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Evidence for a role of nerve injury in painful intervertebral disc degeneration: A cross-sectional proteomic analysis of human cerebrospinal fluid

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Abstract

Intervertebral disc degeneration (DD) is a cause of low back pain (LBP) in some individuals. However, while >30% of adults have DD, LBP only develops in a subset of individuals. To gain insight into the mechanisms underlying non-painful versus painful DD, human cerebrospinal fluid (CSF) was examined using differential expression shotgun proteomic techniques comparing healthy controls, subjects with non-painful DD, and patients with painful DD scheduled for spinal fusion surgery. Eighty-eight proteins were detected, 27 of which were differentially expressed. Proteins associated with DD tended to be related to inflammation (e.g. cystatin C) regardless of pain status. In contrast, most differentially expressed proteins in DD-associated chronic LBP

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patients were linked to nerve injury (e.g. hemopexin). Cystatin C and hemopexin were selected for further examination by ELISA in a larger cohort. While Cystatin C correlated with DD severity but not pain or disability, hemopexin correlated with pain intensity, physical disability and DD severity. This study demonstrates that CSF can be used to study mechanisms underlying painful DD in humans, and suggests that while painful DD is associated with nerve injury, inflammation itself is not sufficient to develop LBP.

Keywords

proteomics; intervertebral disc degeneration; low back pain; cystatin C; hemopexin

Introduction

Chronic **low back pain (LBP)** is a highly prevalent, costly, and disabling condition which is undertreated.⁵⁵ In some individuals, LBP arises from the intervertebral discs, which may undergo degeneration, resulting in alterations in volume, shape, structure and composition.¹² However, the link between intervertebral **disc degeneration (DD)** and chronic LBP is unclear, as 30% of adults without LBP have signs of DD,^{7, 51} associated with normal aging.⁷¹ Because of this, magnetic resonance imaging studies are unable to differentiate painful from non-painful degenerating discs.⁴⁸ Furthermore, while radiating pain is often associated with LBP, nerve compression is not commonly observed by magnetic resonance imaging techniques.³⁶ Other factors not detectable by imaging must therefore contribute to the pathophysiological mechanisms of painful vs. non-painful intervertebral DD. A greater understanding of these factors will identify mechanisms that should be targeted for pain relief.

Biochemical indices of intervertebral DD can be observed in **cerebrospinal fluid (CSF)**, as the spinal canal where CSF is obtained is in close proximity to the intervertebral discs. For instance, previous studies have observed altered protein expression in the CSF of patients with lumbar disc herniation.^{10, 11, 62, 105} Our goal was to examine CSF for differences between individuals with painful and non-painful DD by quantitative shotgun proteomics. Shotgun proteomics has the advantage of providing an unbiased account of protein expression, allowing for the identification of proteins that would not have been predicted *a priori* to be involved in the disease pathology.

In CSF there is a large dynamic range between the most abundant (e.g. albumin; 130–350 mg/L) and the least abundant detectable proteins (ng/L).⁸² Since highly abundant and large proteins produce many peptide fragments which can mask the signal of potentially more interesting, low abundance proteins, we employed two methods of sample enrichment prior to proteomic analysis: immunodepletion and acetonitrile precipitation. **Isobaric tags for relative and absolute quantification (iTRAQ)** were then used in an exploratory cohort to compare relative protein abundances in the CSF of: **i)** normal controls, **ii)** subjects with non-painful moderate to severe DD, **iii)** patients with DD-associated chronic LBP that were scheduled for spinal fusion surgery.

The inclusion of the pain-free DD group allowed us to distinguish between processes involved in painful vs. non-painful disc-degeneration, permitting the identification of pain-specific changes in protein expression. After the initial proteomic screen, two candidate proteins were selected for quantification by ELISA in a larger cohort. Based on the patterns of differential protein expression observed in the proteomics screen, we hypothesized that Cystatin C, a marker for inflammation, would be associated with disc degeneration but not with pain or disability. In contrast, we hypothesized that hemopexin, a protein previously linked to nerve injury, would be correlated with pain, disability and disc degeneration.

Methods

Overview of Experimental Design

In painful intervertebral DD, changes in CSF levels of proteins may be a reflection of the DD itself or of alterations related to the pathophysiological mechanisms driving pain. In order to distinguish between these two possibilities, a group of subjects with asymptomatic, pain-free DD was recruited to act as an internal control, allowing us to differentiate between biochemical indices of asymptomatic versus symptomatic DD. The inclusion of a pain-free group with DD as an internal control to differentiate between biochemical indices of asymptomatic and symptomatic DD is a key feature of this study.

Healthy subjects without DD, pain-free subjects with moderate to severe DD, and patients with chronic LBP linked to intervertebral DD (selected to undergo spinal fusion surgery to treat LBP) were recruited for the study between March 2006 and August 2008. Lumbar MRIs were obtained and CSF was collected and pooled for exploratory proteomic analysis.

CSF was analyzed by mass spectrometry using two different sample preparation protocols for removal of abundant proteins. Removal of abundant proteins is important because of the large dynamic range present in CSF, which extends at least 10 orders of magnitude.¹⁰³ Currently, isotopic labeling techniques are limited in that they can only determine changes in relative abundance over a dynamic range of 2 to 3 orders of magnitude.⁸⁶ Samples were digested with trypsin and labelled with iTRAQ for analysis by 2D liquid chromatography (LC) tandem mass spectrometry (MS/MS). A flow chart describing the experimental protocol of the exploratory proteomics experiment is shown in Figure 1.

Based on the results of the initial proteomic screen, two proteins (Cystatin C and Hemopexin) were selected for further analysis by ELISA. Finally, the relationship between these two proteins and pain intensity, physical disability and DD severity were examined.

All procedures were approved by the institutional review boards of the University of Minnesota (Protocol #0407M62061), Allina Health Hospitals & Clinics (Protocol #1885) and McGill University (Protocol #A02-M29-07B). Informed consent was obtained from each subject. CSF collection, MRIscoring, and all biochemistry were performed by individuals blind to experimental group.

Study Participants

Three groups of participants were included in this study: i) pain-free subjects without DD, ii) pain-free asymptomatic subjects with DD, and iii) patients with chronic LBP with DD. Questionnaires, physical examinations and CSF collection were performed at the University of Minnesota General Clinical Research Center. Lumbar MRIs were collected at the Fairview University Medical Center or the Twin Cities Spine Center. Descriptive statistics for the three groups are shown in Table 1.

General exclusion criteria for all subjects included complicating medical factors such as previous spine surgery, pregnancy, lactation, meningitis, hepatitis, scoliosis, osteoporosis, neuropathies, and neurological conditions (e.g. psychosis, dementia, Parkinson's, etc.). Subjects were asked to refrain from strenuous exercise 3 days before the pain assessment and CSF collection.

Pain-free healthy male and non-pregnant female volunteers ages 21–65 were recruited by advertisement. Individuals were considered for inclusion if they had no history of chronic pain of any type and no LBP over the last three months. Volunteers were excluded if they were using prescribed steroids or narcotics for chronic medical conditions, if they refused to discontinue anti-inflammatory and analgesic medications for 72 hours prior to physical exam and CSF collection, or if they were using antidepressants and had not been on a steady dose for at least 2 months. Lumbar MRIs were scored by an observer blind to participant status to determine if DD was present. Subjects with lumbar discs scoring 3 on the 5-point Thompson scale¹⁰⁸ were placed in the control (no pain or DD) group. Those with at least one lumbar disc scoring 4 or 5 on the Thompson scale were placed in non-painful DD group. The 5-point (1–5) Thomson scale was developed with cadaveric samples and grades gross morphological changes, taking into account the shape of the NP, AF, endplate and vertebral body. Lumbar discs scoring 4 or 5 have clefts in the nucleus, focal disruptions in the annulus, irregularity and sclerosis of the endplate, and osteophytes at vertebral body margins.

For inclusion in the symptomatic painful DD group, patients aged 21–65 years with chronic LBP associated with diagnosed DD were recruited at the Twin Cities Spine Centre. Only patients with a minimum of 6 months of severe pain, scoring 4 or 5 for at least one disc on the Thompson scale, and selected for disc removal and spinal fusion, were recruited. MRI imaging was used by the clinical staff to determine the subject's suitability for surgery. These MRIs were then re-evaluated by a blind observer together with the MRIs from the pain-free volunteers as described above. Patients were excluded unless they belonged to one of the following three medication regimens: non-steroidal anti-inflammatory drug (NSAID) and opioid, NSAID and steroid, opioid and steroid. Analgesic use was not withheld. The potential contribution of other structures (e.g. facet joints, ligaments, muscles) to chronic LBP in this population was not evaluated.

All subjects were assessed for perceived pain intensity using a **visual analogue scale (VAS)** out of 100. Pain-free subjects were included in the study if they reported scores of 10, and LBP subjects were included if they reported scores 25. Subjects also completed the short form of the **McGill pain questionnaire (MPQ)**.^{73, 74} The **Oswestry low back disability**

index (ODI) version 2.0³⁴ was used to evaluate how chronic LBP affected subjects' perceived ability to perform daily activities.

Lumbar MRI scores were missing from three subjects in the control group and four subjects in the painful DD group. Only the available data was analyzed, and missing values were not replaced with replacement values, uncertain values, or modeled data.

Sample Collection and Storage

CSF was collected with a 25 gauge Whitacre spinal needle under i.v. sedation with midazolam. The needle was introduced to the spinal canal at L3/4 according to standard practice using surface landmarks and CSF was collected by passive drip until either a) the 20 ml cutoff was reached or b) the CSF stopped flowing freely. At the time of collection, the quality of the tap and the visual appearance of the CSF were recorded (clear, cloudy, yellow or bloody). No traumatic taps were recorded and all but 4 samples were clear. One of the 4 was excluded due to high protein content, the remaining 3 became clear after the initial tap and produced protein concentration and expression values in range with their respective experimental groups (2 pain-free with no DD and 1 pain-free with DD). Collected CSF was chilled, centrifuged at 250 g for 10 minutes to remove any cellular or other contamination, the supernatant flash frozen in liquid nitrogen and stored at -80°C.

Safety

A maximum of 20 mL of CSF was collected to reduce variability from rostro-caudal concentration gradients and to minimize the risk of post-dural headache. Following CSF collection, each subject was monitored closely for 1–2 hours for any post-dural puncture complications. During this interval, the patients received i.v. fluids at a rate of 200 ml/hr to help minimize the development of post-dural headache. Once the patient was stable and feeling well, standard post-procedure instructions were given to the subjects, and they were released to a friend or family member who escorted them home and stayed with them overnight. Only one case of a possible post-dural headache was reported. However, this individual did not follow post-procedure instructions and consumed excess amounts of alcohol the evening following CSF collection, and may have been experiencing hangover symptoms. The individual declined follow-up treatment.

Creation of the Exploratory Cohort

For the initial proteomics screen, a subset of samples was selected to create an exploratory cohort balanced for sex and age to maximize the chances that observed differences in protein expression were due to disease state rather than variations within the subject pool. This was necessary because of the age differences between groups. The experimental design, in which pain-free individuals were recruited and then separated into 'with' or 'without DD' groups based on MRI, did not allow for age matching as the grouping was performed post hoc based on DD, which increases with age.

Samples were pooled for proteomic analysis into 1) subjects without DD or LBP, 2) subjects with DD but without LBP, 3) subjects with both DD and LBP. Pooling began with the thawing of equal volumes of CSF from each subject and mixing all the samples from each

group together into a single vessel. The vessel was then vortexed briefly to ensure homogenous mixing. Prior to pooling total protein content of the CSF from each individual was determined using the Bradford protein assay (Pierce Biotechnology).

Gel Electrophoresis

Gel electrophoresis was performed with 8–16% polyacrylamide precast Ready Gels (BioRad). Gels were stained with silver nitrate and developed with sodium carbonate for visualization. Polyacrylamide gels were then digitized with a gel reader.

ProteoPrep Immunodepletion of Abundant Proteins

CSF immunodepletion was performed with a ProteoPrep 20 plasma immunodepletion column (Sigma-Aldrich) in 100 µl increments. Two cycles of immunodepletion were performed on CSF to fully deplete abundant proteins. To prevent order effects, the order of immunodepletion between samples was controlled by latin square randomization. The flow through was concentrated before the second round of immunodepletion. This was accomplished using a centrifugal evaporation unit (Eppendorf vacufuge). Flow through CSF was then immunodepleted again using the ProteoPrep 20 column, again accounting for order effects by latin square randomization. The second flow through was then desalted and concentrated with a Vivaspin 500 5 kD nominal molecular weight cutoff ultrafiltration unit (Sartorius Stedim). 10 µg of depleted CSF protein from each group was transferred to a new microcentrifuge tube for digestion and iTRAQ labeling. The removal of abundant proteins was confirmed by gel electrophoresis (Figure 2A).

Precipitation of Abundant Proteins with Acetonitrile

1.5 ml of pooled CSF from each group was thawed on ice. Each sample was dialyzed for 24 hours against MilliQ H₂O using an Ettan Mini-dialysis unit (GE Healthcare) at 4°C to remove biological salts. Acetonitrile precipitation was performed by adding a 1.5× volume of acetonitrile (Fisher Scientific) to each unit of dialyzed CSF. The mixture was then vortexed for 10 seconds and incubated for 1 hour at room temperature. Samples were then centrifuged at 15,000g for 10 minutes. Supernatant was then completely evaporated in a centrifugal evaporation unit to remove acetonitrile. The lyophilized acetonitrile soluble proteins were reconstituted in MilliQ H₂O. Protein quantification was performed with the Bradford protein determination method (Pierce Biotechnology). 10 µg of depleted CSF protein from each group was transferred to a new microcentrifuge tube for digestion and iTRAQ labeling detailed below. This precipitation procedure was performed twice. In one run, peptides were concentrated with an SCX ziptip (Millepore), and in the other run, peptides were not ziptipped. The results of these two runs were combined in the data analysis phase. The separation of proteins by molecular weight proteins was confirmed by gel electrophoresis (Figure 2B).

Trypsin Digestion and iTRAQ Labeling

iTRAQ reagents are a set of isobaric reagents that allow for the identification and quantitation of up to four different samples simultaneously.⁹⁴ Manufacturers protocols were followed (Promega Corporation).

2D LC-MS/MS

Samples were analyzed at the Genome Quebec Proteomics Platform. The sample was reconstituted in 10% ACN:0.1% TFA. Samples were diluted with 30 μ l of 3% ACN:0.1% FA. A fraction of the sample was injected onto a Zorbax Bio-SCXII 50 $\times$ 0.8mm strong cation exchange column (Agilent). Elution from the SCX column was done by stepwise 40 μ l injections of 10, 20, 30, 40, 50, 65, 80, 100, 125, 150, 180, 250 and 500 mM NaCl:0.1% FA: 3% ACN. Eluted peptides were trapped and desalted with a Zorbax 300SB-C18 (5 $\times$ 0.3 mm) at 15 μ l/min of 3% ACN:0.1% FA for 20 min. Nanoflow chromatography separation of peptides was performed with an 1100 series nanoHPLC system using a Biobasic C18 (10 $\times$ 0.075 mm) integras frit column (New Objective). Peptides were eluted using a gradient of solvent A (0.1% FA) and solvent B (95% ACN:0.1% FA) starting at 5% B, reaching 20% B after 29 min, 40% B after 84 min and finally 90% B after 90 min at a flow rate of 200nl/min.

Eluted peptides were analyzed by tandem MS with a QTRAP 4000 (Sciex-Applied Biosystems). Enhanced MS scans in the 375–1500 m/z range were acquired at a 4000 amu/sec scan speed using an active Dynamic Fill time. Information-dependent MS/MS analysis was performed on the 3 most intense 2+, 3+ or 4+ charged ions. Charge determination was done by additional Enhanced Resolution scans of each candidate precursor ion at a speed of 250 amu/sec. A dynamic exclusion was used to limit resampling of previously selected ions to a maximum two events within 180 sec. Three scans were summed, MS/MS scans for each precursor were acquired between 100–1600 m/z at a scan speed of 4000 amu/sec. Fixed fill time was set at 20 ms with Q0 trapping and rolling collision energy of +5 eV.

Database Search

Immunodepleted CSF and acetonitrile soluble CSF was searched using a UniProt human protein database obtained on March 2008 containing 71371 sequences.⁸⁹ Spectral processing included peak smoothing and centroiding without de-isotoping and peak picking for peaklist generation was done with the Mascot distiller ver.2.1 (Matrixscience) algorithm using tryptic peptides with up top 1 miscleavage, methylthiocysteine as fixed modification, methionine oxidation, iTRAQ modified N-terminus, lysine and tyrosines as the variable modification with a 1.5 Da precursor and 0.8 MS/MS fragment tolerances was used to search the databases.

Protein quantitation was done using the Interrogator algorithm of ProQuant ver1.4 (Applied Biosystems) using the same database used for the Mascot searches. N-term iTRAQ and methylthiocysteines were used as fixed modifications. Methionine oxidation and iTRAQ modified lysine and tyrosine were used as variable modifications. Search tolerances were identical to those used for Mascot. Protein grouping was performed with the Progroup software (Applied Biosystems).

To minimize false positive protein identifications, a protein was only considered if a minimum of two peptides belonging to the protein were identified (ProtSc 2).

Expanded Cohort for ELISA

Two proteins from the initial proteomic screen were selected for follow up for validation by ELISA and analyzed individually. Cystatin C was chosen because the initial proteomics screen determined that it was increased in both symptomatic and asymptomatic DD and because of its association with inflammation.⁸³ In addition, cystatin C was previously proposed as a putative CSF biomarker for pain,^{44, 67} although this claim is controversial.³¹ Hemopexin was selected because the initial proteomic screen found that it was significantly increased in painful DD, but not pain-free DD. Furthermore, hemopexin is of interest because of its association with nerve injury.⁵²

Due to the constraints of our study design, ie. a proteomic screen followed by validation studies with ELISA, it was not possible to estimate sample sizes *a priori*. The “1-Way ANOVA Pairwise, 2-Sided Equality” calculator on powerandsamplesize.com was used to perform a post-hoc power analysis (power, $1 - \beta = 0.7$, type 1 error rate, $\alpha = 10\%$), using values based on the results of the magnitude in change of protein levels, coupled with literature values of means and variance for cystatin C⁹⁰ and hemopexin⁹². Calculated sample sizes per pairwise comparison was 31 for cystatin C and 39 for hemopexin.

ELISA Assays

Commercial ELISA kits for cystatin C and hemopexin were used. Cystatin C human ELISA was purchased from Biovendor R&D (Asheville, NC; Cat# RD191009100, Lot E13-093). Human hemopexin ELISA was purchased from Immunology Consultants Laboratory, Inc (Portland, OR; Cat# E-80HX, Lot 12). Manufacturers’ protocols were followed without modification. Placement of samples on the ELISA microplates was randomized to minimize any location and order biases. All samples were processed in duplicate using a microplate reader (Spectramax M2E, Molecular Devices, Sunnyvale, CA).

Statistical Analysis

In the exploratory proteomic analysis of CSF, peptides were identified by mass spectrometry, and the ratio of iTRAQ isobaric reporter tags between groups was measured. Multiple peptides from the same parent protein may be observed. The p-value denoted in Tables 2 and 3 reflects the consistency of the ratio of iTRAQ tags on the peptides belonging to the same protein.

The unused ProteinScore (ProtSc) is a statistical calculation of protein confidence. It denotes the number of peptides observed that are unique to a particular parent protein. A completely unique peptide sequence increases ProtSc by 1, and shared sequences increase ProtSc by fractional amounts. In this study, a minimum ProtSc cutoff of 2 was selected, corresponding to 99% confidence of protein identification.

To control for multiple comparisons in the proteomic analysis, the Benjamini-Hochberg procedure was utilized.⁵³ The Benjamini-Hochberg critical value for a false discovery rate (FDR) was set to 0.02.

The ELISA data was analyzed and graphed using Prism (Graphpad). Comparisons between groups were made using one-way ANOVA followed by Bonferroni post-hoc tests comparing

all groups to each another. Correlation analysis was performed using the non-parametric Spearman's rank correlation coefficient on data from all groups (healthy controls, pain-free DD, painful DD).

Results

Study Participants

A total of 54 participants were enrolled in the study following recruitment at the TC Spine Clinic or by telephone screening, consisting of 39 pain-free subjects and 15 LBP patients with intervertebral DD. Of the 39 pain-free subjects which were enrolled, only 38 subjects who completed the study met the inclusion criteria after examination (one subject had a ventriculoperitoneal shunt and was excluded). Following MRI grading, 25 pain-free subjects were determined to not have intervertebral DD, while the other 13 were found to have pain-free DD. In the LBP patient group, only 13 of the 15 patients met inclusion criteria (one patient was excluded because of an intervertebral disc herniation, and another was excluded due to hepatitis C infection). Table 1A shows the characteristics of the subject and patient populations in the study. Because pain-free individuals were recruited and then separated into 'with DD' and 'without DD' groups based on post hoc MRI results, age differences were expected and were observed between groups. The characteristics of the exploratory cohort created for the initial proteomic screen (formed to reduce expected age and sex differences between painful vs nonpainful DD groups) are provided in Table 1B.

CSF Sample Preparation: Immunodepletion (Exploratory Cohort)

The ProteoPrep 20 immunodepletion column is designed to remove the top 20 most abundant proteins in plasma. As there was no depletion column designed specifically for CSF, the ProteoPrep 20 immunodepletion spin column was adapted for use with CSF. The ProteoPrep 20 immunodepletion column removes: albumin, IgG, transferrin, fibrinogen, IgA, alpha-2-macroglobulin, IgM, alpha-1-antitrypsin, complement C3, haptoglobin, apolipoprotein A1, apolipoprotein A2, apolipoprotein B, acid-1-glycoprotein, ceruloplasmin, complement C4, complement C1q, IgD, prealbumin, and plasminogen. All of these proteins are found in CSF.

The ProteoPrep 20 immunodepletion successfully removed abundant proteins as shown by gel electrophoresis in Figure 2A. After immunodepletion, a number of protein bands were no longer visible while other bands increased in intensity (arrows), demonstrating that the removal of highly abundant proteins resulted in an increase in the relative abundance of the low abundance proteins. The majority of albumin was removed in the first depletion step as shown by the removal of the large band at 66 kDa. The second depletion removed additional albumin among other proteins.

A total of 56 proteins were detected by mass spectrometry after immunodepletion (Figure 3A, Supplemental Table 1. 18 of these proteins were differentially expressed compared to pain-free controls without DD (Figure 3B, Table 2).

CSF Sample Preparation – Acetonitrile Precipitation (Exploratory Cohort)

Acetonitrile precipitation was also used to remove abundant proteins from CSF. Acetonitrile precipitates larger proteins before smaller proteins, and thus, highly abundant large proteins can be separated from smaller proteins by this method.¹⁶ An additional benefit to this method over immunodepletion is that it causes smaller peptides to be released from larger carrier proteins.⁷⁵ In contrast, immunodepletion can result in the loss of small peptides if they are associated with immunodepleted carrier proteins such as albumin.

The silver stained gel electrophoresis of acetonitrile precipitated CSF demonstrates the separation of high molecular weight proteins from low molecular weight proteins (Figure 2B). Notably, the precipitation removed the majority of albumin (MW = 66.5 kDa), the most abundant CSF protein, from the sample. This resulted in an increase in the relative abundance of smaller (<35 kDa), low abundance proteins (arrows).

The number of proteins identified by mass spectrometry in the acetonitrile precipitated samples was 32 (Figure 3A, Supplemental Table 2). 9 of these proteins were differentially expressed compared to pain-free controls without DD (Figure 3B, Table 3).

Identification of Differentially Expressed Proteins in CSF from Individuals with Painful and Pain-Free Intervertebral Disc Degeneration compared to Controls without Disc Degeneration (Exploratory Cohort)

The majority of proteins detected in this study were not significantly different between individuals with either painful or pain-free DD compared to controls without pain or DD. The proportion of proteins that showed differential changes in expression are depicted by volcano plot (Figure 3C).

A total of 15 proteins were identified that were differentially expressed between pain-free individuals with or without DD. These proteins are prostaglandin-H2-D-isomerase, cystatin C, α -1-antichymotrypsin, serine/cysteine proteinase inhibitor clade G, gelsolin, chromogranin A, superoxide dismutase, extracellular superoxide dismutase, neural cell adhesion molecule L1-like protein, amyloid-like protein 1, albumin, calsynthenin-1, orosomucoid-1, apolipoprotein A1, and α -2-HS glycoprotein (Tables 2&3). Of these 15 proteins, 6 were similarly dysregulated in patients with painful DD.

A total of 8 proteins were identified that were differentially expressed in LBP patients but not in pain-free individuals with DD compared to controls. These proteins are hemopexin, apolipoproteins A-IV, D, and E, insulin-like growth factor II, prosaposin, proSAAS, and β -2-microglobulin (Tables 2&3).

CSF Levels of Cystatin C Correlate to Intervertebral Disc Degeneration Severity but not to Pain Intensity or Physical Disability (Full Cohort)

The iTRAQ exploratory experiments with both ProteoPrep 20 immunodepleted and acetonitrile precipitated CSF identified cystatin C as increased in pain-free and in painful DD compared to controls without DD. Cystatin C is a protein associated with inflammation.⁸³ Additionally, CSF cystatin C levels have previously been shown to correlate with pain status,^{44, 67} although this idea has been at the center of some controversy.³¹

To further explore and validate the association between cystatin C levels and non-painful DD, levels of cystatin C were measured by ELISA in the full cohort. The ELISA results confirmed that levels of cystatin C were significantly greater in the pain-free DD group when compared to healthy individuals ($p < 0.05$; Figure 4A). A Spearman nonparametric correlation found no association with cystatin C and pain intensity as determined by the visual analogue scale (VAS) ($r = 0.007$; $p = 0.96$; Figure 4B). CSF levels of cystatin C were also examined for correlation with the scores on the McGill Pain Questionnaire (MPQ) and no significant correlation was found ($r = 0.05$; $p = 0.71$; Figure 4C). Similar analysis between cystatin C levels and disability, as determined by the Oswestry Disability Index (ODI), also demonstrated no correlation ($r = 0.05$; $p = 0.72$; Figure 4D). However, cystatin C levels significantly correlated with the severity of DD as determined by the maximum score of one or more lumbar discs according to the Thompson scale ($r = 0.31$; $p = 0.04$; Figure 4E). The correlation between cystatin C levels and DD severity is consistent with the results of the initial proteomics screen, and suggest that cystatin C levels are associated with DD but not pain.

CSF Levels of Hemopexin Correlate with Pain Intensity, Physical Disability and Intervertebral Disc Degeneration Severity (Full Cohort)

The iTRAQ exploratory experiments with ProteoPrep 20 immunodepleted CSF identified hemopexin as differentially expressed in painful but not pain-free DD. Hemopexin is a protein that has been found to be increased after nerve injury.⁵²

To further explore the association between hemopexin and painful DD, hemopexin levels in CSF were quantified by ELISA in the full cohort. Levels of hemopexin were significantly greater in the painful DD group when compared to controls without DD ($p < 0.01$; Figure 5A). Conversely, hemopexin levels in the pain-free DD group were not significantly increased when compared to healthy controls. CSF hemopexin levels and pain intensity (as measured by the VAS) were significantly correlated ($r = 0.34$; $p = 0.01$; Figure 5B). Likewise, CSF levels of hemopexin correlated with total MPQ ($r = 0.31$; $p = 0.03$; Figure 5C), as well as the sensory ($r = 0.30$; $p = 0.03$) and affective subscales ($r = 0.34$; $p = 0.02$) of the MPQ (data not shown). In addition, a significant correlation was observed between hemopexin levels and physical disability as measured by ODI ($r = 0.36$; $p = 0.01$; Figure 5D). Finally, hemopexin levels correlated with maximum lumbar intervertebral DD severity, as measured by the Thompson scale ($r = 0.39$; $p = 0.008$; Figure 5E). Overall, CSF hemopexin levels were associated with painful DD, consistent with the results from the exploratory proteomic screen.

Discussion

Differential shotgun proteomics was performed on CSF from control subjects, subjects with non-painful DD, and LBP patients with painful DD. Pain-free individuals with DD or herniation have not been included in previous human pain studies examining CSF for pathophysiological markers. Indeed, this is the first study to include such a group, thus allowing for identification of pain-specific biochemical markers through specific comparison of pain-related and non-pain-related components of DD.^{4, 11, 59, 60, 62, 117}

Differential Protein Expression in CSF suggests that Inflammation is Associated with Intervertebral Disc Degeneration but not with Chronic Low Back Pain

There were 15 proteins identified that were differentially expressed in the presence of DD regardless of pain state, many of which are associated with inflammation. Proteins that were increased in DD included: cystatin C, alpha-1-antichymotrypsin, gelsolin, chromogranin-A, neural cell adhesion molecule L1-like protein, and amyloid-like protein 1. Proteins that were decreased in DD included prostaglandin-H2 D-isomerase, serine/cysteine proteinase inhibitor clade G, superoxide dismutase, extracellular superoxide dismutase, calyculin-1, serum albumin, orosomucoid 1, and alpha-2-HS-glycoprotein.

Cystatin C—Cystatin C is a small secreted cysteine protease inhibitor.¹ CSF concentrations of cystatin C have been proposed to be indicative of pain,^{19, 67} although this has been disputed.³¹ In this study, cystatin C levels were higher in both DD groups compared to the group without DD. These results agree with the body of work suggesting that cystatin C is a marker for inflammation but is not specific to pain.³³ Because cystatin C was observed using both separation methods and was previously proposed as a marker for inflammation, it was selected for additional analysis by ELISA. In agreement with the proteomics screen, cystatin C correlated with the severity of DD but not with pain or disability (Figure 4).

Alpha-1 antichymotrypsin—Alpha-1 antichymotrypsin is a serine protease inhibitor which is a type of protein that is increased in response to inflammation¹³. It was increased by DD and is also increased in inflammatory conditions such as rheumatoid arthritis.¹⁵ It exhibits anti-inflammatory activities, cleaving proteases such as cathepsin G²⁹ and chymases³⁸ found in immune cells.

Gelsolin—Gelsolin is important for inflammatory cell migration.¹¹⁵ Gelsolin knockout mice exhibit blunted responses to inflammation due to attenuated infiltration, chemotaxis and apoptosis.⁸⁵ Increased gelsolin expression in patients with DD suggests ongoing inflammation and immune cell migration.

Chromogranin-A—Chromogranins are expressed ubiquitously in secretory cells of the immune, nervous, and endocrine systems.⁴⁵ Chromogranin A is increased in other conditions involving inflammation, such as rheumatoid arthritis.²⁷ Chromogranin A levels correlate with soluble tumor necrosis factor receptors, which are markers of systemic inflammation.²⁰

Prostaglandin-H2 D-isomerase—Prostaglandin-H2 D-isomerase, also known as prostaglandin D-2 (PGD2) synthase, was reduced in both painful and non-painful DD. PGD2 suppresses inflammation via inhibition and blockade of nuclear factor- κ B kinase,^{95, 9899} resulting in reduced expression of proinflammatory cytokines interleukin-1 β , interleukin-6, tumor necrosis factor- α secretion and iNOS from macrophages.^{3, 91, 113} In PGD2 synthase knockout animals, inflammation is more severe and resolution of inflammation is impaired.⁸⁸ Resolution of inflammation may be similarly impaired in individuals with DD.

Serum albumin—Serum albumin CSF levels were reduced in DD. Hypoalbuminemia is commonly observed in patients with inflammatory diseases such as pneumonia, rheumatoid arthritis, bacterial infection⁸¹ and in hemodialysis patients.⁵⁴ Reduced serum albumin levels have also been observed in patients following surgery.^{97, 109} Reduced albumin levels in DD are therefore consistent with inflammation.

Superoxide dismutase and extracellular superoxide dismutase—Superoxide dismutase catalyses the conversion of the superoxide anion to hydrogen peroxide, and plays an essential role in antioxidant defense systems. Increased superoxide dismutase expression helps resolve inflammation in colitis,¹⁰¹ inflammatory arthritis,¹¹⁶ pulmonary fibrosis,³⁹ emphysema,³⁵ and hypoxia induced brain injury.¹¹⁸ Superoxide dismutase levels in DD were decreased compared to healthy controls, which may reflect ongoing oxidative stress and chronic inflammation in intervertebral DD. In agreement, animal models have demonstrated that superoxide dismutase levels decrease in age-related intervertebral DD.⁴⁹

Serine/cysteine proteinase inhibitor clade G—Serine/cysteine proteinase inhibitor clade G, also known as C1 inhibitor, is a complement inhibitory protein that irreversibly binds to and inactivates components of the C1 complex of the complement pathway.¹¹⁹ C1 inhibitor levels were reduced in DD when compared to healthy controls; this reduction could enhance complement activity and contribute to disc inflammation. Indeed, study of human disc tissues have demonstrated enhanced complement activation through the classical pathway in degenerated discs.⁴²

Orosomucoid 1—Orosomucoid 1 has immunosuppressing effects, including inhibition of leukocyte rolling and adhesion, migration, chemotaxis,⁷⁶ and proliferation.³² CSF levels of orosomucoid 1 were reduced in DD, suggesting that reduced basal immunosuppression by orosomucoid may be contributing to ongoing intervertebral DD.

Alpha-HS-2-glycoprotein: Alpha-HS-2-glycoprotein, also known as fetuin-A, has anti-inflammatory activities.¹¹⁴ Additionally, expression of Alpha-HS-2-glycoprotein is known to be negatively regulated by proinflammatory cytokines such as interferon- γ ,⁵⁷ interleukin-1 β and interleukin-6.²⁴ The reduced levels of alpha-HS-2-glycoprotein observed in CSF of subjects with intervertebral DD is consistent with the suggestion that ongoing inflammation is occurring during intervertebral DD.

Other proteins—Levels of neural cell adhesion molecule L1-like protein, amyloid like protein-1, and calstn-1 were differentially regulated in CSF by intervertebral DD. Neural cell adhesion molecule L1-like protein is involved in neuron-neuron adhesion and neurite outgrowth.⁴⁶ Amyloid like protein-1 is postulated to play a role in NMDA receptor homeostasis.²¹ Calstn-1 is a transmembrane protein which plays a role in anterograde axonal transport.¹¹² Alpha-2-HS-glycoprotein is a protein whose function is poorly understood. It is thought to act as a functional antagonist of transforming growth receptor-beta, bone morphogenic proteins,²⁶ and insulin receptor tyrosine kinase.⁷⁰

Differential Protein Expression in CSF suggests that Chronic Low Back Pain Associated with Intervertebral Disc Degeneration has a Neuropathic Component

There were 8 proteins identified that were increased in painful DD compared to pain-free controls without DD. They included apolipoprotein E, D, and A-IV, hemopexin, ProSAAS, β -2-microglobulin, prosaposin, and insulin-like growth factor II. Many of these proteins have been previously linked to nerve injury.

Hemopexin—After peripheral nerve injury, hemopexin is expressed by Schwann cells, endoneurial fibroblasts, and invading macrophages.¹⁴ Hemopexin levels are elevated in injured nerves,⁶⁵ remain elevated in states of ongoing Wallerian degeneration,^{66, 107} and have been shown to negatively regulate inflammatory Th17 T cell responses.⁹³ Likewise, nerve injury also alters T cell responses.^{69, 79} Consistent with our observation of increased hemopexin in CSF of LBP patients, others have observed a reduction in Th17 T cells in LBP patients and in chronic neuropathic pain patients.^{63, 64} The observation that the increase in hemopexin levels in the CSF from patients with painful DD but not in pain-free individuals with DD suggests that nerve injury may play a role in the pathophysiology of painful DD. Due to the association between nerve injury and hemopexin levels, hemopexin was selected for further analysis by ELISA. Consistent with the exploratory proteomics screen, hemopexin levels were found to correlate with pain intensity, physical disability and DD severity (Figure 5).

Apolipoproteins—Compared to pain-free controls without DD, apolipoproteins A-IV, D, and E were all significantly increased in the painful DD group but not in the pain-free DD group. Apolipoproteins are important in remyelination, which occurs after axonal injury. Apolipoproteins A-IV,⁹ D,⁸ and E,⁹⁶ accumulate in nerves after peripheral nerve injury. As Schwann cells attempt to remyelinate, apolipoprotein-D and -E expression are induced.⁴⁰ The finding that apolipoproteins are increased in the CSF of patients with painful DD is further evidence supporting the hypothesis for a neuropathic component to the pathophysiology of painful DD.

Insulin-like growth factor II—Insulin-like growth factor II was increased in the CSF in patients with painful but not pain-free DD. Insulin-like growth factor II is increased in nerves after nerve injury.⁴¹ Thus, its upregulation in the CSF of patients with painful DD is consistent with the hypothesis that nerve injury is playing a role in the generation of discogenic pain.

Prosaposin—Prosaposin was upregulated in the CSF of the painful DD group. Prosaposin is a myelinotrophic protein that is secreted by neurons after nerve injury.¹¹¹ It acts within nerves to provide trophic support to neurons and promote regeneration.⁴⁷ Elevated CSF levels of prosaposin further suggests that nerve injury contributes to painful DD. Importantly, there was no increase in prosaposin levels in the CSF of the pain-free DD group.

Beta-2-microglobulin—Beta-2-microglobulin is a component of the class I major histocompatibility antigens, and is normally expressed on immune cells,²² including

microglia.⁴³ It was increased in the CSF of patients with painful DD. CSF beta-2 microglobulin levels are increased in inflammatory conditions within the CNS, such as multiple sclerosis,⁵ Alzheimer's disease,²⁵ HIV dementia,⁷² bacterial and viral meningitis,¹⁰⁶ and spinal cord injury.¹⁰² Nerve injury induces central sensitization and central inflammation,⁵⁶ which is consistent with the observation of increased beta-2-microglobulin levels in painful DD.

ProSAAS—ProSAAS was found to be increased in the group with painful DD using both the ProteoPrep 20 and the acetonitrile precipitation methods. ProSAAS is a neuroendocrine peptide that inhibits pro-hormone convertase 1/3 at nanomolar concentrations.³⁷ Pro-hormone convertases are involved with the proteolytic cleavage of proteins to mature peptides, including the opioid peptides β -endorphin and dynorphin.^{28, 84, 120} Neuropeptide processing enzymes are also differentially regulated in CSF from patients with painful lumbar herniated discs⁶¹ and in disc injury animal models,⁷⁸ suggesting a role for altered neuropeptide processing in the mechanisms underlying painful DD.

Implications

Intervertebral DD includes a significant inflammatory component.¹¹⁰ We observed that a number of inflammatory markers were increased in the CSF of patients with DD, regardless of pain status, suggesting that inflammation alone is not sufficient to produce chronic LBP. While animal models have demonstrated increases in inflammatory mediators following disc injury or compression,⁷⁷ these increases are transient,⁷⁸ and therefore may not be crucial for the development of pain. Studies in human discs found no correlation between pro-inflammatory cytokines IL-1 β and IL-6 in lumbar herniated discs and sciatic pain.² Our work supports the idea that disc inflammation alone is insufficient for the development of LBP.

Our analysis yielded a number of differentially expressed proteins in individuals with painful DD. A number of these – hemopexin, various apolipoproteins, insulin-like growth factor II, prosaposin, and fibronectin – are increased after nerve injury. These findings are consistent with the hypothesis that nerve injury plays an important role in chronic LBP associated with DD.^{50, 77}

While differential protein expression in the CSF was observed in this study, the source(s) are unknown. One possibility is that mediators move from the disc or surrounding vertebral bones to the CSF. After nerve injury, both the blood-spinal barrier³⁰ and the blood-nerve barrier⁵⁸ become disrupted, which may allow movement of peripheral molecules and leukocytes into the CSF. Other explanations include the possibility that nerve injury creates an edematous state resulting in a flow of molecules from injured nerves to CSF,⁸⁷ or that CSF alterations reflect peripherally-driven changes in the central nervous system such as central sensitization and neuroinflammation.¹⁰⁰

A limitation of CSF proteomics is the challenge of detecting low abundant proteins. In this study, two sample preparation techniques, immunodepletion and protein precipitation were used to remove abundant proteins. Immunodepletion was carried out with a column designed to remove abundant proteins from serum. The development of CSF specific

immunodepletion tools will likely improve the ability to examine low abundance proteins in CSF. Likewise, the acetonitrile precipitation procedure was adapted from serum proteomics experiments. It is possible that the use of other organic solvents, or optimization of the precipitation procedure to CSF proteins, would yield different results. Indeed, while proteomic studies allow for an unbiased account of disease-related changes in protein levels, the information gained from these studies must be validated using additional approaches and models.

An additional limitation of this study is that it was not sufficiently powered to examine correlations of either cystatin C and hemopexin specifically within the painful DD group. Adequately powered studies should evaluate this in the future.

Conclusions

The presence of a neuropathic component contributing to discogenic LBP suggests that LBP may respond to treatments targeting neuropathic mechanisms, even in the absence of sciatica. This is consistent with evidence that medications typically used for neuropathic pain, such as tricyclic antidepressants and antiepileptic medications, have efficacy for LBP.¹⁷ In addition, this study builds on the data from animal models demonstrating that pain associated with intervertebral DD has neuropathic components.⁷⁷ Our results also add to the growing body of evidence linking inflammation to intervertebral DD.⁸⁰ However, our study suggests that inflammatory mechanisms are not central to pain genesis, providing a possible explanation for the lack of consistent benefits from treatments targeting disc inflammation, such as intradiscal steroid injection.¹⁰⁴

Finally, these results provide evidence that CSF can be used to study mechanisms underlying painful DD in humans. Future studies may uncover additional clinically-relevant mechanisms underlying LBP that could be targeted for treatment and may provide clues into why some individuals have pain and others do not.

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Perspective

Cerebrospinal fluid was examined for differential protein expression in healthy controls, pain-free adults with asymptomatic intervertebral disc degeneration, and low back pain patients with painful intervertebral disc degeneration. While disc degeneration was related to inflammation regardless of pain status, painful degeneration was associated with markers linked to nerve injury.

- Intervertebral disc degeneration induces differential protein expression in CSF
- Pain-free disc degeneration is associated with biochemical markers of inflammation
- Painful disc degeneration is associated with biochemical markers of nerve injury
- Mechanistic insights can be gained by probing for biochemical alterations in CSF

Figure 1.

Overview of experimental design. Healthy subjects and LBP patients were recruited. Lumbar MRIs were taken, pain and disability assessments were recorded, and CSF was collected. Healthy subjects were segregated into a control group without DD, or a group with pain-free DD. For the initial proteomic study, an exploratory cohort was created to balance age and sex differences. This was performed in order to reduce variation and to maximize the likelihood that changes in protein levels were due to the presence of DD and/or pain. CSF was pooled and subsequently fractionated by two different sample

preparation techniques. In one analysis pathway, CSF was immunodepleted using a Proteoprep 20 spin column. In the other analysis pathway, acetonitrile was used to precipitate and remove large molecular weight proteins. Samples were then subjected to iTRAQ labelling for quantitative analysis. Two differentially expressed proteins were then selected for further analysis by ELISA in the full cohort.

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Figure 2.

(A) CSF immunodepletion by the Proteoprep 20 spin column. Lane 2 shows CSF before depletion with the Proteoprep 20 immunodepletion column. Lanes 3 and 4 show CSF proteins that were removed by serial passes through the immunodepletion column. The 5th lane shows the immunodepleted CSF. Arrows denote new protein bands that are now visible after immunodepletion. (B) CSF fractionation by acetonitrile precipitation. Lane 2 shows CSF before acetonitrile precipitation. Lane 3 shows CSF proteins that were precipitated by acetonitrile. The 4th lane shows the CSF proteins that were soluble in acetonitrile and were used for analysis in this study. Arrows denote new protein bands that are now apparent in CSF after abundant protein depletion by acetonitrile precipitation.

Figure 3.

Number of proteins detected and differentially expressed in CSF. (A) The total number of proteins identified by the two different sample preparation methods is shown. (B) The number of differentially expressed proteins is shown. Using different sample preparation techniques, distinct differentially expressed proteins were identified from CSF. (C) A volcano plot of iTRAQ quantification ratio in pain-free and painful intervertebral disc degeneration is shown. Points above the horizontal line represent proteins with significant ($p < 0.05$) differences when compared to healthy controls, with points with $FDR < 0.02$ marked as “x”.

Figure 4.

Cystatin C levels in CSF are increased in subjects with degenerative disc disease. (A) CSF Cystatin C levels were compared between healthy, asymptomatic DD, and painful DD subjects. A statistical difference was observed between groups by ANOVA ($p = 0.04$). Bonferroni multiple comparison tests determined that the healthy group was statistically different from the asymptomatic DD group ($p < 0.05$). (B) Spearman nonparametric correlation analysis was used to test for a correlation between CSF cystatin C levels and visual analogue scale (VAS) values; no correlation was observed. (C) Similarly, no significant correlation was observed between CSF cystatin C levels and total McGill Pain Questionnaire (MPQ) score. (D) No significant correlation was observed between CSF cystatin C levels and the Oswestry low back disability index (version 2.0). (E) In contrast, CSF cystatin C levels and maximum lumbar intervertebral disc degeneration were significantly correlated ($r=0.31$; $p=0.04$) when tested with Spearman's rank correlation coefficient.

Figure 5.

Hemopexin levels in CSF are increased in subjects with LBP. (A) CSF Hemopexin levels were compared between healthy, asymptomatic DD, and painful DD subjects. A statistical difference was observed between groups by ANOVA ($p = 0.004$). Bonferroni multiple comparison tests determined that the healthy group was statistically different from the painful DD group ($p < 0.01$). (B) Spearman nonparametric correlation was used to test for a correlation between CSF hemopexin levels and visual analogue scale (VAS) values. A significant correlation was observed ($r=0.34$; $p=0.014$). (C) Similarly, a significant correlation was also observed between CSF hemopexin levels and the total McGill Pain Questionnaire score ($r=0.31$; $p=0.03$). (D) CSF hemopexin levels also significantly correlated with the Oswestry low back disability index ($r=0.36$; $p=0.01$). (E) Spearman's nonparametric correlation was also used to test for a correlation between hemopexin levels and maximum lumbar intervertebral disc degeneration according to the Thompson scale. A significant correlation was observed ($r=0.39$, $p=0.008$).

Table 1

A. Characteristics of the full subject and patient pool (used in the ELISA analysis)

	N	M:F	Age	Maximum disc degeneration score (/5)	Total disc degeneration score (/25)	ODI % disability	VAS	Total MPQ score	Total protein concentration (µg/ml)
Control (no pain or disc degeneration)	25	13:12	32 ± 2	2.7 ± 0.1	12.0 ± 0.6	0 ± 0	0.8 ± 0.6	0 ± 0	238 ± 14
Non-painful disc degeneration	13	10:3	48 ± 3 ***	4.2 ± 0.1 ***	17.8 ± 0.8 ***	1 ± 0.5 ***	3 ± 2	0.5 ± 0.3	311 ± 24 ***
Painful disc degeneration	13	6:7	42 ± 3 *	4.0 ± 0.3 ***	16.4 ± 0.8 **	45 ± 2 *** ###	54 ± 6 *** ###	17 ± 2 *** ###	301 ± 30

B. Characteristics of the exploratory cohort (used in the proteomic analysis)

	N	M:F	Age	Maximum disc degeneration score (/5)	Total disc degeneration score (/25)	ODI % disability	VAS	Total MPQ score	Total protein concentration (µg/ml)
Control (no pain or disc degeneration)	6	3:3	41 ± 2	2.8 ± 0.2	13.3 ± 1.1	0.3 ± 0.3	1.8 ± 1.5	0 ± 0	283 ± 27
Non-painful disc degeneration	8	6:2	46 ± 4	4.1 ± 0.2 ***	17.3 ± 1.0 *	0.5 ± 0.5	0.4 ± 0.4	0.1 ± 0.1	320 ± 30
Painful disc degeneration	8	5:3	41 ± 3	4.4 ± 0.3 ***	17.4 ± 0.6 *	47 ± 3 *** ###	66 ± 7 *** ###	18 ± 3 *** ###	290 ± 29

All values listed as mean ± SEM.

ODI: Oswestry low back disability index version 2.0

VAS: Visual analogue scale

MPQ: McGill Pain Questionnaire

Compared to control

* = p < 0.05,

** = p < 0.01,

*** = p < 0.001

Compared to non-painful disc degeneration

= p < 0.001

Table 2

Differentially expressed proteins from proteoprep 20 immunodepleted CSF

Protein name	Accession number	ProtSc	Ratio Pain-free DDD vs Control	Ratio Painful DDD vs control	Postulated Mechanistic Association	References
Apolipoprotein E precursor	P02649	24.38	1 P=1 [#]	1.07 P<0.001 [*]	Peripheral nerve injury	8, 40
Hemopexin precursor	P02790	12.36	1.08 P=0.18 [#]	1.12 P<0.05 [*]	Peripheral nerve injury	14, 65, 66
Apolipoprotein D precursor	P05090	7.45	1.08 P=0.32 [#]	1.14 P=0.032 [*]	Peripheral nerve injury	8, 40
Apolipoprotein A-IV precursor	P06727	5.49	1.04 P=0.54 [#]	1.16 P=0.023 [*]	Peripheral nerve injury	9
ProSAAS precursor	Q9UHG2	5.54	1.08 P=0.062 [#]	1.17 P<0.0001 [*]	Neuropeptide processing	28, 37, 84, 120
Beta-2-microglobulin precursor	P61769	2.04	1.18 P=0.091 [#]	1.24 P=0.05 [#]	Microglial activation	6, 22, 23, 43
Prostaglandin-H2 D-isomerase precursor	P41222	10.12	0.86 P<0.0001 [*]	0.87 P<0.0001 [*]	Inflammation	88, 95, 98, 99
Cystatin-C precursor	P01034	8.21	1.08 P<0.001 [*]	1.13 P<0.0001 [*]	Inflammation	1, 19, 31, 33, 67
Alpha-1 -antichymotrypsin precursor	P01011	7.92	1.18 P<0.001 [*]	1.27 P<0.0001 [*]	Inflammation	13, 15, 29, 38
Serum albumin precursor	P02768	5.09	0.82 P=0.036 [*]	0.96 P=0.681 [#]	Inflammation	54, 81, 97, 109
Serine/cysteine proteinase inhibitor clade G member 1 splice variant 2	Q5UGI6	4.26	0.90 P<0.05 [*]	0.96 P=0.44 [#]	Inflammation	18
Gelsolin	Q5T0I2	3.99	1.16 P<0.05 [*]	1.17 P<0.01 [*]	Inflammation	85, 115
Chromogranin-A precursor	P10645	3.4	1.44 P=0.001 [*]	1.51 P=0.001 [*]	Inflammation	20, 27, 45
Superoxide dismutase	P00441	4.57	0.78 P<0.001 [*]	0.78 P=0.069 [#]	Free radical scavenger	49, 68
Extracellular superoxide dismutase [Cu-Zn] precursor	P08294	3.05	0.89 P<0.01 [*]	0.93 P=0.11 [#]	Free radical scavenger	49, 68
Neural cell adhesion molecule L1-like protein precursor	O00533	7.02	1.13 P<0.05 [*]	1.14 P=0.06 [#]	Cell adhesion	46

Protein name	Accession number	ProtSc	Ratio Pain-free DDD vs Control	Ratio Painful DDD vs control	Postulated Mechanistic Association	References
Amyloid-like protein 1 precursor	P51693	5.78	1.14 P<0.05*	1.17 P<0.05*	NMDA receptor homeostasis	21
Calsyntenin-1 precursor	O94985	2.75	0.96 P<0.05*	1.01 P=0.69#	Axonal transport	112

ProtSc (Protein Score) is a statistical calculation of protein confidence reflecting the number and uniqueness of the observed peptides that are attributed to a particular parent protein.

The p-values reflect the consistency of the observed ratio of iTRAQ tags on peptides belonging to the same parent protein.

* denotes P-values which are Benjamini-Hochberg significant at false discovery rate < 0.2.

denotes P-values which are not Benjamini-Hochberg significant at false discovery rate < 0.2.

Table 3

Differentially expressed proteins from acetonitrile precipitated CSF

Protein name	Accession number	ProtSc	Ratio Pain-free DDD vs Control	Ratio Painful DDD vs control	Postulated Mechanistic Association	References
Apolipoprotein A1	Q9Y355	4.02	0.65 P<0.05*	1.03 P=0.82#	Peripheral nerve injury	9
Insulin-like growth factor II precursor	P01344	2.52	0.99 P=0.93#	1.23 P<0.05*	Peripheral nerve injury	41
Prosaposin	Q53FJ5	2.01	1.09 P=0.20#	1.29 P<0.05*	Peripheral nerve injury	47, 111
ProSAAS precursor	Q9UHG2	9.58	1.06 P=0.28#	1.15 P<0.01*	Neuropeptide processing	28, 37, 84, 120
Cystatin-C precursor	P01034	14	1.18 P=0.0001*	1.21 P<0.0001*	Inflammation	1, 19, 31, 33, 67
Serum albumin precursor	P02768	12	0.81 P<0.0001*	0.90 P<0.0001*	Inflammation	54, 81, 97, 109
Prostaglandin-H2 D-isomerase precursor	P41222	9.64	0.92 P<0.05*	0.96 P=0.38#	Inflammation	88, 95, 98, 99
Orosomucoid 1	Q5T539	4.01	0.49 P<0.05*	0.84 P=0.26#	Inflammation	32, 76
Alpha-2-HS-glycoprotein precursor	P02765	2.13	0.67 P<0.05*	0.75 P=0.25#	Inflammation	24, 57, 114

ProtSc (Protein Score) is a statistical calculation of protein confidence reflecting the number and uniqueness of the observed peptides that are attributed to a particular parent protein.

The p-values reflect the consistency of the observed ratio of iTRAQ tags on peptides belonging to the same parent protein.

* denotes P-values which are Benjamini-Hochberg significant at false discovery rate < 0.2.

denotes P-values which are not Benjamini-Hochberg significant at false discovery rate < 0.2.