Biomarkers for Better Understanding of the Pathophysiology and Treatment of Chronic Pain

Investigations of Human Biofluids

ANNE-LI LIND
Chronic pain affects 20% of the global population, causes suffering, is difficult to treat, and constitutes a large economic burden for society. So far, the characterization of molecular mechanisms of chronic pain-like behaviors in animal models has not translated into effective treatments.

In this thesis, consisting of five studies, pain patient biofluids were analyzed with modern proteomic methods to identify biomarker candidates that can be used to improve our understanding of the pathophysiology chronic pain and lead to more effective treatments.

Paper I is a proof of concept study, where a multiplex solid phase-proximity ligation assay (SP-PLA) was applied to cerebrospinal fluid (CSF) for the first time. CSF reference protein levels and four biomarker candidates for ALS were presented. The investigated proteins were not altered by spinal cord stimulation (SCS) treatment for neuropathic pain. In Paper II, patient CSF was explored by dimethyl and label-free mass spectrometric (MS) proteomic methods. Twelve proteins, known for their roles in neuroprotection, nociceptive signaling, immune regulation, and synaptic plasticity, were identified to be associated with SCS treatment of neuropathic pain. In Paper III, proximity extension assay (PEA) was used to analyze levels of 92 proteins in serum from patients one year after painful disc herniation. Patients with residual pain had significantly higher serum levels of 41 inflammatory proteins. In Paper IV, levels of 55 proteins were analyzed by a 100-plex antibody suspension bead array (ASBA) in CSF samples from two neuropathic pain patient cohorts, one cohort of fibromyalgia patients and two control cohorts. CSF protein profiles consisting of levels of apolipoprotein C1, ectonucleotide pyrophosphatase/phosphodiesterase family member 2, angiotensinogen, prostaglandin-H2 D-isomerase, neurexin-1, superoxide dismutases 1 and 3 were found to be associated with neuropathic pain and fibromyalgia. In Paper V, higher CSF levels of five chemokines and LAP/TFG-beta-1 were detected in two patient cohorts with neuropathic pain compared with healthy controls.

In conclusion, we demonstrate that combining MS proteomic and multiplex antibody-based methods for analysis of patient biofluid samples is a viable approach for discovery of biomarker candidates for the pathophysiology and treatment of chronic pain. Several biomarker candidates possibly reflecting systemic inflammation, lipid metabolism, and neuroinflammation in different pain conditions were identified for further investigation.

Keywords: chronic pain, neuropathic pain, lumbar radicular pain, amyotrophic lateral sclerosis, radiculopathy, fibromyalgia, pathophysiology, spinal cord stimulation, mechanism of action, disc herniation, cerebrospinal fluid, plasma, biomarker, human, protein, chemokines, cytokines, inflammation, neuroinflammation, mass spectrometry, proximity ligation assay, proximity extension assay, antibody suspension bead array, protein profiling, molecular medicine, personalized medicine

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What we perceive at a given moment is thus confined to an extremely small part of the flood of information about our world that flows in through the sensory organs.

Manfred Zimmermann
To those with chronic pain
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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV **Anne-Li Lind**, David Just, Maria Mikus, Claudia Fredolini, Marina Ioannou, Björn Gerdle, Emmanuel Bäckryd, Lars Tannum, Torsten Gordh, Peter Nilsson, Anna Månberg. Affinity Proteomics Applied to Patient CSF Identifies Protein Profiles Associated with Neuropathic Pain and Fibromyalgia. *Manuscript*

V Emmanuel Bäckryd, **Anne-Li Lind**, Måns Thulin, Anders Larsson, Björn Gerdle, Torsten Gordh. High Levels of Cerebrospinal Fluid Chemokines Point to the Presence of Neuroinflammation in Peripheral Neuropathic Pain - A Cross-Sectional Study of Two Cohorts of Patients Compared to Healthy Controls. *Manuscript*

* These authors contributed equally to the work.

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PhD student’s contributions

This work has been performed in close collaboration with the colleagues presented in the author list of the papers in the acknowledgement section.

I  Worked hands on with study design and the cerebrospinal fluid and plasma sample logistics, centrifugation, aliquoting, biobanking, and logging. Worked directly with the development and set up of the proximity ligation assay (PLA) panel, which included probe conjugations, making standard curves, pilot experiments, performing the final multiplex solid phase PLA experiments, and data evaluation steps on several sets of patient samples. Writing of the manuscript.

II  Worked with the study design, sample logistics, centrifugation, aliquoting, bio banking, and logging. Performed mass spectrometry (MS) sample preparation, MS sample analysis, and interpretation of the results, as well as the writing of the manuscript. Corresponding author.

III Planning, project coordination, sample aliquotation, sample handling, and manuscript writing.

IV Study design. Performed sample aliquotation and logistics. Participated in some of the antibody suspension bead array (ASBA) experiments and data evaluation. Main coordinator and author of the manuscript.

V Study design. Worked with sample collection, aliquotation, and logistics. Participated in the writing of the manuscript.
Related publications not included in the thesis

Bäckryd E, Tanum L, **Lind AL**, Larsson A, Gordh T. Evidence of both systemic inflammation and neuroinflammation in fibromyalgia patients, as assessed by a multiplex protein panel applied to the cerebrospinal fluid and to plasma. J Pain Res. 2017 Mar 3;10:515-525.


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Acknowledgements

References
**Abbreviations**

ALS        Amyotrophic lateral sclerosis
APOC1      Apolipoprotein C1
ASBA       Antibody suspension bead array
BDNF       Brain derived neurotrophic factor
CNS        Central nervous system
CSF        Cerebrospinal fluid
CWP        Chronic widespread pain
DML        Dimethyl labeling
EGF        Epidermal growth factor
ENPP2      Ectonucleotide pyrophosphatase/phosphodiesterase family member 2
FDR        False discovery rate
GABA       Gamma-aminobutyric acid
IASP       International association for the study of pain
IL         Interleukin
LAP TGF    Latency associated peptide transforming growth factor
LBP        Low back pain
LC         Liquid chromatography
LF         Label-free
LPA        Lysophosphatidic acid
MCP        Monocyte chemoattractant protein
MS         Mass spectrometry
NSAID      Nonsteroidal anti-inflammatory drug
OPLS-DA    Orthogonal partial least squares discriminant analysis
PCR        Polymerase chain reaction
PEA        Proximity extension assay
PLA        Proximity ligation assay
S1P        Sphingosine 1-phosphate
SCS        Spinal cord stimulation
SP-PLA     Solution phase proximity ligation assay
TENS       Transcutaneous electric nerve stimulation
TLR4       Toll-like receptor 4
TNF-α      Tumor necrosis factor alpha
VEGF       Vascular endothelial growth factor
YLD        Years lived with disability
Introduction

Acute pain
The International Association for the Study of Pain (IASP) defines pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" 1. Acute pain serves the organism by eliciting protective behaviors in response to actual or threatening tissue damage. Rare congenital disorders with insensitivity or indifference to pain often lead to inadvertent self-mutilation, such as biting or chewing on the tongue or hands, and repeated fractures 2-4, exemplifying the adaptive value of pain. Although unpleasant, acute pain is not a big problem from a scientific point of view, because it typically responds well to pharmacological agents including paracetamol, nonsteroidal anti-inflammatory drugs (NSAID), opioids, and/or local anesthetics.

Chronic pain
Sometimes acute pain does not resolve. Instead, the pain persists and transitions, into a chronic condition by mechanisms that we do not clearly understand. Pain outlasting expected healing time by 3 to 6 months is usually considered chronic 5. Generally, chronic pain cannot be relieved by the treatments that relieve acute pain; rather, it is a disease in its own right 5, 6. Chronic pain is associated with stigmatization, disability, disturbed sleep, immobility, poor appetite, dependence on medication, high dependence on family and health care, sexual dysfunction, isolation, turning inward, anxiety, bitterness, frustration, depression, and suicide 6, 7. The biopsychosocial model of illness 8 has been adopted to pain medicine 9 to describe the complexity of chronic pain. A few quotes illustrate the diverse personal challenges faced by people with chronic pain (Figure 1).
Epidemiology and societal costs

Approximately 20% of the world’s population suffers from chronic pain. Pain and chronic pain are the two of the primary reasons why patients seek health consultation. Globally, chronic low back pain (LBP) alone causes more years lived with disability (YLD) than any other health condition, increasing by 61% in the last two decades. After surgery, 10-50% of the patients develop persistent pain, and 2-10% of those patients develop severe pain. As might be expected, the prevalence of chronic pain increases with age and is associated with markers of social disadvantage. In Sweden, 7% of population has a large need for healthcare due to chronic pain and the societal costs of chronic pain reach 87.5 billion SEK annually. In the UK, back pain alone costs 26–49 billion USD yearly. In the USA, 100 million adults suffer from chronic pain, with annual economic losses due to chronic pain estimated to 600 billion USD. Based on individual and societal costs alone, chronic pain needs to be regarded as a public health priority. As the population ages, the suffering and societal economic burden of chronic pain can be expected to increase.

Current treatments

Chronic pain is notoriously difficult to treat. Pharmacological treatments are successful in approximately 30% of chronic pain cases. Current pharmacological treatments of chronic pain include paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), opioids, tricyclic anti-depressants, anti-
epileptics, glucocorticoids, local anesthetics, benzodiazepines, alpha2-receptor agonists, neuroleptics, and topical administration of capsaicin\textsuperscript{41-43}. Alternative treatments are transcutaneous electric nerve stimulation (TENS), neurostimulation (including spinal cord stimulation (SCS)), and psychological treatment such as cognitive behavior therapy and acceptance commitment therapy. Multi-professional health care teams demonstrate comparatively good results in treating chronic pain\textsuperscript{5,44}.

Classification of pain conditions

Classification of pain conditions is not straightforward. Scientists and clinicians refer to chronic pain conditions by mechanistic classifications as inflammatory\textsuperscript{4}, or neuropathic (meaning caused by a lesion or disease of the somatosensory nervous system)\textsuperscript{45}, and others as dysfunctional\textsuperscript{4}. However, because the pathophysiological basis of most human chronic pain conditions remains occluded, classification of chronic pain conditions has led to considerable debate. For example, the 2011 redefinition of neuropathic pain left several pain conditions, e.g., fibromyalgia, complex regional pain syndrome, some musculoskeletal pain conditions, and visceral pain disorders, without a pain classification\textsuperscript{46}. Therefore, the adoption of a new, third pain descriptor encompassing the conditions referred to as nociplastic, algopathic, or nocipathic was recently advocated\textsuperscript{46}.

Theories of pain pathophysiology

In this section central concepts reflecting our current understanding of pain pathophysiology are introduced.

The gate control theory

The gate control theory, first described in 1965\textsuperscript{47}, stipulates that the spinal cord has a gate through which the pain signal must pass. Activity in large touch sensation fast conducting Aβ-fibers can inhibit the nociceptive signal in small slower conducting Aδ- and C-fibers via a common interneuron (Figure 2). This concept provided the first explanation for why similar injuries can generate varying pain sensations, basically depending on whether the gate is open or closed.
The gate control theory. The input from large fast conducting $A\beta$-fibers (L) can inhibit the input from the smaller (S) slow conducting $A\delta$-and $C$-fibers via a common interneuron that inhibits the projection neuron (P).

The theory led to the development of TENS treatment, in which nerves are stimulated through the skin. In 1967 areas in the brain stem were shown to exert tonic inhibition over nocireponsive neurons in the spinal cord. Disinhibition and thus loss of this gate control was suggested to contribute to pathological pain states. Today, the spinal inhibitory gate is described as inhibitory neurotransmitters, primarily gamma-aminobutyric acid (GABA) and glycine, released spinally by local circuitry interneurons and descending fibers from the brain acting pre- and post-synaptically to inhibit nociceptive signaling. Recently, a mouse model was used to characterize the molecular identity of the inhibitory neurons that exert the spinal gate control of pain-like behaviors in response to mechanical stimuli.

Central sensitization

Central sensitization was first described for nociceptive circuits in the spinal cord of animal models in 1983, as the process by which peripheral input makes higher order (central) neurons more sensitive to subsequent input. Today, central sensitization is defined by the IASP as “increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input”, which includes other mechanisms of sensitization than the ones described in the early publications and refers to any form of pain sensitization arising within the CNS. For example, central sensitization can be caused by alterations in: peripheral input, descending inhibition from the brainstem, neuronal activity, glial activity, connectiv-
ity, or loss or inhibitory neurons. In many clinical pain conditions there is a spreading of pain. Although it has been debated, central sensitization mechanisms are considered contributors to the spread of pain and the pathophysiology for many pain states, including neuropathic pain, fibromyalgia, post-surgical pain, temporomandibular joint disorders, osteoarthritis, musculoskeletal disorders with generalized pain hypersensitivity, headache, dental pain and visceral pain hypersensitivity disorders. The psychological and emotional aspects of chronic pain, altered activity in the brain regions processing mood and anxiety, may also contribute to central sensitization or result from it. Methods for assessing central sensitization in humans are emerging, and some are being evaluated in clinical pain practice.

Glial cells and neuroinflammation

Glial cells respond to nerve impulses, injury and regeneration, but traditional neuroscience and pain research has primarily focused on neurons. In the last decade, technical advances, and animal model research has shifted this view. Today glial cells are considered as key contributors to development and maintenance of chronic pain-like behaviors in animal models. For example, peripheral nerve injury induces microglial activation in the spinal cord and in brain regions involved with pain processing. As catalysts for acute pain turning chronic, glial cells are believed to contribute to increased inflammation in the central nervous system (CNS).

Inflammation in the nervous system, neuroinflammation, is characterized by infiltration of immune cells, activation of glial cells and production of immune mediators including cytokines and chemokines. After nerve or CNS lesions, the recruitment of immune cells to injured nervous tissue is adaptive for repair and tissue regeneration, but as cytokines, chemokines, and immune cells gather in the otherwise relatively immune sheltered nervous tissue, the neuroinflammation can lead to central sensitization of pain signaling neurons. The blood-brain-barrier, which consists of endothelial cells that are in connection with astrocytes, has been shown to be disrupted in models of chronic pain contributing to the exposure of CNS cells to blood-borne immune mediators.

Although there is little doubt of the importance of glial cells and neuroinflammation in animal models of chronic pain, glial reactions in response to peripheral nerve injury vary between species, and evidence for gliopathy in human pain conditions is scarce. A post-mortem tissue study showed evidence for astrocytic (but not microglial) activation in the dorsal horn of pain-positive human immunodeficiency virus (HIV)-affected spinal cord compared with pain-negative human HIV-affected spinal cord, together with elevated levels of pro-inflammatory cytokines (tumor necrosis factor α (TNFα) and interleukin-1β (IL1β)). In addition, a recently published
Spinal cord stimulation for the treatment of neuropathic pain

From the gate control theory followed the idea electrical stimulation of the large non-nociceptive (A-\(\beta\)) fibers could close the pain gate and hinder ascending pain signals from reaching the brain. Trials with low-intensity stimulation via implanted electrodes and with SCS, were conducted and SCS subsequently became an important treatment option for neuropathic pain (Figure 3). The response rate for neuropathic pain patients to present day SCS is 50-70%\(^{117-121}\). This response rate is high considering that the average SCS eligible patient has had pain for 5-10 years and has failed to sufficiently respond to pharmacological treatments\(^{122-124}\). SCS is a relatively safe, cost-effective treatment. Although this has been negated\(^{126}\), SCS is considered by many investigators to show good long-term efficacy, where 47-60% of patients have >50% pain relief at long-term follow-ups\(^{124, 127-130}\).

Figure 3. Spinal cord stimulation (SCS). Left: the components of a spinal cord stimulator. Right: patient with peripheral neuropathic pain controlling SCS via a remote control. The placement of the electrodes in the epidural space is optimized to provide relief in the painful area.

Neuropathic pain patients with SCS treatment report prolonged pain relief that commonly outlasts the actual electrical stimulation by several hours\(^{131}\) and in some cases the pain has improved with continued treatment\(^{133, 134}\). SCS treatment practice has recently been enriched with new modes includ-
ing high frequency and burst stimulation, these modes may have different mechanisms of action compared to conventional stimulation \(^{135}\).

SCS requires surgery, a team of specialized physicians and nurses, and frequent patient contact. Thus, in spite of its benefits, SCS is not a first line treatment and SCS is not available globally, to all neuropathic pain patients. A clearer understanding of the SCS mechanism would improve current SCS treatment and patient selection but also assist drug discovery efforts for more readily available treatments.

Because SCS is performed by stimulating over the entire spinal cord through the dura mater, any of the spinal fibers in the dorsal column, i.e. ascending or descending dorsal horn fibers, local spinal level circuitry, or even fibers from the anterior horn of the spinal cord may be important for the effect \(^{136}\).

Research on animal models of SCS and neuropathic pain has demonstrated SCS-mediated increases of mechanical withdrawal thresholds and reduced spinal astrocyte and microglia activation \(^{137}\), increases of spinal cord levels of serotonin, substance P \(^{138}\), acetylcholine \(^{139}\), glycine \(^{140}\), GABA \(^{141}\) \(^{142}\) \(^{143}\), and decreased levels of spinal cord excitatory amino acids \(^{142}\). Results from animal model research suggest a central role for the GABA system in the SCS mechanism of action \(^{142-145}\). Whereas spinal excitatory neurotransmitter levels have been found to remain decreased for at least 90 minutes \(^{142}\), SCS-reduced allodynia has been shown to last up to 3 days post-stimulation \(^{137}\) \(^{142}\). This prolonged effect suggests the possibility that has potential effects on gene regulation and protein translation. If there were such effects, it is plausible that the protein content of the cerebrospinal fluid (CSF) would reflect them \(^{146}\). This expectation was recently confirmed in a human study in which analysis of CSF levels of monocyte chemoattractant protein 1 (MCP-1), brain derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF) revealed increased VEGF when the SCS was in use \(^{147}\). Another investigation of pain patients, measured the temporal summation in the affected and unaffected leg during SCS and found reductions only in the affected leg accompanied by a >50 % pain relief. This report indicates that SCS works by reducing temporal summation and central sensitization \(^{148}\).

In **Paper II**, we contribute proteomic information that may help elucidate the mechanisms of SCS. We hypothesized that SCS alters many other proteins in the spinal cord of the neuropathic pain patient and that some of these can be reflected in the CSF and quantified by mass spectrometry (MS). This regulation may shed light on the analgesic mechanism of SCS.
Lumbar radicular pain after disc herniation

Approximately 2-6 % of people worldwide suffer from a subcategory of LBP called lumbar radicular pain, or sciatica pain, a radiating pain that usually follows the dermatome of the affected nerve-root (Figure 4). About 90 % of these cases are caused by herniation of the intervertebral disc and subsequent nerve-root compression (Figure 4). Most patients recover spontaneously after disc herniation, but 1 year after onset only 30 % are totally pain-free. The pathophysiology is commonly considered to be a combination of nerve-root compression and inflammation initiated by content leaked from the nucleus pulposus into the spinal canal. Sciatic pain after disc herniation may include neuropathic features, a symptom screening study of 8000 LBP patients suggested that 37 % had predominantly neuropathic pain. However, there are no widely accepted biomarkers for the pathophysiology and treating lumbar radicular pain remains challenging.

Figure 4. The lumbar spine and sciatic nerve are two of the most common anatomical sites of pain chronification. Left: Schematic overview of the anatomy of the lumbar spine and sciatic nerve. Upper right: Illustration of intervertebral disc herniation, spinal column transection view. Lower right: Common sciatic pain distribution.

Fibromyalgia and chronic widespread pain

Approximately 2 % of the general population have fibromyalgia, and the prevalence is higher in women (4.0 %), than in men (0.01 %). Fibromyalgia is characterized by chronic widespread pain (CWP), palpation tenderness and is typically accompanied by sleep disturbances, fatigue, headache and cognitive/mood disturbances and reduced quality of life. It has been
possible to quantify sensory dysfunction in patients with fibromyalgia for at least two decades. Still, many patients with fibromyalgia and generalized pain suffer from stigmatization, likely in part, because of the poor understanding of the pathophysiology among clinicians and in society. Recent imaging studies of fibromyalgia patients have demonstrated altered structure, connectivity and activity in brain areas important for pain processing. There is evidence that endogenous pain inhibition is altered in patients with fibromyalgia. Previous work has shown that fibromyalgia patients have elevated CSF levels of IL8 indicative of central inflammation and possibly an enhanced innate immune response. Research supports sex differences in responses to inflammatory challenge, for example the descending pain modulation appears to be less activated in women than in men. These results shed light on a plausible reason for the female predominance in chronic pain conditions, a predominance that is perhaps most obvious in fibromyalgia. Other aspects of fibromyalgia pathophysiology have been demonstrated including a genetic polymorphism predisposing for central sensitization, small-fiber neuropathy, and post-traumatic stress response to adverse events in childhood, and altered emotional modulation of pain.

Neuropathic pain
Neuropathic pain is caused by a lesion or disease of the somatosensory nervous system, and affects 7–10% of the population, most commonly in the lower back, lower limbs, upper limbs and neck. Patients often describe neuropathic pain as burning, tingling or prickling. Common symptoms are evoked pain, spontaneous pain attacks, and numbness. The available treatments of neuropathic pain are unsatisfactory. It is currently believed that excitatory and inhibitory signaling imbalance, ion channel modifications, and central sensitization is part of neuropathic pain pathophysiology. In the current scientific view of clinical neuropathic pain there is not much emphasis on glial signaling or neuroinflammation. However, although neuropathic pain is obviously not an inflammatory condition such as rheumatoid arthritis, a compelling list of scientific publications from animal models and human studies indicate that the role of neuroinflammation and gliopathy in neuropathic pain may have been underestimated.

Chronic pain drug development
In the 1970s to 2000s, the pharmaceutical industry pain research programs focused on animal models, assuming that rodent models would predict, reasonably well, the drug effect and safety in human pain conditions.
There was hope of a silver bullet treatment to manage all or at least broad sub-categories of chronic pain conditions just as morphine manages nociceptive pains of different etiologies. This opportunity proved to be difficult to realize. Many clinical trials failed due to limited CNS target access and severe side effects caused by unsatisfactory target selectivity. Although some treatment principles with strong preclinical evidence were successful in humans, e.g. spinal opiate administration, cyclooxygenase (COX)-2 inhibitors, and pregabalin, these drugs were modifications of pre-existing drugs or were adopted from other medical disciplines, which may explain why none of them became a real breakthrough treatment.

To conclude, none of the treatment principles for chronic pain come from pain research; rather, they come from traditional medicine or from other medical disciplines (e.g. the antidepressants duloxetine and milnacipran, as well as the anticonvulsant pregabalin).

There are novel analgesic treatment principles under evaluation including anti-nerve growth factor (NGF) treatment, conotoxin derivatives, angiotensin II type 2 (AT₂) receptor antagonists, modulators of toll-like receptor 4 (TLR4) and glial modulators. Anti-NGF treatment of osteoarthritic pain first appeared successful, but trials were halted in 2010 due to occurrences of osteonecrosis and re-negotiated in 2012. New anti-NGF trials are now ongoing, but the historical track record of most new promising analgesic compounds has prompted some caution in the expectations.

The problem remains

Although, in the academic community, chronic pain has attained the status of disease in its own right with numerous mechanisms described and a significant number of potential treatment targets emerged, in the real world, pain patients still struggle to get their pain problems acknowledged, diagnosed, and treated. What is the reason for this discrepancy?

There are regulatory limitations contributing to the hindrance of analgesic development, but there are also purely research-related explanations. First, the poor translation from animal models to human pain has been a bottleneck. The lack of translation has been attributed to publication bias, lack of transparency and poor quality of preclinical data. There are also in-
herent limitations in the predictive ability of rodent models to mimic human chronic pain and its treatments, as the pain system has many species differences. Second, our lack of understanding of chronic pain pathophysiology in humans has led to a pain conditions classifications which are insufficiently mechanism-based. This has generated large clinical, pharmacological and pathophysiological heterogeneity, even within a diagnostic group, in clinical trials. The heterogeneity may have concealed positive treatment effects on the group level, and caused some clinical trials to report false negative outcomes. Third, the hope of a simple solution has placed too much focus too early on a single marker or candidate. This thesis aimed to address these three issues by investigating human pain pathophysiology, in well-defined patient groups, measuring biofluid protein profiles instead of single markers.

Biomarkers for pain pathophysiology

A biological marker, biomarker, is “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” By definition, a biomarker need not be a biofluid protein profile (as in this thesis), it may also be measured in tissues, or with genetic tests, images, physiological measurements, and neurophysiological recordings.

Although the analysis of biomarkers is a corner stone in diagnosis and monitoring in most medical disciplines, pain medicine lacks these molecular tools, and typically relies purely on medical history and clinical examination.

There are at least three ways in which biomarkers would benefit chronic pain patients. First, clinically biomarkers could be used to identify those at risk for developing chronic pain, improve diagnostic accuracy, prognostication, as well as selection and monitoring of treatments. Second, scientifically biomarkers could be used to provide mechanistic understanding and discover new treatment targets. Third, biomarkers would aid pharmaceutical companies by providing a tool for better sub-classification of patients and provide surrogate endpoints for effective clinical trials.

Biofluids for biomarker discovery

The biofluid most commonly used for biomarker discovery is blood (often serum or plasma). The human plasma proteome has been suggested to hold the potential to revolutionize patient diagnostics and treatment monitoring. Because interesting disease biomarkers become diluted in biofluids, sensitive analytical techniques are of interest in for biomarker discovery studies. Another way of improving the chances of finding biomarkers is by analyzing
biofluids specifically enriched with molecules from the tissue of interest, the CSF is one such enriched biofluid.

Cerebrospinal fluid
Because of the close contact, and continuous exchange between the CSF and the nervous tissue of the brain and spinal cord, the CSF can reflect ongoing physiological and pathological processes of the CNS. In fact, the CSF has been used for diagnostic purposes for more than 100 years. The CSF proteome appears to be stable in non-pathological conditions. The CSF is contained in the subarachnoid space making it less accessible than blood. The CSF is most commonly sampled by lumbar puncture at the vertebral interspaces L3-L4, L4-L5, or L5-S1, or from the ventricles.

Most of the CSF is produced by the choroid plexus. An average adult has 100-150 mL of CSF circulating the brain, spinal cord, and the four ventricles, and this volume is replaced three to four times daily. The CSF is an ultrafiltrate of plasma that is combined with CNS-specific proteins and molecules. About 20 to 56% of the proteins in CSF are directly derived from the CNS. Compared with blood, the healthy CSF contains very few cells (0-4 cells/μL) and about 100-200 times less protein (0.35(0.05-0.8) mg/mL). The top eight most abundant CSF proteins (albumin, prostaglandin D-synthase, immunoglobulin G, transthyretin, transferrin, alpha-1-antitrypsin, apolipoprotein A, and Cystatin C) make up over 85% of the total protein content.

Research history and thesis scientific context
The first studies aimed at distinguishing pain patient biofluid samples from those of controls were published in the 1990s. One, or a few, molecules of interest were investigated in each study. High concentrations of CSF interleukin-8 (IL8) were reported in patients with herniated discs. CSF and blood levels of arginine and vasopressin were found to be higher in chronic pain patients compared with healthy controls.

In the early 2000s, three publications in PAIN, the official journal of the IASP, spurred discussion about the role of biomarkers in pain medicine. The first study suggested, based on animal experiments and a human labor pain study, that cystatin C was a potential biomarker for human pain. The article concluded that a profile of biochemical markers of nociception could be used to assess pain in situations when pain measurements using conventional methods are difficult. The following year, the validity of the finding was disputed. The authors of a second larger study of healthy volunteers, women in labor, and patients with chronic neuropathic pain reported that the highest concentrations of cystatin C were found in the CSF of chronic pain patients with an indwelling intrathecal catheter. This result suggested that increased cystatin C levels marked inflammation or
some other phenomenon related to the catheter \(^{284}\) rather than pain. The second study was accompanied by an editorial stating that calling cystatin C a biomarker for human pain was oversimplified and that the purpose of a pain biomarker must not be to overrule a patient’s statement with a biological test. Instead the purpose should be to inform about the pathophysiology underlying the actual pain experience, to help treat it more effectively. This is type of usage of biomarkers is the long term vision of the biomarker discovery studies in the present thesis.

During the 2000s, fibromyalgia/CWP patients were reported to have reduced CSF levels of neurotrophic factors \(^{285}\), and signs of systemic inflammation \(^{286}\). Inflammatory blood profiles of patients with painful neuropathies were shown to differ significantly from those of patients with painless neuropathies and healthy controls \(^{287, 288}\).

**Omics**

Parallel with the advances in our understanding of pain-like behavior in animal models and early human biofluid investigations, there has been a veritable revolution in the biological technologies. Perhaps the most obvious starting point was the sequencing of the human genome \(^{289, 290}\). Equally important was subsequent development of the other omics sciences including, proteomics, transcriptomics and metabolomics, and the resulting integrated omics projects such as the human protein atlas \(^{291}\). These methods provide holistic tools for understanding profiles, and “omes” rather than single biomarker molecules. The last decade has also seen the innovation of new high-throughput affinity-based technologies for sensitive multiplex analyses \(^{292-296}\), which can also be considered “proteomic” although this term traditionally referred to MS-based methods. These modern omic methods have been successfully used to identify biomarker candidates for many disease types including cancers and neurodegenerative diseases.

**Applying modern protein detection methods in pain research**

The present thesis is part of a recent and rapid evolution of the human pain pathophysiology research. Ten years ago, proteomic methods had not been introduced to clinical pain research, although one publication predicted this development \(^{297}\). In 2010, when the present work was initiated, there were less than 20 original research publications addressing the topic of pain research on human biofluids, and none using highly multiplexed or proteomic methods. Only 6 years later proteomics was described as an integral part of research in human pain pathology \(^{249}\). In retrospective, it may seem only natural to combine proteomic methods and pain research. However, early critique pointed out that compared with diseases like cancer and degenerative diseases, chronic pain is 1) caused by more subtle nervous tissue changes, markers of which are likely to be too diluted for detection in CSF or blood, 2) subjective, and 3) highly complex (biopsychosocial). In light of
these challenges, the quest for clinically useful biomarkers for pain medicine is appropriately summarized by Emmanuel Bäckryd, “The task is immense. So is the need.” 298.
Aims

The aim of this doctoral thesis was to identify chronic pain biomarker candidates in human biofluid samples. The specific aims of the present investigations were:

I To determine whether applying purpose-designed affinity-based protein panels such as multiplex SP-PLA to patient CSF is a viable approach to CNS biomarker discovery.

II To use dimethyl labeled and label-free MS to investigate SCS mechanism of action by comparing the CSF proteomic profiles when SCS had been turned off for 48 h with when it had been in use for 3 weeks, in 14 SCS-responsive neuropathic pain patients.

III To compare, using a new 92-plex PEA pane, the serum inflammatory protein profile of 23 patients with high lumbar radicular pain levels and 22 patients with none or low pain levels one year after disc herniation.

IV To investigate the CSF protein profiles of patients with neuropathic pain and fibromyalgia using a 100-plex ASBA targeting 55 proteins.

V To determine, using a 92-plex PEA panel, the CSF inflammatory protein profile for two cohorts of neuropathic pain patients.
Methods

This section describes the four main methods used for detection and relative quantification of proteins in human biofluids in the present thesis.

Proximity Ligation Assay (PLA)

In Paper I, the multiplex proximity ligation assay (PLA) was applied, for the first time, to CSF samples (Figure 5). PLA is a DNA assisted antibody-based method for sensitive protein detection developed at the unit of Molecular Tools at the Department of Immunology Genetics and Pathology at Uppsala University. In PLA, the antibodies are coupled to DNA oligonucleotides (approximately 20 bases, single-stranded DNA molecules) to create PLA probes that can bind the protein for which the antibody has affinity. When two antibodies bind the same target, their DNA oligonucleotides are close (proximal) enough for them to hybridize to another DNA oligonucleotide to form a template for enzymatic ligation, allowing the two arms form a template for amplification using real-time PCR. There are several variants of PLA, in the solid-phase PLA (SP-PLA), the target protein is first captured via an immobilized antibody on a solid support before the probes are added and PLA is performed, which provides increased specificity due to requirement of three recognition events. In Paper I, a multiplex SP-PLA was used. Here, the sample is first incubated with a mixture of magnetic beads covered separately with 47 different polyclonal antibodies against the target proteins, the capture step. After the target proteins have been captured, the excess samples are washed away, the probes are added, and incubated to allow binding to their target protein. The final signal coming from the PCR amplification is thereby ensured to result only if three binding events to the same protein have taken place, giving this method superior selectivity compared with other antibody-based techniques in which one or two target binding events suffice to generate a detection signal.
Mass spectrometry-based proteomics

The second main method used in this thesis is MS-based proteomics. MS is a powerful analytical tool that can determine the identity and quantity of ionized proteins and peptides (and many other compounds) in a complex sample based on their mass to charge ratio (m/z). The field of proteomics is concerned with the proteome, meaning the entire expressed protein set available in a certain tissue or body fluid. In contrast to disease designated antibody based panels, such as the PLA panel that we created for Paper I, the approach used in Paper II is unbiased, without a pre-existing hypothesis regarding which proteins will be altered by SCS. Rather than confirm or refute a hypothesis, the aim in that study was to generate hypotheses for the SCS mechanism. There is a large number of mass spectrometric proteomic approaches. In the following sections the principles and choices behind the specific MS approaches used in Paper II are described (Figure 6).

Sample preparation

Complex sample solutions, such as human biofluids, are usually not directly infused into the mass spectrometer for optimal analysis. To reduce the high abundance proteins that otherwise saturate the capacity of the mass analyzer an immunoaffinity fractionation strategy (sometimes called depletion) was used. The immunoaffinity columns used for the label-free (LF) and dimethyl label (DML) experiments were different but serving the same purpose. The sample was optimized for MS by removing salts and primary amines. In bottom-up proteomics, the proteins are first enzymatically cleaved (digested) into peptides before they are separated, ionized, and introduced into the mass analyzer. In Paper II, trypsin was used to digest the proteins. Trypsin cleaves the protein after basic residues arginine and lysine, (if not next to proline) into tryptic peptides.
Figure 6. Workflow of MS proteomics used in Paper II. Labeling was done for one of the two sample sets. Ping Sui acknowledged for contributions to the illustration.

**Separation**

To increase the sensitivity of MS detection, the sample complexity is typically further reduced by introduction of a separation step prior to MS. In Paper II, reversed phase nano liquid chromatography (nLC) was used. This miniaturized LC consumes less sample volume and has a higher efficiency than conventional columns. Reverse phase LC uses an aqueous moderately polar mobile phase to transport the sample though the LC column containing a non-polar stationary phase. Complex peptide mixtures are separated by the LC system based on their polarity before entering the ionization step.

**Ionization**

A basic requirement for all applications of MS-based proteomics is the generation of gas phase ions. Coupling electrospray ionization (ESI) to the MS (Figure 7), as was done in Paper II, is one way of meeting this requirement. ESI is a comparatively soft ionization method that allows proteins and peptides to be ionized without breaking them apart. ESI ionizes analytes in three steps. First, by forming charged droplets at the tip of the separation capillary. Second, by gradual droplet shrinkage until actual separate gas phase ions are formed.
Third, by transporting the newly formed gaseous ions from the ion source (which is at atmospheric pressure) into the mass spectrometer (which operates at high vacuum). Tryptic peptides will get protonated on their N-terminal amine and any lysine, arginine, and histidine residues as these are bases, and form singly or doubly charged ions. The aim of the ionization step is to ionize as many of the peptides as possible. The big voltage difference between the capillary and the MS, allows ions to fly through the electrical field. Once the peptides are ionized, they can be detected by the mass analyzer.

**Mass spectrometric analysis**

In **Paper II**, a linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (LTQ FTICR-MS) from Thermo Scientific was used. With a strong magnetic field (7 Tesla), the FTICR traps the ions and holds them circulating inside the ICR cell. The frequency at which the ionized peptides circulate (the cyclotron frequency) is unique to their m/z, which then becomes the basis of their identification. The output from the MS is a mass spectrum generated by Fourier transformation.

**Identification**

In **Paper II**, we performed protein identification using peptide spectra recorded in MS/MS mode. MS/MS mode means that after the first MS, the peptide ions are fragmented by collision induced dissociation and MS is again performed on the resulting smaller ions. The purpose of this fragmentation is to reveal the amino acid sequence of the proteins in order to identify them. In **Paper II**, the protein identities were obtained by using one of the commonly
used search engines, Mascot, to match the experimentally acquired MS/MS spectra to theoretically generated spectra for each peptide in a database called SwissProt.

**Relative quantification**

In MS-based proteomics proteins can be quantified in an absolute manner, provided that a stable isotopically labeled standard is spiked into the sample. Absolute quantification is therefore suitable for single or few proteins at a time, as it would be too expensive and complicated to spike in standards for hundreds of proteins. Relative quantification can be done on the entire proteome and the relative quantity of hundreds of proteins can be determined in one experiment. Relative quantification can be performed using LF or labeled approaches. The quantifications in Paper II were performed in MS mode. The LF MS relative quantification can be achieved by counting the number of spectra acquired for a certain peptide ion as an indicator of how much of that peptide is present. Alternatively, the peak intensity of the peptide in the spectrum from the case sample can be compared with the peak intensity of the corresponding peptide in the spectrum of the control sample. LF relative quantification always involves an inter-run comparison, which is less reliable than an intra-run comparison, which is possible for labeled samples. After the mass spectrometric analysis the raw data files of the LF sample are typically imported into a software (in Paper II we used the software DeCyder MS2.0) for peak detection and alignment to enable comparison of the amounts of protein between the case and control sample.

**Labeling**

There are many labeling approaches to choose from: isobaric tag for relative and absolute quantification (iTRAQ), tandem mass tags (TMT), and dimethyl labeling (DML). DML was chosen in Paper II because the combination of DML and FTICR outperformed other methods available in our lab in a previous comparison. The DML method is relatively fast, simple to execute and inexpensive compared with other labeling methods. We used two labels, the light (-CH₃) and the medium (-CHD₂) labels (Figure 6). After the MS experiment is performed, the raw data files are usually imported into an automated peak quantification software (in Paper II MSQuant was used). The peak intensities can then be log-transformed for later statistical analysis.

**Data analysis**

To control the quality of MS proteomic data different types of data analysis can be used. In Paper II, hierarchical clustering analysis was used, an R-Squared (R²) value was calculated for each pair (ON and OFF), and all low quality (R² ≤ 0.85) sample pairs were removed before further analysis. The peak in the ON state was then subtracted by the corresponding peak in the OFF state and normalized. Thereafter, moderated paired t-statistics were
used, linear models were fitted to the data, and p-values and fold changes were calculated for each peak. To quantify the difference between the ON and OFF state, peptides were selected that were present in more than 50% of the patients, had a fold change of at least 0.3, a p-value <0.05 and a Mascot score >25. These peptides were then combined into proteins and median p-values, fold changes, and Mascot scores; thereafter, Fisher’s p-values were calculated for each protein. The proteins were subsequently ranked in a top protein list. Because the data from MS-based proteomics are complex, it is common to perform a clustering or enrichment analysis to visualize the result. In Paper II, an enrichment analysis was performed.

Proximity Extension Assay (PEA)

The third method used in this thesis was the proximity extension assay (PEA) (Figure 8). A PEA panel of 92 inflammatory proteins was used to analyze serum samples from pain patients following disc herniation in Paper III. In Paper V the same PEA inflammation panel was applied to CSF samples from neuropathic pain patients and controls. Similar to PLA, PEA employs antibody-based probes with an attached oligonucleotide, and with the difference that in PEA the DNA oligonucleotides are hybridized to each other and the ligation step is replaced with a polymerization step to create a double-stranded DNA template for PCR-based amplification.

Figure 8. The principle of proximity extension assay (PEA).

In multiplex PEA the levels of 92 proteins are measured in one microliter samples using a microfluidic PCR readout, allowing simultaneous analysis of 92 target proteins and 4 controls.

Antibody Suspension Bead Arrays (ASBA)

In ASBA\textsuperscript{295, 296} (Figure 9), the detection antibody is coupled to a magnetic color-coded bead containing three internal fluorescent dyes. The relative abundance of the dyes in each bead conveys the bead identity. When the antibodies have been immobilized on (coupled to) the bead, as many as 384
bead identities can be mixed in the same suspension and used to analyze the same number of protein targets in a biofluid using only a few µL of the sample. The sample is prepared by dilution, biotin labeling of protein content, another dilution, and a heating step to improve epitope exposure. The sample is then incubated with the arrays. After incubation, the unbound proteins are washed away and relative protein abundances can be measured with the help of a streptavidin-conjugated fluorophore.

The relative protein abundances are displayed as median fluorescent intensities (MFIs) that can be used for further statistical analysis. In Paper IV, a unique 100-plex ASBA panel was designed, targeting 55 proteins, based on the results generated in Paper II to verify and extend the findings using a different technique with high throughput and small sample consumption. Other purpose-designed ASBA panels have revealed disease specific protein profiles in patient plasma and CSF.

Figure 9. The principle of antibody suspension bead array (ASBA).
Main results

Paper I: A Multiplex Protein Panel Applied to Cerebrospinal Fluid Reveals Three New Biomarker Candidates in ALS but None in Neuropathic Pain Patients.

- Nineteen of the 47 proteins were detectable in more than 95% of the 72 controls.
- None of the 21 proteins detectable in CSF from neuropathic pain patients was significantly altered by SCS.
- The levels of the four proteins, follistatin, interleukin-1 alpha, interleukin-1β, and kallikrein-5, were significantly lower in the ALS group compared with the age-matched controls.
- The results demonstrated that a multiplex PLA panel can be successfully used for CNS biomarker research by detecting several proteins in the human CSF.


- The levels of 86 proteins were statistically significantly different between the SCS on and SCS off states in the CSF of neuropathic pain patients with satisfactory SCS treatment.
- Functional enrichment analysis of the 86 altered proteins revealed that they are previously known for involvement in platelet degranulation, wound healing, complement/coagulation, and neuropeptide signaling.
- The top 12 altered proteins are involved in neuroprotection (clusterin, gelsolin, mimecan, angiotensinogen, secretogranin-1, amyloid β A4 protein), synaptic plasticity/learning/memory (gelsolin, apolipoprotein C1, apolipoprotein E, contactin-1, neural cell adhesion molecule L1-like protein), nociceptive signaling (neurosecretory protein VGF), and immune regulation (dickkopf-related protein 3).
- These findings in the CSF of neuropathic pain patients expand the scope of SCS effects on the neurochemical environment of the human spinal cord.
Paper III: Inflammatory Serum Protein Profiling of Patients with Lumbar Radicular Pain One Year after Disc Herniation.

- A clear overall difference in the serum cytokine profile between the chronic and recovered patients was demonstrated.
- Given a false discovery rate (FDR) of 0.10 and 0.05, we identified 41 and 13 proteins, respectively, which were significantly upregulated in the patients with severe pain 1 year after disc herniation.
- The three proteins with the highest increase were C-X-C motif chemokine 5 (CXCM5, 217 % increase), epidermal growth factor (EGF, 142 % increase), and monocyte chemotactic protein 4 (MCP-4, 70 % increase).

Paper IV: Affinity Proteomics Applied to Patient CSF Identifies Protein Profiles Associated with Neuropathic Pain and Fibromyalgia.

- Apolipoprotein C1 (APOC1) levels were found to be higher in CSF of neuropathic pain patients when compared with controls.
- There was a non-significant trend for increased levels in the CSF of fibromyalgia patients.
- Ectonucleotide pyrophosphatase (ENPP2/Autotaxin) levels were higher in the CSF of fibromyalgia patients when compared with controls and with neuropathic pain patients.
- Multivariate analysis confirmed the APOC1 and ENPP2 findings.
- Multivariate analysis also revealed partially overlapping and partially distinct CSF profiles in neuropathic pain patients compared with fibromyalgia and controls for several other proteins including angiotensinogen (AGT), prostaglandin-H2 D-isomerase (PTGDS), neurexin-1 (NRXN1), superoxide dismutase 1 (SOD1) and superoxide dismutase 3 (SOD3).

Paper V: High Levels of Cerebrospinal Fluid Chemokines Point to the Presence of Neuroinflammation in Peripheral Neuropathic Pain: A Cross-Sectional Study of Two Cohorts of Patients Compared to Healthy Controls

- CSF levels of chemokines CXCL6, CXCL10, CCL8, CCL11, and CCL23, as well as protein latency associated peptide transforming growth factor (LAP TGF)-beta-1 were significantly higher in both the neuropathic pain cohorts than in the healthy controls.
- Increased CSF levels of chemokines and other markers indicate an ongoing neuroinflammation in neuropathic pain patients.
Discussion

Method comparisons and complementarity

We used four main methods for protein analysis in this work (PLA, MS, PEA, and ASBA). Here the differences between the methods will be highlighted.

The protein identification in MS based on the amino acid sequence and the unique m/z of each peptide. The PLA, PEA, and ASBA, on the other hand are antibody-based techniques, i.e. the detection of targeted proteins is performed using antibodies coupled to an entity that can be detected. Because antibodies, even affinity-purified antibodies, have affinity for other molecules than their target, referred to as off-target or unspecific binding, the protein detection is less selective than with MS. In PLA and PEA this is partially compensated for by requiring several recognition events for detection signal compared with standard assays such as enzyme-linked immunosorbent assay (ELISA). In the ASBA panel used in Paper IV this selectivity is partially compensated for by incorporating several different antibodies in the panel with affinity for the same target. One advantage of the single binder and direct labeling employed in ASBA the multiplexed analysis of analytes as well as samples. In Paper IV, the uncertainty of antibody-based detection was exemplified as two of the three ENPP2 antibodies did not appear to bind ENPP2; the third antibody with high affinity for ENPP2 may also have had affinity for other proteins, which could be identified with immunoprecipitation followed by MS, although this is more likely to be background signal. Although unspecific binding of antibodies in a complex matrix can be a problem, it is not necessarily detrimental in the discovery stage of biomarker studies, as follow-up experiments with MS can determine the identity of the protein, and more selective antibodies can then be tested for further studies. Also, even if the theoretical protein identification with MS is highly specific, technical variation such as unstable spray, overlapping peaks, and complex data interpretation (for LF methods) can add uncertainty to the final MS results as well.

The antibody-based techniques (PLA, PEA, and ASBA) typically have higher throughput, allowing for faster processing of large numbers of samples. Antibody-based techniques consume relatively little sample, 5-100 μL, of CSF or blood compared with the MS protocol we used which requires 500 μL. Low sample consumption allows several replicates and serial investiga-
tions of the same sample set. On the other hand, MS methods usually detect several hundreds of proteins, which is more than the average antibody-based panels we used which were all below 100 targets. The sample consumption and runtime can be put in relation to the purpose of the study.

In biomarker discovery, a disadvantage with antibody-based multiplex panels compared with MS-based proteomics is the requirement of preexisting hypotheses about candidate markers for a relevant panel composition. MS methods allow for open-ended research questions, not based on previous biomarker candidates, allowing for truly novel disease targets to be discovered. This thesis clearly illustrates this point. In Paper I, our selection of protein targets based on the literature appeared irrelevant to the investigated SCS mechanism, but with an untargeted MS method like the one used in Paper II we could find several protein alterations in the same sample set. PLA has been recommended for biomarker validation. The original plan was to start with MS and follow-up with PLA. For practical reasons, this was not possible, but it would have been a more logical order. Instead, we later used the list of interesting proteins composed from the MS results in Paper II to select antibodies for the ASBA panel in Paper IV. That approach produced at least two new biomarker candidates for chronic pain conditions, confirming that it is a productive way to combine MS and antibody-based methods. Particularly noteworthy was the lack of overlap between the proteins detected in the SCS CSF samples with PLA in Paper I and with MS in Paper II, suggesting that perhaps PLA detected lower abundant proteins than MS. Thus, our results appear to support the notion that antibody-based techniques are able to detect lower abundant proteins than MS although this is not applicable for targeted MS methods.

Antibody-based methods use relatively light sample preparation compared with the immune fractionation and digestion common in MS methods. Antibodies can usually find their targets (although with some unspecific binding as discussed above) in complex matrices, which is highly beneficial in that it reduces the contribution of sample preparation to technical variation in the final result. Instead of sample preparation sample dilution, buffer composition, background variation and heating steps can contribute technical variability. These parameters can also affect MS results. Also the MS instrumentation is more complex than the ones used for PLA, PEA, and ASBA, requires more attendance (such as continuous filling of nitrogen to cool the magnet), and more frequently needs technical support and repair.

The commercial PEA method is reported to have a coefficient of variation (CV) below 10% for most proteins within the dynamic range of the assay. PEA is a solution phase assay, i.e., the captured antibody is not coupled to a solid support. In assays such as SP-PLA and ASBA, the capture antibody is coupled to a solid phase magnetic bead, which usually increases the unspecific binding and entails a washing step that contributes to the variation. The 92-plex PEA used in Paper III and Paper V has benefited from thorough
commercial optimization of several assay parameters (e.g., the choice of DNA polymerase and reduced manual handling steps with no requirement for washing steps compared to solid-phase PLA we applied in our first study (Paper I). PEA reports lower CVs for many of the protein targets compared with the SP-PLA used in Paper I but the two methods were not directly compared with the same targets and that can also contribute to differences in CVs.

In Paper II we used two different MS methods, label free and labeled samples. There were also other differences between the two protocols. The immunoaffinity column used in the LF experiment has affinity for the top 14 most abundant proteins, and therefore reduces the amount of these proteins remaining in the samples, whereas the one used for DML has affinity for the top 7 most abundant proteins. This difference can explain some of the differences in the results seen by LF and DML studies in Paper II.

The benefit of labeling the samples with stable isotope labels (commonly $^{15}$N, $^{18}$O, $^2$H, or $^{13}$C) is the possibility of mixing the case and the control sample, a strategy that dramatically halves the analysis time and eliminates the problem of run-to-run variations in the efficiency of the ESI (the spray is not always stable) and LC retention time. LF approaches lack the benefit of being able to combine the case and the control sample into one. Therefore, it is more sensitive to variations over time such as spray instability and shifts in retention time due to LC memory (carryover) effects and gradual increased packing or saturation of the column. One option for minimizing the effect of these variations on the final result is to run the case sample directly after the control sample, and vice versa as we did in Paper II. The requirement on expert data processing for normalization and other handling of this variability is higher in LF quantification approaches compared with labeled ones.

In summary, the studies presented in this thesis support the view that the combination of antibody-based and MS methods can answer important research questions in pain biomarker discovery, perhaps best starting with MS-based proteomics and following up with a dedicated PLA, PEA, or ASBA panel based on the MS findings.

Biofluid samples

One challenge in CSF research is the scarcity of samples from healthy volunteers. Blood samples can be obtained from consenting volunteers, but to sample CSF lumbar puncture is required. Lumbar puncture is rarely performed on healthy individuals because of the discomfort, requirement of trained expertise, and small but existing risk of side effects. We attempted to handle this in different ways in the four papers comprising this thesis.
In Paper I, II, and IV, we addressed this issue using a longitudinal intra-individual study design comparing pain patients using SCS to themselves when not using SCS. In Paper IV, we analyzed samples from 200 individuals of which 25 were patients with neuropathic pain, 40 with fibromyalgia, and 135 were controls. The neuropathic pain verification set (NP2, n = 11) and their controls (C2, n = 11) were collected at the same clinic to enhance reliability in the comparison between controls and neuropathic pain patients. The other samples were collected at different clinics in Cluj (Romania) (C1, C3), Uppsala (NP1), and Oslo (fibromyalgia). Lifestyle and clinical practices in Norway and Sweden are comparable. Differences in the Nordic-Romanian comparison may be larger, however. The CSF sample collection and handling protocols were the same and the person performing the sample collections in the Cluj site was trained in Uppsala. Still, we saw that the C1 and C3 sample sets, which were collected at the Romanian site, differed significantly in several protein levels from the C2 sample set collected in Sweden. This discrepancy could be caused by different factors, including differences in lifestyle, health status (minor urology surgery patients vs. healthy volunteers), and sample handling. We addressed this problem in Paper IV by disregarding all biomarker candidates that were significant in the control-control comparison. The sample set from the patients with minor urology surgery naturally contained more males than females and all the fibromyalgia CSF samples were from female patients only. Yet, they were compared with samples from mixed sex groups. However, sex has not proven to influence any CSF proteins in our previous investigations, except for the expected difference in PSA. Moreover, we found no sex bias in the ENPP2 result and the fibromyalgia patients had significantly higher ENPP2 levels compared to all other groups even when all males were removed from the analysis (p-values; FM vs. C2 <0.001, FM vs. NP2 <0.001, FM vs. C3 0.005).

Another consideration is that most patients in the study are taking medications for their pain. In Paper III the patient group was also taking more anti-inflammatory medication than the controls for natural reasons. It therefore seems plausible that the effect of medication reduces rather than increases the difference in inflammatory serum proteins. Although there are indications that drugs do not interfere with the pain protein profile in the CSF, interference cannot be ruled out. These factors contribute to the uncertainty of the results. Validation of the biomarker candidates in additional cohorts is required.

The apparently low levels of follistatin, IL-1 alpha, and Kallikrein-5 (KLK5) in CSF found in ALS patients in Paper I may be caused by high levels of many proteins in the CSF control samples which were demonstrated with ASBA in Paper IV when we had access to CSF samples from healthy volunteers for comparison. The 19 protein control concentration values, four of which (sortilin, CCL16, cystatin B, KLK5) had not previous-
ly been reported for adult individuals without neurological disease should be interpreted with caution. Still, this limitation pertains to the selection of control samples and does not affect the basic conclusion of the paper, which is the utility of multiplex SP-PLA panels as a tool for research on neurological biomarkers.

Because plasma concentrations of several proteins are associated with age, we matched the groups for age in Paper I-V. The effect of sex bias is also an important parameter as this can affect protein levels in both the blood and CSF. In Paper I, measurements of all 47 proteins were compared between males (n = 20) and females (n = 20) in the ALS patients and controls using the Mann-Whitney U test. The analysis demonstrated no sex effect on any of the markers, except prostate specific antigen (PSA), which is to be expected. In Paper II, patients (males and females) were only compared with themselves and sex bias is not applicable. In Paper III, the samples were also sex-matched. The fibromyalgia group in Paper IV consisted of only females, reflecting the real-life demography of this disease. In our study the female fibromyalgia patients were mostly compared with a group of patients, in which male sex was overrepresented, but again we saw no sex bias in any of the groups for ENPP2 and ENPP2 signals were significantly higher than those for all other females. In Paper V, male-female ratio was similar in the different groups.

A recent report has also highlighted the importance of sample storage time. Some of the samples we compared in Paper I, IV, and V were stored for durations differing by several years. In Paper IV the largest difference in sample storage times was 15 years. This factor may influence the results. Enroth et al. analyzed plasma samples with the same PEA panel that was used for the CSF analysis in Paper III and V. They reported that for 10% of the proteins, sample storage time explained 5-35% of the observed variance in protein level. For LAP TGF-beta-1, which we found to be upregulated by approximately 20% in neuropathic pain patients in Paper V the percentage of the variance explained by storage time was 10%, suggesting that part of this result may be explained by differences in sample storage time. However, the sample storage time of the sample groups in Paper V differed by less than one year as compared with 26 years in the Enroth study, suggesting that this discrepancy is minor. The storage time effects may also be different in plasma samples than in CSF, since plasma samples contain 100-200 times more protein. In Paper III, a prospective study design was used, with serum samples collected continuously at the same site. Such a design also reduces potential confounders.
Data evaluation and statistics

For any type of research analysis, data evaluation will have an influence on the result. Data analysis is even more central in the large data sets in omics research. The normalization and statistics applied to the raw data will determine which markers end up on the reported list of biomarker candidates. For example, in Paper II we applied median normalization, but in hindsight, the lack of verification with ASBA in Paper IV suggests that spike in normalization may have been preferable.

In Paper I-V, we analyzed multiple markers in the same experiment. When analyzing many variables, the multiple testing problem is a concern. In theory, when using the common significance level of 0.05, and if $k$ is the number of comparisons, the risk of at least one false positive is $1 - 0.95^k$. In Paper I, we measured levels of a maximum of 47 proteins and conducted multiple comparisons, which were corrected using the Bonferroni method by dividing $\alpha=0.05$ by the total number of tests ($n=47$), i.e. $0.05/47 \approx 0.001$. Thus, all p-values reported were adjusted using this approach. Since then it has come to our attention that Bonferroni correction may be too conservative. This is because p-value-based corrections assume independence between the measured variables (in this case proteins or peptides). Such an assumption is incorrect because peptides (and proteins such as cytokines and chemokines investigated in Paper III and V) are correlated. In Paper II, we applied commonly used evaluation methods of mass spectrometric data. Instead of a p-value cut-off, several factors were considered and the findings were weighted. Essentially, we choose the risk of false positives over the risk of losing potential biomarkers. Such a strategy, however, requires a more targeted method to validate the discovery, as was attempted in Paper IV. The lack of verification with ASBA, make the results of Paper II unconfirmed, but not falsified (see “Biomarker verification and validation” section below).

In Paper III and Paper V, for our main comparison between cohorts 1a and 1b, we reasoned that in a discovery phase rather than a biomarker validation study some false positive results are acceptable and we controlled the FDR using the procedure of Benjamini and Hochberg. In Paper III we used FDRs of 0.10 and 0.05, meaning that up to 1/10 and 1/20, respectively, of the findings may be false positives. Because 13-41 of the inflammatory proteins were upregulated, the results still provide a strong case for overall increased inflammation in these patients, even if a few findings are false positives. This reasoning was also supported by linear discriminant analysis, which completely separated the two groups when an FDR of 0.1 was used.

In Paper V, we examined 92 proteins. Given an FDR of 0.1, up to 1/10 of the findings are likely to be false positives. Hence, although a few false positive markers may be present, it seems unreasonable to conclude our results are a massive type I error. The possibility of medication, comorbidities, or
both being confounding factors should be acknowledged for those results obtained in Paper III and V. In Paper V, as a complement to FDR and confirmation, we used orthogonal partial least squares discriminant analysis (OPLS-DA). This multivariate method includes all variables simultaneously, taking the correlation structure of the data set into consideration 325. OPLS-DA, is a less common method, but it has been used for a series of peer-reviewed publications 326–331 and is in accordance with the principles maintained by Wheelock and Wheelock 332. Extraordinarily, the 11 proteins we found by OPLS-DA when comparing the first neuropathic pain cohort with healthy controls were the same as those found using multiple univariate testing with control of FDR. We argue that the measures taken to control for the multiple testing dilemma in Paper III and V are appropriate for discovery-oriented studies. Because all the investigations in this thesis are discovery studies investigating moderate-sized sample sets, all results require further validation.

In Paper IV, we used principal component analysis to obtain an overview of the data and detect and remove strong outliers before further analysis. We then tested for differences using the Wilcoxon rank sum test (univariate analysis). P-values <0.05 after FDR were considered significant. We also applied OPLS-DA (multivariate analysis) as described above. Uni- and multivariate analyses largely converged on the same biomarker candidates.

Compared with confirmative studies or studies focusing on one marker with a long research tradition, discovery studies have fewer previous publications to help understand and interpret the findings. It follows that in publications from the omics field, long lists of proteins without much attempt to interpret potential disease relevance are common. Biofluid samples also do not carry detailed information of the source of the analyzed biomarker candidate, which is because the fluids circulate entire organs, including many sites and cell types resulting in broader mechanistic interpretations.

Verification and validation

The certainty of a biomarker candidate finding can be increased by verification, which usually refers to the re-analysis of the same sample set with a different method. A more crucial divider on the road from a biomarker candidate to a fully useful clinical biomarker is the validation study. This is where the biomarker candidate is measured in a new material (e.g., another patient sample cohort) to validate the finding from the discovery study. Large follow-up studies fall outside the scope of this thesis. Thus, what we present here are biomarker candidates. Nonetheless, the measures already taken to verify and validate the findings are described below.

Validation of the ALS biomarker candidates reported in Paper I was not attempted. In Paper II we attempted to verify and improve the quantifica-
tion of protein levels in CSF by using two complimentary MS approaches, LF and DML. We could not verify the MS SCSON-SCSOFF findings from Paper II when using the ASBA technique (Paper IV) for the selected 55 proteins. This lack of verification brings some uncertainty to the findings, although it does not falsify them for the following five reasons. First, although the two studies centered on the same cohort of 15 individuals, there is not an exact overlap of patients between the studies. Second, the sample preparation steps are different in the two methods. Third, MS detection is performed on peptides derived from an intact protein by tryptic digestion, whereas the ASBA detects intact heated proteins. Fourth, the magnitudes of the fold changes between the SCSON and OFF state detected in the MS study may have been too small to be detected by the ASBA technique. At least two studies have had partial success in verifying mass spectrometric findings with ASBA (4 of 12 attempted proteins \(^{308}\) and 11 of 52 attempted proteins \(^{307}\)). Both studies used inter-individual comparisons and overall had higher fold changes in the MS result than we reported. Fifth, in our MS study we used the leverage of two complementary MS techniques, with one of these labeled to improve quantification, but we did not use spike-in normalization, which has previously been found to result in better reproducibility between the MS and ASBA results \(^{307}\).

The study design of Paper III was prospective but we only analyzed samples from the 12-month time point. Therefore, we do not know whether the inflammation we detected in pain patients is a state or a trait. However, a previous study showed that all the patients had high levels of IL6 and IL8 at the acute time point \(^{333, 334}\), which is suggestive of inflammation in both groups at the outset. In Paper IV, we used two separate cohorts of patients with neuropathic pain and two separate sets of controls. We could see a confirmative trend of the APOC1 finding though the result did not reach statistical significance.

In Paper V we included two neuropathic pain cohorts and found partially (55 %) overlapping protein profiles with signs of neuroinflammation in both groups when compared with the same healthy control group (n = 11). This finding is a validation within the study, although the sample sets are limited (n = 11 and n = 16). One potential contribution to the lack of complete overlap is that the long-term use of SCS might have altered the CSF inflammatory profile of the second cohort.
The biomarker candidates and their potential role in the pathophysiology of chronic pain

Apolipoproteins

One recurrent protein in two of our studies is APOC1. The results from Paper II suggest that APOC1 concentration levels in CSF levels are reduced by satisfactory neuropathic pain treatment. In Paper IV, we found that APOC1 was upregulated in the CSF of neuropathic pain patients. If neuropathic pain patients have higher levels of APOC1, it is noteworthy that a satisfactory treatment can reduce these levels. To our knowledge, there are no other published results directly linking APOC1 to persistent pain or its treatments. APOC1 can enhance the TLR4-mediated inflammatory response 335, affect hippocampal volume and function 336, 337 (which is altered in animal models of chronic pain and chronic pain patients 173, 177, 338-345), and is involved in lipid metabolism that is important for CNS homeostasis. There are reports of other apolipoproteins associated with the pathophysiology of chronic pain 327, 346, 347. The results in Paper II suggest that satisfactory SCS treatment of neuropathic pain altered levels of APOE, APOA1, APOA4 and APOH. Based on our results the apolipoprotein system appears worthy of further exploration for chronic pain mechanisms.

ENPP2 and lipid signaling

The increased levels of ENPP2 seen in fibromyalgia patient samples in Paper IV may indicate a role for ENPP2 in fibromyalgia pathophysiology. Although we did not see any increases of ENPP2 in neuropathic pain patients, ENPP2, synonymous with extracellular lysophospholipase D (LysoPLD), was the third most important protein for distinguishing neuropathic pain patients CSF samples from control samples in a recent mass spectrometric study 327, supporting a role for ENPP2 in neuropathic pain as well. One of the products of ENPP2, lysophosphatidic acid (LPA) has been implicated as a key mediator of neuropathic pain 348, 349. Because ENPP2 catalyzes the formation of two inflammatory lipid mediators (sphingosine 1-phosphate (S1P) and LPA with reported effects on pain modulation, the ENPP2 result is consistent with a neuroinflammatory component in fibromyalgia. This is consistent with previous studies suggesting a role for altered inflammation in fibromyalgia and CWP 184, 350, 351. Our results suggest that a more extensive investigation of lipid mediated signaling in fibromyalgia may be worthwhile.
LAP TGF-beta-1.

Five of the six markers significantly associated with neuropathic pain in Paper V were chemokines, classical inflammatory mediators. The sixth protein associated with neuropathic pain in both cohorts, LAP TGF-beta-1, was perhaps more unexpected. To our knowledge, there are no previous reports of increased levels of LAP TGF-beta-1 in neuropathic pain patient CSF. LAP TGF-beta-1 is the latency-associated peptide complex of TGF-beta-1. It is noteworthy that LAP TGF-beta-1 was one of the proteins associated with lumbar radicular pain in Paper III, as well as in a recent fibromyalgia publication using the same PEA panel \( ^{352} \). Recent results from animal models of chronic pain provide a basis for interpretation of this finding in the pain context. Intrathecal infusion of TGF-beta-1 was recently reported to suppress the nerve injury-induced inflammatory response (cytokine expression) and glial activation in the spinal cord \( ^{353} \) and has been shown to inhibit neuropathic pain-like behavior in a murine chronic constriction model of neuropathic pain \( ^{354} \). TGF-beta latency-associated peptide has been shown to block TGF-beta-1 biological effects \( ^{355}, ^{356} \) and modulate the half-life of TGF-beta-1. In the experimental cell culture setting exposure to pro-inflammatory conditions increased the LAP TGF-beta-1 and TGF-beta-1 activity in hippocampal neurons \( ^{357} \). Based on these and our results a hypothesis is that the high CSF levels of LAP TGF-beta-1 we detected in neuropathic pain patients mirror a response to high pro-inflammation and, in fact, are part of an elevated but insufficient anti-nociceptive mechanism in chronic pain patients. Because many studies of pain conditions indicate a role for this protein, it should be further investigated.

Broad inflammatory profiles and support for glial activation in human pain conditions

Some of the protein changes we detect in Paper II are consistent with an effect of SCS on inflammatory processes. In Paper III, we found evidence to support a role for inflammatory processes in chronic sciatic pain after disc herniation. In Paper IV, and V we report protein changes consistent with an ongoing neuroinflammation in the CSF of neuropathic pain, and fibromyalgia patients.

Systemic inflammation in chronic sciatic pain after disc herniation

Previous work has demonstrated changes of a handful of proteins in blood samples of chronic pain patients suggestive of inflammation (chronic widespread pain \( ^{286} \), fibromyalgia/chronic pain \( ^{288}, ^{358} \), and painful neuropathies
Recent evidence from lipopolysaccharide (LPS) stimulation of healthy volunteers supports the theoretical link between systemic inflammation and weakened pain regulation. In Paper III, we report an inflammatory profile consisting of 41 of 92 analyzed proteins (including IL6 and IL8) in the serum of patients with sciatic pain 1 year after disc herniation. The novelty in Paper III is the broad inflammatory profile combined with a prospective study design. IL6 and IL8 had been measured by ELISA in the same samples before and were found to be upregulated in all the disc hernia patients in the acute phase. It appears these interleukin levels normalize in those who recover without pain, whereas they remain elevated in those who develop chronic pain. These results are in line with previous findings. Overall, our results support the hypothesis that unresolved systemic inflammation contributes to chronic pain development after disc herniation.

Future concept comparing protein profiles of fibromyalgia and lumbar radicular pain

Recent studies using the same multiplex PEA panel as in Paper III and Paper V have also shown remarkable overlap in results in patients with chronic widespread pain. If we allow a 0.05 FDR in the inflammatory plasma profile of the disc herniation pain patients and compare it to the profile of serum from fibromyalgia patients analyzed with the same panel we see that some markers are shared (STAMPB, Casp-8, TGF-beta-1, CXCL5, MCP-4, MCP-2), some are solely elevated in the serum of patients with lumbar radicular pain (EGF, IL-15-R-alpha, CCL4, CXCL10/IP10, VEGF-A, M-CSF/CSF-1, MCP-3/CCL7) and others are uniquely elevated in the plasma of fibromyalgia patients (SIRT2, CD40, AXIN1, IL-7, CXCL6, CXCL1, 4EBP1, CD244, CXCL11, STIA1, TNFSF14, ADA, MMP-1, IL-8, ENRAGE). Changing the FDR affects these lists. These data are not optimal for comparison because plasma and serum are different and the results are not yet confirmed in additional studies. Nonetheless, comparing biomarker profiles in this manner may become a real future clinical tool, guiding diagnosis, treatment selection, and response monitoring.

Signs of neuroinflammation in neuropathic pain and fibromyalgia patients

Neuroinflammation and glial activation are already considered important concepts in modern pain medicine and one of the possible mechanisms underlying human neuropathic pain. Extensive analysis of animal models of chronic pain in the 1990’s and 2000’s suggested that chemokines and
inflammatory proteins play a role in the pathophysiology of neuropathic pain. However, the clinical evidence for this has been moderate. **Paper V** is, to our knowledge, the first report of an extensive CSF inflammatory profile of patients with severe peripheral neuropathic pain. Five of the six proteins upregulated in both neuropathic pain cohorts, in **Paper V** (CXCL6, CXCL10, CCL11, CCL23, and LAP TGF-beta-1), were also recently demonstrated to be higher in the CSF of fibromyalgia patients using the same PEA panel, whereas CCL8 was not. The CSF control group in **Paper V** and the fibromyalgia study is the same, but the overlapping results are nonetheless striking. In **Paper IV**, we reported altered CSF levels of APOC1 in neuropathic pain patients and ENPP2 in fibromyalgia patients. APOC1 has been shown to modulate glial activation and neuroinflammation that is triggered by glial activation is a hallmark of chronic pain development according to animal model research.

Most of the evidence of central neuroinflammation in chronic pain has come from animal experiments. For instance, microglial release of chemokine CCL2 (MCP-1) and involvement of chemokine CX3CL1 (fractalkine) were reported in neuropathic pain development and neuropathic animals have high CSF levels of CX3CL1. In **Paper V**, we showed that CX3CL1 was significantly upregulated in cohort 2 and upregulated with a rather high VIP value in cohort 1a (VIP = 1.06), demonstrating for the first time this result in patients with severe neuropathic pain. However, the main finding in **Paper V** is the broad picture indicating an extensive and ongoing process of chronic neuroinflammation in neuropathic pain patients and perhaps that some classical factors (IL6 and BDNF) were not significantly different between patients and controls. The central neuroinflammation that is visualized in **Paper V** is a possible contributing mechanism to chronic pain development and maintenance. We cannot exclude that the proteins we measured in CSF are derived from plasma, but it is reasonable to assume that the present findings might mirror inflammatory activity in the CNS. Future studies relating the inflammatory fingerprints of CSF and blood will probably cast light on this matter.

Our study does not answer the question of whether the inflammatory CSF fingerprint is a pre-existing risk factor or is a consequence of the pressures associated with having chronic pain over a long duration (e.g., aspects of the sickness syndrome, depression, poor sleep, stress, physical inactivity, and obesity).
Conclusions

- The biofluid protein profiles of chronic pain patient differ significantly from those of subjects without chronic pain.
- Combining proteomic mass spectrometric methods and multiplex antibody-based protein panels allows for discovery of candidate biomarkers of chronic pain pathophysiology and therapeutic mechanisms.
- SCS treatment of neuropathic pain appears to have previously unknown effects on levels of proteins involved neuroprotection, nociceptive signaling, immune regulation, and synaptic plasticity.
- Our results support the presence of ongoing inflammation and neuroinflammation in chronic pain patients, which have not been characterized in this detail before.
- We present several candidate biomarker profiles of human pain conditions for further investigation:
  - Elevation of up to 41 inflammatory serum proteins, including CXCM5, EGF, and MCP-4, one year after disc hernia specifically in those patients who had developed lumbar radicular pain.
  - Partially overlapping and partially distinct CSF profiles in neuropathic pain patients compared with fibromyalgia and controls for proteins including AGT, PTGDS, NRXN1, SOD1, and SOD3.
  - Increased levels of ENPP2 in fibromyalgia patient CSF.
  - Increased CSF levels of APOC1, CXCL6, CXCL10, CCL8, CCL11, and CCL23, LAP TGF-beta-1 neuropathic pain patients.
  - Biomarker profiling of human biofluids is a promising addition to current tools in pain medicine and pain research.
Future perspective

Further efforts are needed to validate and extend these and other biomarker candidates for pain pathophysiology.

In future research on biofluid biomarkers of chronic pain pathophysiology it will be imperative to continue to specify unique and common markers for different pain conditions. One task will be to disentangle contributions from pain and its comorbidities (depression, anxiety, stress, and poor sleep) to the inflammation that appears to be ongoing in chronic pain patients.

Concrete approaches to mechanism-based diagnosis and treatment selection have recently been suggested. Hopefully future pain medicine includes a fortified diagnostic tool box with biofluid biomarker panels similar to those presented in this thesis.

New therapeutic opportunities for chronic pain that target excessive systemic and neuroinflammation (not necessarily restricted to drug treatments) are already being investigated and may be part of a future pain treatment portfolio. A better understanding of chronic pain pathophysiology should also be paired with more research on protective factors. Hopefully this could also result in better awareness on how to prevent chronic pain from developing in the first place.
Kronisk smärta drabbar ca 20 % av befolkningen, är svårbehandlad, och orsakar mycket lidande. I Sverige uppgår de direkta och indirekta kostnaderna för kronisk smärta årligen till 87,5 miljarder SEK. Kronisk smärta lindras sällan tillfredsställande av de läkemedel som lindrar akut smärta, den kroniska smärtan tycks upprätthållas av egna mekanismer. Trots decennier av forskning vet vi för lite om dessa mekanismer.

Inom smärtmedicinen är patientintervju och klinisk undersökning central för diagnostiken, men därutöver finns ett stort behov av biomarkörer, metoder för att mäta de biologiska processer som orsakar den kroniska smärtan. Den rådande bristen på biomarkörer inom smärtmedicin försvårar preoperativ riskbedömning, behandlingsval samt utveckling av nya effektivare behandlingar och preventiva strategier. Många smärttillstånd vars orsaker vi idag inte förstår, kan påvisa med röntgenbilder eller andra traditionella metoder, har ansetts vara av psykologisk natur och icke biologiskt mätbara. Även med mycket känsliga metoder har det bedömts osannolikt att biomarkörer ska gå att detektera i prover från smärtpatienter. Många patienter med oförklarliga smärttillstånd upplever utöver sin smärta, en stigmatisering, och att deras smärta inte tas på allvar.

Målet med den här avhandlingen var att identifiera biomarkörkandidater som kan bidra till att förklara den kroniska smärtans biologiska mekanismer. Det långsiktiga målet är att biomarkörer ska leda till effektivare behandlingar.

I den här avhandlingen användes för första gången masspektrometri och nyutvecklade känsliga multiplexa antikroppbaserade metoder systematiskt för att utforska proteinmönster i blodprover och prover av ryggmärgsvätskan, den vätska som omger hjärnan och ryggen för många olika typer av smärtpatienter. Proverna har donerats av personer utan kronisk smärta (kontroller), samt av patienter med 1) kronisk smärta efter diskbråck, 2) perifer neuropatisk smärta (smärta som beror på en skada eller sjukdom i det perifera nervsystemet), och 3) fibromyalgi (smärta och ömhet över stora delar av kroppen).

I den första studien rapporterades för första gången att en ny känslig analysmetod, multiplex solid-phase proximity ligation assay (SP-PLA), kan en användas för att mäta tiotals olika proteiner i ryggmärgsvätska parallellt. Prover av ryggmärgsvätska från en grupp patienter med amyotrofisk lateral

I den andra studien analyserades samma ryggmärgsstimuleringsprover som i första studien med masspektrometri. Masspektrometrin analyserar proteiner förutsättningslöst och med denna metod detekterades förändringar i nivåerna av 86 proteiner varav 12 var speciellt intressanta. Dessa proteiner har inte tidigare kopplats till ryggmärgsstimulering, men är tidigare kända för att kunna skydda nervceller, bidra i smärtsignalering, immunförsvar och reglera synaptisk plasticitet. Att halten av dessa proteiner förändras av ryggmärgsstimulering var inte känt sedan tidigare och det pekar på helt nya typer av mekanismer för denna behandlingsform.

I den tredje studien mättes nivåerna av 92 inflammationsproteiner med proximity extension assay (PEA) parallellt i blodprover från patienter som ett år tidigare hade haft ett smärtsamt diskbråck. Mätningarna visade för första gången att de personer som hade utvecklat kronisk smärta hade förändrade halter av fler än 30 olika proteiner i sitt blod jämfört med de som läkt normalt och blivit smärtfria. Detta visar för första gången en omfattande inflammationsprocess mätbar i blodet hos dem som utvecklar kronisk smärta efter diskbråck.

I den fjärde studien användes en annan, ny måttmetod, antibody suspension bead array (ASBA) för att analysera ryggmärgsvätska från flera olika patientgrupper, två grupper av patienter med neuropatisk smärta, en grupp med fibromyalgi och två kontrolgrupper utan smärta. Patienter med neuropatisk smärta hade förhöjt eller förändrad proteinnivåer skiljde mellan de olika grupperna (angiotensinogen (AGT), prostaglandin-H2 D-isomerase (PTGDS), neurexin-1 (NRXN1), superoxide dismutase 1 (SOD1) and superoxide dismutase 3 (SOD3)). Dessa resultat visar för första gången på distinkta och delvis gemensamma proteinprofiler i ryggmärgsvätska från patienter med fibromyalgi och neuropatisk smärta jämfört med kontroller.
I den femte studien mättes nivåerna av samma 92 inflammationsproteiner som i den tredje studien, men denna gång i ryggmärgsvätska från två grupper av patienter med nervsmärta jämfört med en kontrollgrupp. Patienterna hade förhöjda nivåer av sex inflammationsproteiner vilket tyder på att de har en inflammation i sitt centrala nervsystem. Neuroinflammation har tidigare påvisats i djurförsök, men denna studie visar för första gången en neuroinflammatorisk profil i ryggmärgsvätskan från patienter med neuropatisk smärta.

Slutsatserna av denna avhandling är att kombinationen av masspektrometri och känsliga antikroppsbasera mätmetoder är användbar för att finna biomarkörkandidater för kronisk smärta. Våra resultat pekar ut nya möjliga mekanismer bakom effekten av ryggmärgsstimulering. Våra fynd tyder på att patienter som utvecklar kronisk smärta efter diskbräck har en pågående omfattande inflammation som går att mäta i blodet ett år efter diskbräcket. Våra fynd tyder också på att inflammatoriska lipidmediatorer relaterade till APOC1 och ENPP2 kan spela en roll i nervsmärta och fibromyalgi respektive. Våra resultat tyder också på att patienter med neuropatisk har en pågående inflammation i sitt centrala nervsystem vilken möjligen kan vara en del av förklaringen till, eller följen av deras smärta. Vidare forskning krävs för att bekräfta och utveckla dessa fynd. Om fynden bekräftas av fler studier kan de tillsammans med andra resultat från smärtforskning innebära nya verktyg inom smärtforskning, smärtvård och för utvecklingen av nya behandlingsstrategier. Förhoppningsvis kan resultaten i denna avhandling bidra till en ökad förståelse av hur smärta blir kronisk, och hur den kan behandlas och förebyggas.
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