proteins in cluster I are muscle fiber components that affect motor activity and cytoskeletal protein binding (actin, microtubule, or intermediate filament cytoskeleton). Cluster II included proteins involved mainly in cellular metabolic process (blue – 18 proteins: ACTB, ALB, ALDOA, APOA1, B2M, CD38, ENO3, GAPDH, HBB, HSPA1A, MYH6, MYH7, NGF, PGM1, PKM, PYGM, SERPINA1, TF, and TPI1). Proteins in cluster II are part of the cytoplasm component that functions as an enzyme/enzyme inhibitor activity, ion/protein binding, and microfilament motor activity. Cluster III was dominated by proteins involved in ATP metabolic process (green – three proteins: OGDH, ATP5A1, and HSPA1A). Proteins in cluster III are all mitochondrial proteins that function as small molecule binders.

new statistical methods); comparative proteomic studies (i.e. studies differentiating between chronic pain and controls); diagnostic proteomic studies (i.e. studies relating the protein pattern to pain aspects, intensity, sensitivity, spreading on the body, etc.), psychological distress (depression, anxiety, etc.), personal characteristics (body mass index, age, and gender/sex)); and monitoring proteomic studies (i.e. treatment and intervention studies). We only included studies in English published in peer-reviewed journals.

2.3. Intervention

No restrictions with respect to interventions were pre-defined.

2.4. Comparison

Proteins that (a) can be differentiated between controls and patients with chronic pain conditions and/or (b) proteins associated with relevant clinical variables.

2.5. Outcome

Proteins in muscle, blood, saliva and cerebrospinal fluid that (a) differentiated between controls and patients with chronic pain conditions (CNSP, CWP including FM, CLBP, and chronic neuropathic pain) and (b) proteins that were associated with relevant clinical variables in these pain conditions.

2.6. Data extraction

Independently the two authors identified relevant articles including type of study, proteins identified, methodology and results from the electronic searches. Any disagreement was resolved by consensus.

2.7. Quality of data assessment

Based on earlier reviews (not systematic) we anticipated the number of identified studies was low [68,71,72]. Hence, we chose to not systematically examine the methodological quality of every study. Instead, we chose the strategy to discuss

overall weaknesses in the studies we identified. Moreover, there is currently no generally accepted method for making quality assessments in proteomics. Thus, we adopted the advices presented by the editors of a special issue of Proteomics: ' … We suggest that as long as the data analysis approach used in an experiment is based on sound scientific principles and appropriate fundamental mathematics and statistics, and it is acknowledged that technical changes in the analysis could affect important conclusions, the method should be considered acceptable and the results should be given due consideration ….' [73].

2.8. Bioinformatics – network analyses

Using the online database tool STRING (version 11), we analyzed the protein-protein association network for the important proteins reported in the identified studies [67]. This was done both for comparative studies and for studies relating the proteome pattern to clinical variables (e.g. pain intensity). Protein accession numbers (UniProt) for the identified important proteins were entered in the search engine (multiple proteins) with the following parameters: organism was *Homo sapiens*; the maximum number of interactions was query proteins only; interaction score was set to minimum required interaction score of high confidence (0.700); and an FDR ≤ 0.05 was used when classifying the Biological Process (GO) of each protein. For each obtained network, PPI enrichment *P*-value was reported. In the network figures, each protein is represented by a colored node, and protein-protein interaction and association are represented by an edge represented by a line. Higher combined confidence scores are represented by thicker lines. The generated network was further investigated to identify a group of proteins that clustered together and the significant biological process for the cluster was identified. All significant (FDR≤ 0.05) biological processes that were identified are listed as a supplementary Excel file 1.

3. Results

The searches identified 27 articles (Supplementary Figure 1). The excluded full-text articles assessed for eligibility are listed in supplementary text file 2.

3.1. Basic characteristics

Basic characteristics of the identified studies are shown in Table 1. Cohorts of neck-shoulder pain (i.e. trapezius myalgia) were investigated in three studies, CWP including fibromyalgia (FM) in 14 studies, neuropathic pain conditions in nine studies, mixed chronic pain conditions (farmers with chronic musculoskeletal pain conditions) in one study, and nonspecific low back pain in two studies.

Five studies investigated muscle in chronic trapezius myalgia and CWP/FM. Saliva was investigated in three studies, which mainly concerned FM. Blood (plasma in all except one study) was examined in six studies of CWP/FM, in one study of mixed chronic pain conditions, and in one study of trigeminal neuralgia. CSF was examined in 12 studies, which focused on CWP/FM, neuropathic pain, and low back pain.

Women were mainly investigated in the studies concerning CWP/FM and trapezius myalgia. The FM cohorts generally consisted of women (Table 1). However, the FM group investigated in Ciregia et al.'s study included a few men [74], and several FM studies had healthy control groups that were mixed [74–76]. Studies of neuropathic pain conditions and low back pain included both sexes. The studies of neuropathic pain were generally mixed both in the patient group and in controls and therefore reasonably balanced [77–80]; however, there were exceptions [76].

Country of origin for the 27 identified studies was Sweden (n = 15), Italy (n = 6), Spain (n = 1), Iran (n = 1), China (n = 1), USA (n = 1), and mixed (n = 2).

The identified studies are briefly summarized below. When appropriate, we present the network analyses of the identified proteins.

3.2. Methodological and comparative studies

3.2.1. Chronic trapezius myalgia – muscle

This pain condition was investigated in three studies.

Olausson et al. investigated microdialysate from the trapezius muscle of two pain cohorts – trapezius myalgia and chronic widespread pain – and from a healthy group [81]. This study, using pooled dialyzate for each group, found that of the 262 identified proteins 48 proteins in trapezius myalgia and 30 proteins in CWP were expressed at least two-fold higher or lower than in controls. The altered proteins pertained to several functional classes (e.g. proteins involved in inflammatory responses) and in processes of pain (e.g. creatine kinase, nerve growth factor, carbonic anhydrase, myoglobin, fatty acid-binding protein, and actin aortic smooth muscle). In both groups of patients, 17 proteins showed alterations – 12 in a similar way and five in a unique way.

Hadrevi et al. investigated trapezius muscle biopsies of female cleaners with chronic trapezius myalgia and pain-free female cleaners [82]; 28 unique proteins of 847 proteins contributed to the separation of the two groups according to a multivariate discriminant analysis. The important proteins were related to the glycolysis, the tricarboxylic acid cycle, the contractile apparatus, the cytoskeleton, and to acute response proteins.

In a continuation study, the authors used proteomics to characterize the phosphorylation pattern of regulatory myosin light chain 2 (MLC2) in chronic trapezius myalgia [83]. MLC2 is a sacromeric protein expressed in several isoforms that regulate Ca^{2+} in muscle. In addition, the study used immune assay to determine the abundance of two other calcium regulatory proteins – calsequestrin and Ca2+ channel protein SERCA-1. The authors found an increased abundance of fast regulatory MLC, no differences in the degree of phosphorylation of MLC2, a higher abundance of SERCA-1 proteins, and a lower abundance of calsequestrin in subjects with trapezius myalgia compared to healthy subjects, findings that indicate difference in the contractile regulation independent of fiber type content, which might affect muscle pain due to an imbalance.

Table 1. Basic characteristics of the identified studies.

Authors	Year	Cohorts	Tissue	Sex	Type of study	Proteomic methods	Comments including pharmacological treatments and wash-out
Muscle							
Olausson et al. [81]	2012	TM (n = 37) CWP (n = 18) CON (n = 22)	Muscle dialysate	F	C	2-DE	Pooled samples used. NSAID medication was avoided the week before the study.
Hadrevi et al. [82]	2013	TM (n = 12) CON (n = 12)	Muscle	F	C	2-DE	Excluded subjects with oral steroids or NSAID drugs.
Hadrevi et al. [83]	2016	TM (n = 12) CON (n = 12)	Muscle	F	C	2-DE	Continuation of the previous article. Excluded subjects with oral steroids or NSAID drugs.
Olausson et al. [84]	2015	CWP/FM (n = 18) CON (n = 19)	Muscle	F	C	2-DE	Excluded subjects with anticoagulatory, continuous anti-inflammatory drug, opioid, or steroidal use.
Olausson et al. [96]	2016	CWP/FM (n = 18) CON (n = 19)	Muscle	F	D	2-DE	Excluded subjects with anticoagulatory, continuous anti-inflammatory drug, opioid, or steroidal use.
Saliva							
Bazzichi et al. [85]	2009	FM (n = 22) CON (N = 26)	Saliva	F	C + D	2-DE	Proportion of patients using drugs potentially inducing xerostomia reported; no washout.
Ciregia et al. [74]	2019	FM (n = 30) RA (n = 30) Migraine (n = 30) CON (n = 30)	Saliva	F & M	C + D	2-DE	Patients were on different pharmacological treatments; no washout.
Bazzichi et al. [98]	2013	FM (n = 40)	Saliva	F	Mo	2-DE	Pooled samples – 20 for each treatment arm. Patients were on different pharmacological treatments; no washout.
Blood							
CWP/FM							
Ruggiero et al. [86]	2014	FM (n = 16) CON (n = 12)	Serum	F	C + D	2-DE	Patients were on different pharmacological treatments; no washout.
Wåhlen et al. [87]	2017	CWP/FM (n = 16) CON (n = 23)	Plasma	F	C	2-DE	Excluded subjects with anticoagulatory, continuous anti-inflammatory drug, opioid, or steroidal use.
Ramirez-Tejero et al. [48]	2018	FM (n = 12) CON (n = 12)	Plasma	F	C	LC-MS/MS	None of the subjects used drugs affecting antioxidative status or were under treatment of corticosteroids, estrogens, analgesics, or anti-inflammatory drugs.
Wåhlen et al. [97]	2018	CWP/FM (n = 15) CON (n = 23)	Plasma	F	D	2-DE	Mainly results concerning CWP/FM. Excluded subjects with anticoagulatory, continuous anti-inflammatory drug, opioid, or steroidal use.
Other chronic pain							
Ghafouri et al. [88]	2016	CP (n = 13) CON (n = 11)	Plasma	M	C	2-DE	Pharmacological treatment/wash-out not mentioned.
Trigeminal neuralgia							
Farajzadeh et al. [77]	2018	Trigeminal neuralgia (n = 13) CON (n = 13)	Plasma	M & F	C + Mo	2-DE	Patients were on different pharmacological treatments; no washout.
CSF							
CWP/FM							
Olausson et al. [89]	2017	CWP (n = 12) CON (n = 13)	CSF	F	C	2-DE	Pharmacological treatment/wash-out not mentioned.
Khoonsari et al. [90]	2019	FM (n = 13) RA (n = 11) OND (n = 8)	CSF	F	C	LC-MS/MS	Pharmacological treatments mentioned for RA; NSAID not allowed 24 h before sampling. For FM, antidepressants not allowed and NSAID not allowed 24 h before sampling.
Khoonsari et al. [75]	2019	FM (n = 39) CON (n = 38)	CSF	FM: F CON: M & F	C	LC-MS/MS	Pharmacological treatment/wash-out not mentioned.
Lind et al. [76]	2019	FM (n = 40) Healthy CON (n = 11) Minor urology surgery CON (n = 28)	CSF	FM: F CON: M & F	C	LC-MS/MS	Pharmacological treatment/wash-out not mentioned.

(*Continued*)

Table 1. (Continued).

Authors	Year	Cohorts	Tissue	Sex	Type of study	Proteomic methods	Comments including pharmacological treatments and wash-out
Neuropathic pain							
Liu et al. [78]	2006	LBP with sciatica (NP) (n = 10) CON (n = 10)	CSF	M & F	C	2-DE	Pharmacological treatment/wash-out not mentioned.
Conti et al. [79]	2005	NP (n = 9) NPN (n = 8) CON (n = 9)	CSF	M & F	C	2-DE	Pharmacological treatment/wash-out not mentioned.
Pattini et al. [92]	2008	NP (n = 8) NPN (n = 8) CON (n = 8)	CSF	M & F	Me	2-DE	Based on data and characteristics of subjects reported in [79]. Pharmacological treatment/wash-out not mentioned.
Cannistraci et al. [93]	2010	NP (n = 7) NPN (n = 8) CON (n = 8)	CSF	M & F	Me	2-DE	Based on data presented in Conti et al. [79] and Pattini et al. [92]. Pharmacological treatment/wash-out not mentioned.
Bäckryd et al. [80]	2015	NP (n = 11) CON (n = 11)	CSF	M & F	C	2-DE	Patients were on different pharmacological treatments; no washout.
Bäckryd et al. [91]	2018	NP (n = 11) CON (n = 11)	CSF	M & F	C + D	2-DE	Patients were on different pharmacological treatments; no washout.
Lind et al. [76]	2019	NP group 1 (n = 14) Minor urology surgery CON (n = 28) NP group 2 (n = 11) Healthy CON (n = 11)	CSF	M & F	C	LC-MS/MS	This study also reported results FM vs. controls – see above Pharmacological treatment/wash-out not mentioned.
Lind et al [99]	2016	NP (n = 14) as own controls	CSF	M & F	Mo	LC-MS/MS	Patients used spinal cord stimulation as treatment. Pharmacological treatment/wash-out not mentioned.
Other pain conditions							
Lim et al [95]	2017	LBP with DD (n = 8) CON with DD (n = 8) CON (n = 6)	CSF	M & F	C + D	LC-MS/MS	It was not obvious that these patients had sciatica. Excluded subjects with steroids, narcotics, anti-inflammatory, or algesic drugs. They also excluded subjects using antidepressants not receiving a steady dose for ≥2 months.
Yuan et al [94]	2002	Idiopathic LBP (n = 3)	CSF	Not reported	Me	2-DE	Pharmacological treatment/wash-out not mentioned.

Cohorts: CWP=chronic widespread pain; FM=fibromyalgia; RA=rheumatoid arthritis; TM=chronic trapezius myalgia; NP=chronic neuropathic pain; NPN= Neuropathy without pain; CP=farmers with chronic musculoskeletal pain; LBP=low back pain; OND=Other Neurological Diseases (i.e., patients with noninflammatory neurological symptoms and without pain).
Sex/gender: F=female gender/sex; M=male gender/sex.
Type of study: Me=methodological; C=Comparative; D=Diagnostic, Mo=Monitoring
Proteomic methods: 2-DE= Two-Dimensional gel electrophoresis; LC-MS/MS=the combination of liquid chromatography (LC) with mass spectrometry (MS). MS/MS is the combination of two mass analyzers in one mass spectrometry instrument.

Both Olausson et al. and Hadrevi et al. identified the following proteins as important for group separation: actin aortic smooth muscle, actin cytoplasmic, serum albumin, carbonic anhydrase 3, beta-enolase, and alpha-1-antitrypsin [81,82].

3.2.1.1. Muscle proteins in chronic trapezius myalgia – network analysis.

Network interaction analysis was performed using the identified proteins from three studies that compared trapezius myalgia to healthy controls [81–83]. The significant protein-protein Interaction (PPI) enrichment analysis separated the identified proteins into three clusters (Figure 1). Proteins in cluster I were associated with muscle fiber component functions such as motor activity and cytoskeletal protein binding (actin, microtubule, or intermediate filament cytoskeleton). Proteins in cluster II consisted of cytoplasm components that inhibit enzyme/enzyme activity, ion/protein binding, and microfilament motor activity. Cluster III included mitochondrial proteins involved in small molecule binding.

3.2.2. CWP/FM – muscle

As mentioned above, Olausson et al. investigated microdialysate from the trapezius muscle of two pain cohorts – trapezius myalgia and chronic widespread pain – and from a healthy group [81].

In addition, multivariate analysis of muscle biopsies revealed 17 proteins of more than 200 proteins that were highly significant and that could be used to differentiate patients from controls [84]. The important proteins were enzymes in metabolic pathways (e.g. glycolysis and gluconeogenesis) and proteins associated with stress, inflammation, muscle damage, and muscle recovery.

Both muscle biopsies and microdialysate found the same altered protein – carbonic anhydrase 3 [81,84].

3.2.2.1. Muscle proteins in CWP/FM – network analysis.

The PPI enrichment analysis of the altered proteins in muscle from CWP/FM compared to controls was significant [81,84], indicating that the proteins were at least partially biologically connected (Figure 2). Three clusters were identified. Proteins in cluster I included extracellular proteins that contribute to enzyme binding and ion binding. Proteins in cluster II are enzymes involved in small molecule metabolic processes and phosphorylation. Proteins in cluster III are involved in the muscle system.

3.2.3. FM – saliva

Three articles investigated saliva samples from patients with FM and two were comparative [74,85].

Bazzichi et al. compared FM patients with sex- and age-matched healthy subjects [85]. In FM, 11 proteins were significantly overexpressed; the strongest over-expression was found for transaldolase and phosphoglycerate mutase I.

Ten years later, Ciregia et al., in a comparison of several patient groups (FM, RA, and migraine) with healthy controls, identified 23 proteins including proteoforms (12 unique proteins) that were significantly differently expressed in FM compared to controls [74]. The best discriminate power was attributed to a combination of alpha-enolase, phosphoglycerate-mutase-1, and serotransferrin.

The common altered proteins in the two studies were transaldolase, protein S100-A8, and phosphoglycerate-mutase-1 [74,85].

3.2.3.1. Saliva proteins in fibromyalgia – network analysis.

The significant PPI enrichment analysis of altered proteins in saliva in FM compared to controls identified three protein clusters (Figure 3) [74,85]. Proteins in cluster I are cytoskeletal proteins that bind actin, proteins in cluster II are secretory enzymes, and proteins in cluster III include secretory proteins that are involved in ion and protein binding.

3.2.4. CWP/FM – blood

We found three comparative studies of blood in FM [48,86,87].

In a preliminary study analyzing serum from FM patients and healthy controls, Ruggero et al. identified three proteins that were significantly increased in FM: transthyretin, alpha1-antitrypsin, and retinolbinding protein 4 [86].

Wåhlén et al., in an analysis of plasma from CWP (mainly FM) and healthy controls, identified 22 proteins (plus proteoforms) of more than 400 proteins that were significantly altered according to the advanced multivariate analyses [87]. The 22 altered proteins were divided into four classes (according to the uniport database): immunity, metabolic, iron ion hemostasis, and inflammatory processes.

Finally, Ramirez-Tejero et al. found a total of 33 proteins differentially expressed in FM compared to controls [48]. Using a network analysis, the authors concluded that the different biological pathways involved in the identified protein profile were related to inflammation. Five dominant pathways (according to their *P*-value) were identified as enriched: LXR/RXR activation, FXR/RXR activation, coagulation system, complement system, and the acute phase response signaling. Haptoglobin and fibrinogen were suggested as potential biomarker candidates.

Both Wåhlén et al. and Ramirez-Tejero et al. found that haptoglobin, serotransferrin, fibrinogen gamma chain, and the protein complement C1s subcomponent were altered [48,87]. Furthermore, both Wåhlén et al. and Ruggero et al. found that transthyretin was altered [86,87].

3.2.4.1. Blood proteins in fibromyalgia – network analysis.

The PPI enrichment analysis of altered proteins in plasma from CWP/FM compared to controls was highly significant [48,86,87], a finding that indicates the proteins were at least partially biologically connected as two large groups (clusters I and II) (Figure 4). Cluster I was dominated by proteins involved in post-translational modifications and in regulation of cellular protein metabolic processes. Cluster II included proteins involved in complement activation. Many proteins involved in the immune system were identified in the whole network, including clusters I and II.

3.2.5. Mixed pain conditions in farmers – plasma

In a plasma study of male farmers with musculoskeletal disorders and healthy controls, Ghafouri et al. found that 15 proteins of more than 200 proteins differed significantly

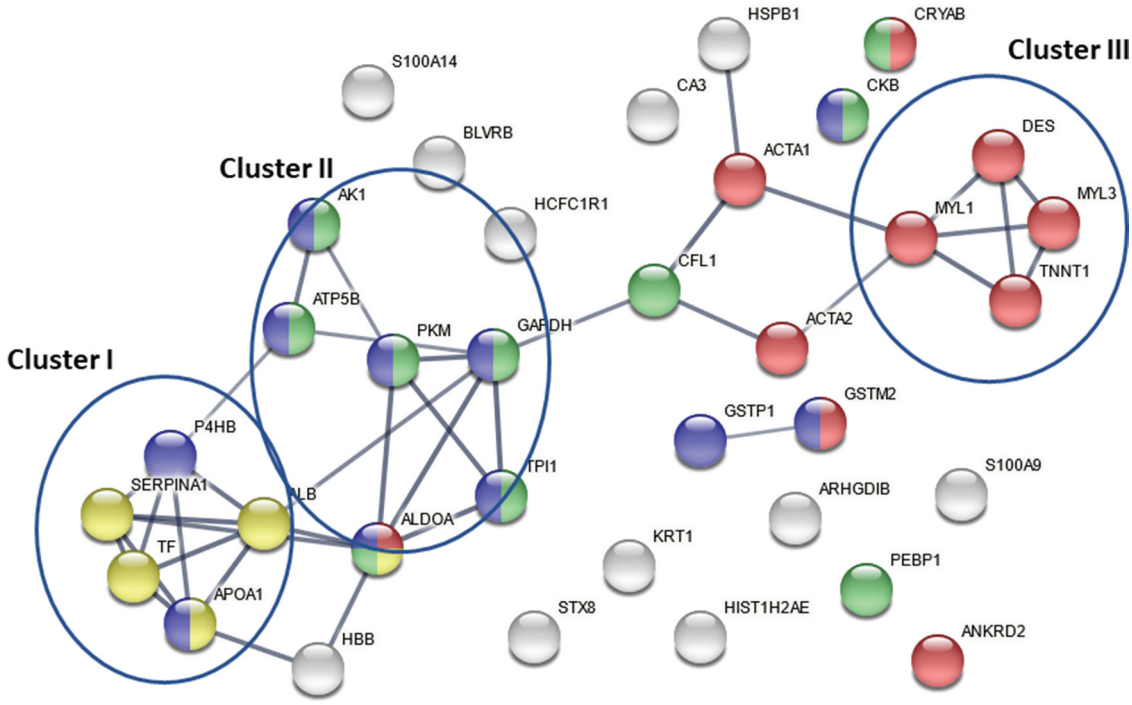

Figure 2. Pathway analysis for altered proteins in muscle from CWP/FM compared to controls [81,84]. The protein-protein interaction (PPI) enrichment analysis had a *P*-value < 1.0e-16, indicating that the proteins are at least partially biologically connected as a group. Three clusters were identified. Cluster I was dominated by proteins involved in platelet degranulation (yellow – four proteins: SERPINA1, TF, APOA1, and ALB). Cluster I included extracellular proteins that affect enzyme binding and ion binding. Proteins in cluster II (AK1, ATP5B, PKM, GAPDH, ALDOA, and TP11) were involved in small molecule metabolic process (blue) and phosphorylation (green). The four proteins in cluster II are enzymes. The proteins in cluster III were s involved in muscle system processes (red: MYL1, MYL3, DES, and TNNT1). Proteins in cluster III are part of the sarcomere, contractile fiber, myosin binding, and cytoskeletal protein binding.

between the two groups and that several of the identified important proteins were mediators or indicators of inflammation [88].

3.2.5.1. Plasma proteins in mixed pain conditions (farmers) – network analysis. The significant PPI enrichment analysis of altered proteins in plasma from farmers with musculoskeletal disorders compared to controls identified two groups of proteins: clusters I and II (Supplementary Figure 2) [88]. Proteins in cluster I were involved in platelet degranulation and proteins in cluster II were involved in transport. Both proteins in clusters 1 and 2 are extracellular proteins that signal receptor binding, transporter activity, and enzyme inhibitor activity.

3.2.6. Trigeminal neuralgia – plasma

Farajzadeh et al., investigating plasma samples in patients with trigeminal neuralgia and controls, identified four significantly altered proteins (upregulated) in the patient group: retinol-binding protein 4, transthyretin (two proteoforms), and alpha-1-acid glycoprotein 2 [77].

3.2.6.1. Plasma proteins in trigeminal neuralgia – network analysis. The PPI enrichment analysis of this study showed that the three proteins were significantly connected to each other (Supplementary Figure 3). The proteins were extracellular proteins involved in neutrophil degranulation and retinol metabolic process.

3.2.7. CWP/FM CSF

Four CSF studies compared CWP/FM with controls [75,76,89,90].

Using advanced multivariate analysis, Olausson et al. found that 48 proteins (of 481 proteins) discriminated between patients (12 females) and controls (13 controls) [89]. The most discriminative proteins were involved in immunity, apoptotic regulation, endogenous repair, and anti-inflammatory and anti-oxidative processes.

Khoonsari et al. published two articles in 2019 that used proteomic techniques [75,90].

In the first exploratory study, the authors identified 176 known pain-related proteins in CSF [90]. From three groups of subjects of FM, RA, and as controls other neurological diseases (i.e. noninflammatory neurological symptoms without pain) they demonstrated that 96 proteins had importance for significantly distinguishing the three groups; ten of these were pain proteins.

Khoonsari et al., in an investigation of 39 female FM patients and 38 non-pain controls (five women and 33 men), reported that the level of changes between patients and controls was relatively moderate [75]: four proteins were associated with FM (three increased and one decreased).

Lind et al. investigated several groups of patients, including FM patients (n = 40). FM was compared with two groups of controls – healthy controls (n = 11) and controls who had undergone minor urological surgery (n = 28) [76]. Lind et al. found highly significant regressions differentiating FM from

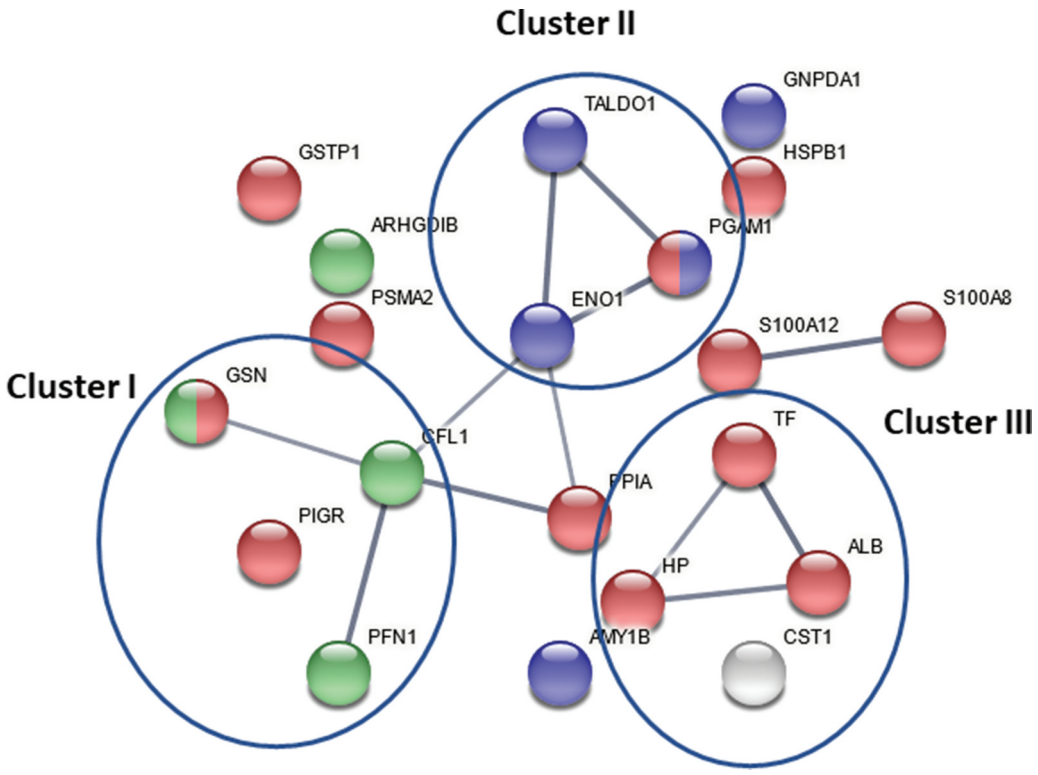

Figure 3. Pathway analysis of altered proteins in saliva in FM compared to controls [74,85]. The protein-protein interaction (PPI) enrichment analysis (*P*-value = 7.81e-09) identified three protein clusters. Cluster I includes proteins involved in regulation of actin cytoskeleton organization (green: PFN1, CF1, and GSN). The biological process that proteins in cluster II were involved include the carbohydrate metabolic process (blue: TALDO1, ENO1, and PGAM1). Cluster III was dominated by proteins involved in transport (red: TF, HP, and ALB). Clusters I and II were connected by a transport protein – PPIA. Proteins in cluster I are cytoskeletal proteins that bind actin. Proteins in cluster II are secretory enzymes, and cluster III includes secretory proteins that affect ion and protein binding.

the two groups of controls. In the analysis comparing FM and healthy controls, seven proteins were important and for FM versus the other control group one protein was important; compared to both cohorts of controls, ENPP2 was increased in FM.

The only overlapping protein from all four studies was malate dehydrogenase.

3.2.7.1. CSF proteins in CWP/FM – network analysis. The significant PPI enrichment analysis of altered proteins in CSF from patients with CWP/FM compared to healthy controls identified a cluster of proteins involved in transport (Figure 5) [75,76,89]. The proteins in this cluster are extracellular proteins involved in signaling receptor binding. Khoonsari et al.'s exploratory study was not included as it did not include health controls [90].

3.2.8. Neuropathic pain conditions – CSF

Five comparative studies [76,78–80,91] and two methodological studies [92,93] were identified.

Liu et al., in a comparison of CSF from patients with sciatica and healthy controls, found that 15 proteins were significantly altered [78]. These proteins were classified into six groups based on their characteristics and functions: (1) signal protein; (2) signal/transport and binding protein; (3) cytoskeletal protein; (4) antioxidant protein; (5) immune-related protein; and (6) transport and binding protein. Most of the differentially expressed proteins had clear relationships with nerve injury, and their changes were consistent with what the literature reports.

Conti et al., in an investigation of neuropathic pain patients, neuropathic pain-free patients, and controls, found four important proteins for differentiating the groups: cystatin C, FAM3 C protein, Human Monoclonal IgM Cold Agglutin, and pigment epithelium-derived factor (three proteoforms) [79]. In the subjects with pain, cystatin C was increased and Human Monoclonal IgM Cold Agglutin was decreased compared to the other two groups.

The study by Pattini et al. is a continued study of Conti et al., using the 2-DE gels from majority of the same subjects presenting an automatized strategy in two-dimensional electrophoresis analysis. The differentially expressed proteins between the groups were published in Conti et al. and in this study they don't present any new information on protein levels with respect to the three groups of subjects investigated [92].

Essentially using the data obtained by Conti et al., Cannistraci et al. presented a method for nonlinear dimension reduction and clustering suited for nonlinear small datasets [93].

Bäckryd et al., comparing CSF from patients with peripheral neuropathic pain with healthy controls, found that 32 proteins of 260 proteins were highly associated with class/group

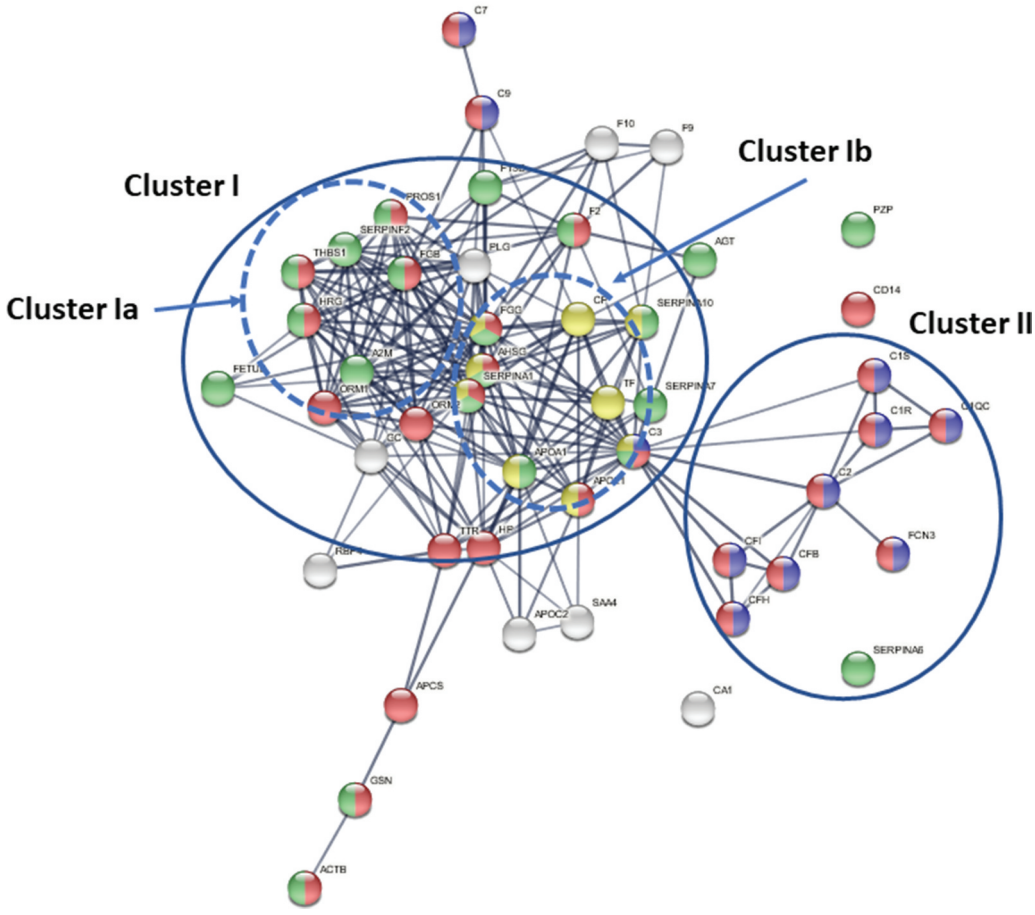

Figure 4. Pathway analysis of altered proteins in plasma from CWP/FM compared to controls [48,86,87]. The protein-protein interaction (PPI) enrichment analysis was highly significant (*P*-value< 1.0e-16), indicating the proteins were at least partially biologically connected as two large groups (clusters I and II). Cluster I was dominated by proteins involved in regulation of cellular protein metabolic process (green: A2M, AHSG, FGB, FGG, GSN, HRG, PROS1, FETUB, SERPINA1, SERPINF2, and THBS1) and proteins involved in PTM (yellow: AHSG, APOA1, APOL1, C3, CP, FGG, SERPINA1, SERPINA10, and TF). The proteins in cluster II were involved in complement activation (blue: C1QC, C1R, C1S, C2, C3, CFB, CFH, CFI, and FCN3). Many proteins involved in the immune system (red: A2M, ACTB, APCS, APOA1, C1QC, C1R, C1S, C2, C3, C7, C9, CD14, CFB, CFH, CFI, F2, FCN3, FGB, FGG, GSN, HRG, ORM1, ORM2, PROS1, RBP4, and THBS1) were identified in the whole network including clusters I and II.

discrimination after controlling for possible age effects [80]. Seven proteins expressed as several proteoforms had very high discriminatory power and the protein with highest discriminatory power was a proteoform of angiotensinogen.

Bäckryd et al. investigating post-translational modifications of the same subjects using 2-DE [91] found that the proteoforms identified in their previous study [80] were glycosylated: N-terminal and C-terminal truncated. They concluded that altered levels of fragments and/or glycosylated isoforms of alpha-1-antitrypsin might mirror pathophysiological processes in the spinal cord of patients with chronic peripheral neuropathic pain [91].

Lind et al. (see above), also investigating neuropathic pain, made two regression analyses to identify differentiating proteins [76]. When comparing their first neuropathic group with minor urology surgery controls, they identified four important proteins. However, they were not able to find a significant regression for group differentiation when using their second neuropathic group versus the healthy controls. They concluded that subtle differences in level of proteins exist between neuropathic pain and controls. However, they found indications that apolipoprotein C1 was increased in neuropathic pain.

Both Conti et al. and Bäckryd et al. found pigment epithelium-derived factor, and both Liu et al. and Bäckryd et al. found prostaglandin-H2 D-isomerase [78–80].

3.2.8.1. CSF proteins in neuropathic pain – network analysis.

The pathway analysis of altered proteins in CSF from patients with neuropathic pain compared to healthy controls is based on three studies (Figure 6) [78–80]. The PPI enrichment analysis was highly significant and identified three groups of proteins: proteins involved in inflammatory responses, proteins involved in immune responses, and proteins involved in metabolic processes.

3.2.9. Other pain conditions – CSF

The CSF of three patients with idiopathic low back pain was investigated by Yuan et al. [94]. This is a methodological study without any comparison group; they identified 22 proteins.

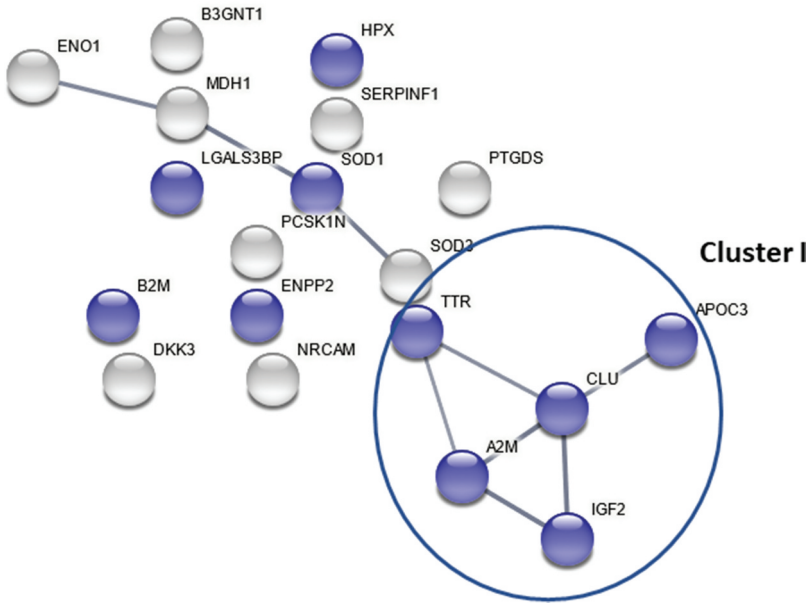

Figure 5. Pathway analysis of altered proteins in CSF from patients with CWP/FM compared to healthy controls [75,76,89]. The protein-protein interaction (PPI) enrichment analysis (*P*-value = 1.17e-07) identified a group of proteins involved in transport (cluster I, blue: APOC3, CLU, TTR, A2M, and IGF2). The proteins in cluster I are extracellular proteins involved in signaling receptor binding.

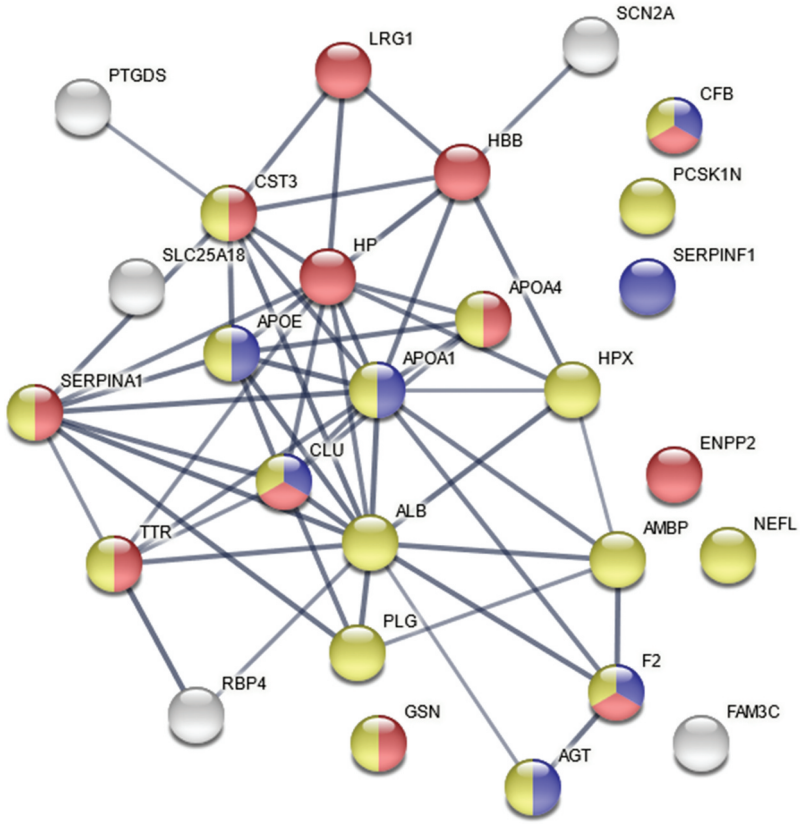

Figure 6. Pathway analysis of altered proteins in CSF from patients with neuropathic pain compared to healthy controls [78–80]. The protein-protein interaction (PPI) enrichment analysis was highly significant (*P*-value < 1.0e-16) and identified three groups of proteins: proteins involved in inflammatory responses (blue), in immune responses (red), and in metabolic processes (yellow). The proteins are extracellular with molecular functions such as enzyme activity, protein/lipid/ion binding, and transporter activity.

Lim et al. – comparing CSF samples of patients with low back pain (LBP) with disc degeneration (DD),[1] pain-free controls with DD, and healthy controls without DD – found 12 proteins that were significantly altered in LBP with DD [95]. Eight proteins were uniquely altered in painful DD but not in pain-free patients versus healthy controls: proSAAS, hemopexin, prosaposin, beta-

2 microgubulin, insulin-like growth factor II, and apolipoproteins A-IV, D, and E. Lim et al. concluded that DD was related to inflammation regardless of pain level, while painful DD was associated with biomarkers linked to nerve injury.

3.2.9.1. CSF proteins in other pain condition – network analysis. The significant PPI enrichment analysis (*P*-value < 1.0e-16) of altered proteins in CSF from patients with low back pain compared to healthy controls grouped the identified proteins as transport proteins (Supplementary Figure 4) [95]. These extracellular proteins have molecular functions connected to cholesterol regulation, enzyme regulator activity, and meta ion and protein binding.

3.3. Protein patterns versus clinical variables

Eight articles analyzed whether the protein pattern obtained correlated with clinical variables (Table 2).

3.3.1. CWP/FM

Olausson et al., investigating the proteome from muscle biopsies of the painful trapezius in CWP/FM, found that 12 proteins were multivariately associated with pain intensity [96]. Sixteen proteins were multivariately associated with pressure pain thresholds (PPTs) in CWP/FM; no significant correlation was evident in the controls. The network analyses of these two variables are shown in Supplementary Figure 5.

Bazzichi et al., using saliva from FM patients, found no significant correlations between the identified proteins and clinical characteristics (Fibromyalgia Impact Questionnaire (FIQ) – Pain intensity and tender points) [85].

Ciregia et al., also investigating saliva from FM, found that proteins with the best discriminate power between groups did not correlate with clinical variables [74].

Ruggero et al., analyzing serum in FM, found increased levels of transthyretin, alpha1-antitrypsin, and retinolbinding protein 4 [86]. No correlations were found with clinical characteristics (i.e. duration, pain intensity, or FIQ).

Wåhlén et al., using the same cohorts as in their study from 2017 and advanced multivariate regressions, reported that the proteomic profile in plasma in CWP/FM correlated with pain intensity and psychological distress [97]. Pain intensity was highly significantly associated with 20 plasma proteins, which were mostly involved in metabolic and immunity processes according to Uniprot (e.g. kininogen-1, ceruloplasmin, and fibrinogen gamma chain). Psychological distress was significantly associated with 18 plasma proteins (including proteoforms) related to iron ion, immunity response, and lipid metabolism (e.g. complement factor B, complement C1r subcomponent, hemopexin, and clusterin). With respect to these two clinical variables, the protein patterns generally differed in CWP/FM. The network analyses of these two clinical variables are shown in Supplementary Figure 6.

3.3.2. Neuropathic pain

Conti et al. – investigating CSF from neuropathic pain patients, patients with neuropathy without pain, and controls – found no correlations between Cystatin C and pain aspects (intensity and duration) [79]. Bäckryd et al., investigating CSF in neuropathic pain, identified proteins from CSF with high fit and predictivity associated with pain intensity and pain duration in the patients [91]. The network analyses of these two variables are shown in Supplementary Figure 7.

Lim et al. – analyzing CSF samples between LBP with disc degeneration (DD), pain free controls with DD, and healthy controls without DD – found a correlation between Cystain C and severity of DD and a correlation between hemopexin and DD severity, pain intensity, and pain experience (McGill Pain Questionnaire (MPQ)) and disability (Oswestry Disability Index (ODI)) [95].

3.4. Protein patterns – interventions

Our review identified three studies that investigated the effects of interventions for patients with chronic pain [77,98,99].

The effects of balneotherapy and mud-bath therapy for patients with FM were investigated [98]. Four proteins showed significant difference of expression (increases): Rab GDP dissociation inhibitor beta, zinc-alpha-2- glycoprotein, trandolase, and phosphoglycerate mutase 1.

In trigeminal neuralgia, four plasma proteins were upregulated (see above) [77]. After microvascular decompression, three of these proteins (retinol-bindning protein 4 and two proteoforms of transthyretin) were downregulated and one (alpha-1-acid glycoprotein 2) did not change.

The proteomic alterations associated with spinal cord stimulation were investigated in neuropathic pain patients [99]. The authors compared a situation with the stimulator turned off for 48 hours and when the stimulator had been used for three weeks; 86 proteins were significantly altered. The most important 12 proteins were involved in neuroprotection, immune regulation, nociceptive signaling, and synaptic plasticity/learning/memory. The authors also performed a network analysis, which was interpreted as spinal cord stimulation; they found that the stimulation affected inflammation and the balance of degeneration and regenerative processes.

4. Discussion

4.1. Major results

- In the field of common chronic pain conditions, 27 relatively small proteomic studies were identified that examined muscle, blood, saliva, and CSF; the number of studies per diagnoses and tissue were few.
- Most studies focused on identifying protein patterns differentiating between chronic pain and healthy controls; the statistical approaches showed prominent differences.
- Few studies investigated the protein pattern's relationship to relevant clinical variables; the methodology, including the statistical methods, showed marked differences.
- Two studies used formal network analyses. The network analyses performed in the present review within each area (diagnosis and tissue) generally identified significant/highly significant PPI enrichment analyses.

Table 2. Studies relating the protein pattern to clinical variables; for details concerning cohorts see Table 1.

Authors	Year	Pain and Tissue	Clinical variables	Statistical analysis	Results and comments
Olausson et al. [96]	2016	CWP/FM – muscle	PI PPT	OPLS	12 proteins correlated significantly with PI in CWP/FM. 16 proteins correlated significantly with PPT in CWP/FM but no significant correlation in CON.
Bazzichi et al. [85]	2009	FM – saliva	PI FIQ TP	Spearman's rank correlation	No significant correlations between clinical variables and transaldolase and phosphoglycerate mutase-1. No correlations reported for the other proteins identified. Note that proteins that differed between groups were used in the analyses.
Ciregia et al. [74]	2019	FM -saliva	PI FIQ FIQR TP FACIT	Spearman's rank correlation	No significant correlations between clinical variables and proteins different in FM. Note that proteins that differed between groups were used in the analyses.
Ruggiero et al. [86]	2014	FM -blood	PI Pain duration FIQ	unclear	No correlations were found between the important proteins (alpha1-antitrypsin, transthyretin and retinolbinding protein 4) and clinical characteristics. Note that proteins that differed between groups were used in the analyses.
Wåhlen et al. [97]	2018	FM/CWP – blood	PI HADS-total Age BMI	OPLS	20 proteins correlated significantly with PI in CWP/FM. 18 proteins correlated significantly with HADS-total in CWP/FM. 12 proteins correlated significantly with HADS-total in CON. 12 proteins correlated significantly with age in CWP/FM. 19 proteins correlated significantly with age in CON. 21 proteins correlated significantly with BMI in CWP/FM. 31 proteins correlated significantly with BMI in CON.
Conti et al. [79]	2005	NP – CSF	PI Pain duration	unclear	The article mentions that no correlations existed between Cystatin C and pain intensity or pain duration (data not shown).
Bäckryd et al. [91]	2018	NP-CSF	PI Pain duration	OPLS	21 proteins correlated significantly with PI in NP. 16 proteins correlated significantly with pain duration in NP.
Lim et al. [95]	2017	LBP-CSF	PI MPQ ODI Thompson scale	Spearman's rank correlation	Cystain C correlated with Thompson scale. Hemopexin correlated with Thompson scale, PI, MPQ, and ODI. Note that they used selected proteins that differed between groups for their analyses.

PI = pain intensity; PPT = pressure pain threshold; OPLS = orthogonal partial least squares regression; FIQ = Fibromyalgia Impact Questionnaire; TP = number of tender points; FIQR = Revised Fibromyalgia Impact Questionnaire; FACIT = Functional Assessment of chronic Illness Therapy-Fatigue; HADS-total = sum of the two subscales of Hospital Anxiety and Depression Scale; BMI = Body mass index, MPQ = McGill pain questionnaire-measures pain experience; ODI = Oswestry Disability. Index: Thompson scale = severity of DD.

4.2. Comparative proteomics: studies differentiating between chronic pain and controls

The comparative studies were exploratory and the largest studies included approx. 40 patients [75,76]. Suppers et al. provide advice on how to optimize the sample size [49]. The importance of large sample sizes (several hundreds of subjects) for obtaining valid results was clearly illustrated in a proteomic study on obesity [100]. However, more pre-analytical parameters can then affect results. For example, dividing the material into different batches is needed to handle the analysis process, which in turn requires a good design for how to randomize the samples into different batches and good control of the variation between different batches. Using larger cohorts of a certain diagnosis, researchers may be able to identify and characterize proteomic mechanisms in subgroups of patients. Hence, larger studies are needed to develop true mechanistic classification of patients with chronic pain.

The patients participating in these studies were generally selected patients as these were part of an exploratory phase of a research area. Future larger studies should include patients from the regular non-selected flow within primary care and/or specialized clinics to reduce volunteer bias, i.e., those willing to participate in traditional research studies may be systematically different from other patients with chronic pain [101].

Most of the identified 27 studies described the patients based on their clinical diagnosis according to ICD10. The most common pain conditions investigated within the frames of this review were CWP/FM and chronic neuropathic pain. Generally, the studies compared one diagnosis with healthy controls. Although several pain diagnoses can identify which changes are general and which are specific, few studies included more than one pain group [74,76,79,81,90,95].

In clinical practice, most pain diagnoses, with the exception for neuropathic pain, are based on duration and anatomical distribution of the pain. The need for mechanism-based classifications of pain conditions (nociceptive, nociplastic, neuropathic, or combinations) in clinical practice is increasingly emphasized. Although the application of mechanism-based classification in clinical practice will be a step forward, both diagnoses (according to ICD) and clinical classifications of the involved pain mechanisms contain prominent subjective elements as they are based on clinical assessments of history and examination findings. That is, the responsible physician subjectively assigns a diagnosis and/or which pain mechanisms best describe the diagnosis.

The interpretation of protein patterns is complicated as chronic pain patients show a wide spectra of associated factors and/or consequences of their pain condition. Patients with a certain chronic pain diagnosis show considerable differences in clinical presentations – e.g. not all patients with chronic pain show psychological distress [102]. For example, using cluster analyses of patients' self-reports has shown that chronic pain conditions are heterogenous and subgroups can be identified. In addition to differences in clinical presentations, these subgroups differ in outcome of treatment and work ability [103–105]. The chronic pain cohorts investigated in proteomic research may be heterogeneous with respect to these comorbidities and consequences. A few studies analyzed here considered the individual clinical presentation (e.g. presence of psychological distress or obesity). Future proteomic studies should more thoroughly report the clinical presentations and their variations. The Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials (IMMPACT) and the Validation and Application of a patient-relevant core set of outcome domains to assess multimodal PAIN therapy (VAPAIN) have listed important variables that can be used to systematically describe pain cohorts [106–108].

The associated factors such as psychological distress, obesity, etc., can per se be associated with proteomic alterations. Proteomic and cytokine studies of major depressive and anxiety disorders have reported associations with inflammation, metalloproteinases, and insulin-related pathways [109–113]. Increased BMI is associated with chronic pain and the two conditions adversely influence each other as well as share comorbidities such as hypertension, anxiety, and depression [114]. Proteomic studies report that obesity is associated with inflammation and weight loss is associated with sustained reduction of low-grade inflammation [100,115]. In addition, insomnia is prevalent in chronic pain conditions and interacts in complex ways with anxiety, pain intensity, and depressive symptoms [116]. In some of the studies, age differed between patients and controls, a situation that makes comparisons difficult as aging, including frailty, shows complex proteomic alterations such as chronic systemic inflammation and alterations in immune responses and in the cardiovascular system [117–119]. Imbalance gender between patient group and control group may also be problematic as parts of the proteome in non-sexual organs and fluids may be sex dependent [120,121]. Several of the identified studies made efforts to control for such sex imbalances. Larger studies should investigate whether differences in proteomic patterns exist between men and women in the pain cohort. This information may help explain why women have a higher prevalence and pain severity of chronic pain [122–125]. Furthermore, consequences such as physical inactivity may be associated with proteomic changes. The above briefly mentioned factors require clearly distinguishing which proteins and/or biological processes are primarily involved in the nociceptive and pain processes and which are responsible for the broader picture (i.e. the clinical presentation of having pain).

Although the effects of pharmacological treatments for chronic pain conditions are small compared with acute pain, many patients are prescribed pharmacological treatments on a trial-and-error basis. As obvious from Table 1, not all studies report presence of pharmacological treatments. Wash-out periods were seldom used or reported for NSAID treatments. Future proteomic studies should systematically report the following: (a) whether certain pharmacological treatments resulted in exclusion; (b) the medications used by the patients and the controls; and (c) whether wash-out periods for certain drugs increase the understanding of proteomic results.

Both peripheral (muscle, saliva, and blood) and central (CSF) tissues have been investigated when differentiating patients and controls. Most published studies find several

proteins or protein patterns that differ significantly between patients and controls. However, when several studies within a field (i.e. diagnosis and tissue) exist, the extent of overlap on individual protein level is limited. This finding is also the case for other clinical areas applying proteomics.

The statistical approaches for identifying proteins differentiating patients from controls differed. The identified studies are generally characterized by the 'small n large *P*' problem. Hence, classic statistical methods such as t-tests, ANOVA (and their non-parametric alternatives), multiple regression, and logistic regression are not applicable for such datasets. Several studies used these classic statistical tests, but these methods do not consider the complex interplay between proteins forming biological networks and are associated with multiple testing problems [49]. Other studies applied advanced multivariate statistical methods capable of handling the characteristics of the datasets typical for proteomic studies. This approach puts less focus on single proteins, but captures various proteins representing one or several biological processes (i.e. a system biology approach). Pros and cons of suitable advanced multivariate methods have been described elsewhere [49,126,127].

4.3. Diagnostic proteomics – studies relating the proteome to pain aspects, psychological distress, and personal characteristics

A diverse overall picture was found with respect to whether clinical variables were associated with certain proteins or protein patterns (Table 2). Different statistical approaches were chosen for these analyses. Some studies used the proteins to differentiate patients from controls and investigated whether these proteins bi-variately correlated with various clinical variables [74,79,85,95]. Except for Lim et al.'s study, these studies failed to show significant correlations. Other studies did not presuppose that using proteins to differentiate between patients and controls was important for identifying associations with clinical variables [91,96,97]. These studies found significant associations between mainly other protein patterns and clinical variables such as pain intensity, pain sensitivity, and psychological distress. The three studies used advanced multivariate regression analyses to handle significant correlation patterns (multicollinearity) among the proteins.

As discussed above, the clinical presentations differ across patients. Understanding how to handle the fact that clinical presentations across patients show considerable variability and that some proteins may be important for both, e.g., pain intensity and psychological distress are issues that need further examination. This examination is complicated because a certain protein may participate in several biological processes and the same biological process can be important for several clinical variables. Eliminating common proteins in the multivariate correlation analyses may be associated with the inability to identify important molecular mechanisms. Acknowledging that chronic pain is a complex condition may necessitate the use of a more complex clinical independent variable than, for example, anxiety, pain intensity, and depressive status. One way forward may be the use of a multivariate measure obtained from the t-scores of advanced principal component analysis of clinically important variables. This new variable may capture the degree of severity in a broad sense so it can be used as the independent variable when regressing the associations with proteins. In a second step, it may be reasonable to separately investigate the proteomic associations with the individual clinical variables (e.g. anxiety, pain intensity, and depressive symptoms). Hence, such an initial analysis may increase the interpretability of such studies.

Clinical experience suggests that chronic pain varies, for example, between visits to the health care system. As noted above, the pain experience is formed at a given moment in a complex interaction between psychological factors, neurobiological factors, and social/contextual factors. Davis and Cheng discuss trait and state pain [128]. The experience of pain is affected by each such individual component but the impact is more complicated than that because there are interactions between the various components which may be stronger and more complex [5]. Two patients may have the same average pain intensity (i.e. same trait) over the previous four weeks but different degrees of variation in the intensity of the pain (i.e. different state) [128]. Together, trait pain and state pain contribute to individual differences (i.e. pain being unique for each individual) and may be part of the explanation for variations in pain intensity and other aspects of having chronic pain. Not only pain intensity shows variations but there is also extreme dynamism of the proteome. Repeated registrations of clinical variables and sampling of blood or saliva for proteomic analyses, during a specific period using multivariate time series analyses might shed further light on the association of clinical variables with specific protein patterns [129].

4.4. Monitoring proteomics – intervention studies

We identified three small intervention studies of different pain conditions that found significant changes for several investigated proteins [77,98,99]. These promising results may indicate that such studies can provide further insights into how treatments act on a molecular level. Within the field of interdisciplinary treatment for patients with chronic pain conditions, panels of cytokines and chemokines show interesting alterations [130,131]. Although outcomes of pain interventions usually are evaluated using Patient Reported Outcome Measures (PROMs), these intervention studies open up the possibility of developing biologically measurable outcomes using different body fluids. Such biomarker panels will be an important tool for further development of treatments for chronic pain patients. However, there is a need for larger and more strictly controlled studies of treatments with respect to protein profiles. Such studies should consider diagnoses, pain mechanisms, additional therapies, and responders/non-responders.

4.5. Network analysis

The studies generally identified proteins differentiating patients from controls both in peripheral tissues and in CSF.

Hence, the included pain conditions appear to be associated with protein changes in the peripheral tissues of CWP/FM patients. These results provide a nuanced approach to the perception of chronic pain states and necessitate a view that is more complex and interacting than the dichotomy of peripheral versus central factors. The results presented in two studies that pain intensity correlates with certain protein patterns or proteins in peripheral tissues need to be confirmed in other studies, but this may challenge the understanding of factors responsible for pain [96,97]. Nonetheless, it is important to discover which biological processes drive the changes in proteome patterns and whether these processes are primary to the clinical picture of pain or reflect secondary consequences.

Network analyses can be used not only to investigate whether and how the identified proteins interact but also contribute to the identification of involved biological processes. In this review, we found only two studies that used network analyses to investigate chronic pain conditions [48,99]. In one of these studies, the authors identified a protein pattern characteristic for FM and concluded that all the identified biological pathways were related to inflammation [48]. In the other study, the authors used network analysis to investigate whether spinal cord stimulation altered expression of proteins [99]. The analysis indicated that the spinal cord intervention decreased inflammation and balanced the degeneration and regenerative processes.

For the single protein level, we only found a few common proteins between similar studies. Depending on the variability in the clinical picture across patients and studies, network analysis may be a more successful approach than focusing on individual proteins.

Within the second aim of this review, we investigated protein pathways in plasma/serum, saliva, CSF, and muscle to find the common and different protein networks in CNSP, CWP/FM, and neuropathic pain. Hence, the analyses were made to investigate whether the identified proteins interact (in whole or in part) and reflect meaningful biological processes. All the present analyses performed for a certain pain condition or tissue as well as for the clinical variables showed significant networks in which the proteins interacted partially or wholly. From the analyses, one can also ascertain, as expected, that proteins can be important for several biological processes (Supplementary Excel file 1). From the lists of the significant biological processes obtained from the network analysis, we present processes characterized by several of the identified proteins interacting together as a cluster (shown in the figures of the network analyses). However, the analyses may also reflect the weaknesses of the included studies, for example, in terms of clinical descriptions, sample size, and statistical methods.

The protein networks that were common in the studies of CNSP [81–83] were muscle contraction, cellular metabolic process, and ATP metabolic process. The studies that investigated CWP/FM in different tissues had more protein networks in common than the CNSP studies [48,74–76,81,84–87,89]. The pathways in CWP/FM were platelet degranulation, small molecule metabolic process, phosphorylation, muscle system processes, regulation of actin cytoskeleton organization, carbohydrate metabolic process, transport, regulation of cellular protein metabolic process, post-translational modifications, complement activation, and immune system. The common protein networks of CSF in neuropathic pain were immune processes, metabolic processes, and inflammatory responses [78–80].

The metabolic pathway was common in all chronic pain conditions. In CNSP, the levels of the enzymes, ion/protein binding, and microfilament motor activity (ACTB, ALB, NGF, TF, ALDOA, ENO3, GAPDH, MYH6, MYH7, and PKM[2]) were downregulated and the enzyme inhibitor SERPINA1 was upregulated. In contrast to the downregulated metabolic enzymes GAPDH, ENO3, ALDOA, and PKM, the levels of three other glycolytic enzymes (TPI1, PGM1, and PYGM) were upregulated in CNSP compared to CON. The results regarding an activated metabolic process in CNSP are consistent with studies reporting elevated concentrations of metabolites (glutamate, pyruvate, and lactate) in muscles [39,40,132]. In CWP/FM, the levels of the enzymes (ENO1, PGAM1, TALDO1, SERPINA1, ALDOA, TP11, ATP5B, GAPDH, PKM, A2M, FGB, FGG, PROS1, THBS1, AHSG, GSN, and SERPINF2) were upregulated. The proteins GAPDH, PGAM1, PKM, ENO1, and ALDOA are enzymes involved in glycolysis. Several microdialysis studies, including studies performed by our group, have shown that there are elevated levels of metabolites and products of glycolysis (lactate, glutamate, and pyruvate) in muscles from patients with CWP/FM compared to healthy subjects [39,40,133,134]. The upregulation of the glycolytic enzymes supports the findings of upregulated metabolites, indicating an increased need for energy to support muscle activity under anaerobic conditions. Based on these upregulated enzymes and the metabolites in MD studies, it might be speculated that there is an upregulated metabolic process in CWP/FM. In neuropathic pain, the enzymes (AGT, APOA4, TTR, SERPINA1, CFB, APOA1, AMBP, and CST3) involved in cellular metabolic processes were upregulated and the proteins that function as binders (PCSK1 N, ALB, APOE, CLU, F2, GSN, HPX, and PLG) were downregulated. These proteins bind to signaling receptors, ions (Ca+, K+), fatty acids, lipids, chaperons, actin monomer, and heme and are involved in clearance of immune complexes. The decreased levels of these 'cleaner' proteins lead to an increase in the immune complexes that might be related to the development of neuropathic pain.

Proteins involved in inflammatory and immune responses were identified both in neuropathic pain and in CWP/FM conditions. An upregulated immune process was more dominated in CWP/FM as the levels of several immunity proteins (A2M, ACTB, APCS, C1QC, C1S, C2, C7, C9, CFH, FGB, FGG, ORM1, ORM2, PROS1, THBS1, RBP4, C1R, C1S, C3, CFB, CFI, F2, FCN3, FGG, and GSN) were upregulated in CWP/FM compared to the controls. Interestingly, the expression levels of APOA1 involved in immune and inflammatory responses were downregulated in CWP/FM but upregulated in neuropathic pain. For neuropathic pain, an increased level of AG, which is involved in inflammatory response, was found. Together, the increased levels of APOA1 and AGT support the reported increased levels of inflammatory cytokines/chemokines in

neuropathic pain [135], which might point to an upregulated inflammatory mechanism in chronic neuropathic pain.

4.6. Validity and translation to clinical practice

Reproducibility is an issue that has gained increasing attention both in biomedical and in social science research [136]. Hence, results must be independently replicated before they can be accepted as true. From other clinical fields using proteomics, several molecular signatures have been proposed, but there is a lack of overlap and several biomarkers have failed validation in independent patient cohorts [137]. This is a problem on the single protein level in the identified studies in this review. Considering the results of the present systematic review, it can be questioned whether it is reasonable to expect reproducibility at the protein level? Focussing on identifying the involved biological processes might be more fruitful at the present stage.

Irreproducibility is often perceived as a disappointment, but it can reflect undiscovered errors and unknown sources of variability. In part, this lack of reproducibility may be related to what was discussed above regarding the clinical diagnoses and these presumed heterogeneities, both in terms of mechanisms and clinical aspects/consequences. Moreover, the tissues chosen for investigation in this review are seldom composed only of one cell type and mixed samples are investigated [72]. Other factors to consider are circadian variations; a recent study has reported prominent alterations in plasma proteins with potential relevance for nociception and pain with respect to circadian variations [138]. When interpreting the results of the proteomic studies, it is important to consider that chronic pain extends beyond the nervous system, that the endocrine and immune systems interact in complex ways, and that sex/gender may affect results [68].

Post-translational modification (PTM) is an important aspect of protein expressions. Proteins may undergo modifications, ranging from quite simple, such as N-terminal acetylation, to more complex additions, such as glycosylation and phosphorylation or proteolytic cleavages that generate the final active product. It has been estimated that PTM occurs in 50–90% of all mammalian proteins. The modifications may change the properties of the proteins and influence the activation state, the localization, and the turnover of proteins as well as the interaction with other proteins. The probability of a modification occurring depends on the primary structure as well as the location of a possible modifying enzyme. Additionally, the three-dimensional structure of the protein affects the accessibility of the modifying enzyme [139]. The advantage of 2-DE technology is the identification of proteoforms caused by PTM. In this review, we found 19 of the 27 studies used 2-DE. Bäckryd et al.'s studies illustrate the usefulness of 2-DE in identifying truncated and glycosylated proteins that correlate to pain intensity in patients with neuropathic pain [80,91]. The next challenging step is to characterize the proteoforms that are differentially altered in the different chronic pain conditions. The presence of different proteoforms is highly important to be considered when comparing results from different studies. Large-scale 'proteoformics' to investigate the PTM patterns in different chronic pain conditions is recommended to gain better insight into the biological mechanisms in chronic pain. In general, the number of proteins identified in those 19 studies is higher than the reported list presented as supplementary for the pathway analysis. The network analyses do not consider different proteoforms of a protein, which is an obvious limitation.

The translation of proteomic biomarkers to clinical practice has been limited and factors such as prefiltering, validation costs, small sample sizes, lack of instrumental standardization, and insufficient statistical analyses and overfitting are general barriers [49,126]. Unfortunately, these factors also characterize the proteomic research in the field of chronic pain conditions according to this systematic review. In addition, individual biomarkers often lack the specificity required for accurate diagnosis; a panel or signature of molecules may be the way forward [140].

4.7. Expert opinion

In this review, we found that several studies applied proteomic technology to investigate expression, function, and regulation of the entire set of proteins in plasma/serum, saliva, muscle, and cerebrospinal fluid. These proteomic studies were used to investigate peripheral and central mechanisms in different chronic pain states. It must be noted that the number of studies per diagnosis and tissue is small – i.e. using proteomics to study chronic pain conditions is in an exploratory phase. The studies in this review use relatively small pain cohorts (sample sizes ≤40). Obviously, larger proteomic studies are needed for chronic pain conditions. More pre-analytical parameters, however, can impact the results and therefore should be carefully considered.

The identified comparative studies reported proteins that significantly differed in expression between patients and controls both in peripheral tissues and in CSF. Only a few studies reported the extent these protein alterations were able to explain group belonging. However, it cannot be excluded that publication bias may be present (i.e. studies that do not find significant alterations in protein patterns are not published). Our network analyses generally found significant PPI enrichment analyses, and thus showed interactions among most proteins. The proteomic results and the associated network analyses necessitate a more complex dynamic view than the dichotomy of peripheral versus central factors with respect to maintenance of chronic pain. It is important to discover which biological processes drive the changes in proteomics patterns and whether these processes are primary for the clinical presentation or reflect secondary consequences.

Most of the identified studies aimed to differentiate a pain diagnosis from a healthy control group. The statistical methods used differed, so applying multivariate methods capable of handling and identifying complex intercorrelated protein patterns can reasonably be asserted as important. Such methods must also be applied when investigating whether patterns of proteins correlate with clinical variables such as pain intensity, pain sensitivity, and psychological distress. The results

from some of the identified studies indicated that proteins other than those differentiating patients and controls affect clinical variables such as pain intensity.

When several studies of a certain diagnosed tissue area exist, the overlap at the level of single proteins is very limited. This observation agrees with other areas applying proteomics. At the present stage, a system biology approach probably necessitates a focus on identifying the involved biological processes. Although numbers of multiple proteins have been identified when discriminating patients with chronic pain and healthy controls, the role of the identified proteins in the pathophysiology of chronic pain needs to be investigated. Future studies should include several tissues simultaneously and perform network-based analysis of such large-scale proteomic datasets to obtain a more comprehensive understanding of the chronic pain condition under investigation. Moreover, as multiple factors (symptoms) characterize chronic pain conditions, proteomic studies should identify a combination of different mechanisms underlying the whole clinical presentation of a certain chronic pain condition.

Chronic pain cohorts may be heterogeneous with respect to comorbidities and consequences. Comparing studies will require standardized descriptions of the pain cohorts investigated. This is certainly not only a problem for proteomics studies but also applies when comparing different studies investigating the outcomes of different interventions. The variables identified by the IMMPACT and VAPAIN initiatives may be the starting point for initiating such as standardization [106–108]. To interpret the proteomic results properly, pharmacological treatment and washout periods used for certain drugs will need to be described in a standardized way.

The use of proteomics in chronic pain research is in its infancy. Despite various challenges, we believe that proteomics has a huge potential to dissect chronic pain conditions for the strategic purpose of predictive and personalized health care. Proteomic pain research can contribute to an increased knowledge about the pathophysiological mechanisms of the complex multifactorial condition of chronic pain. This review suggests that in chronic pain (CNSP, CWP/FM, and neuropathic pain) a highly distinct set of molecular changes reflects different combinations of underlying mechanisms. Many of these alterations might be involved in the generation and/or maintenance of each type of pain, and so the 'molecular signature' of each pain state might be important for the development of therapeutics. The reason that different processes have been identified in the identified studies may be due to differences in the grade of complexity, including comorbidities, in the included cohorts of subjects.

Proteomics in combination with bioinformatics have a potential to identify previously unknown panels of proteins involved in chronic pain that may be relevant factors when devising new pain control strategies. Identifying multiple proteins instead of a single protein leads to an identification of a combination of different biological processes involved in pain that is inconsistent with the whole clinical presentation of chronic pain.

Notes

1. It was not reported that the pain group had sciatica.
2. See Supplementary Excel file 1 for explanation of abbreviations.

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