Proteomic Analysis of Human Cerebrospinal Fluid from Patients with Painful and Non-painful Degenerative Disc Disease

Tony K.Y. Lim, BSc

Department of Pharmacology and Therapeutics
Faculty of Medicine

McGill University, Montreal

Submission: October 2008

A thesis submitted to McGill University in partial fulfillment
of the requirements of the degree of Master of Science

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1.1 ABSTRACT

One of the primary causes of persistent lumbar back pain is degenerative disc disease (DDD). In most persons, DDD is a normal process occurring with natural age, while in others, DDD results in chronic pain. While imaging techniques can be used to detect degenerative changes, there is a low correlation between the extent of degenerative changes and the pain found upon physical evaluation, suggesting biochemical factors may be involved with the persistent pain state. The purpose of this study was to examine human cerebrospinal fluid (CSF) for changes in protein expression using high throughput proteomics technology, which would help to identify biochemical factors involved with DDD and low back pain. Differences at the protein level were observed in the CSF of persons with asymptomatic and painful degenerative disc disease. Markers of inflammation were altered in patients with degenerative disc disease. In the case of painful degenerative disc disease, our results suggest altered neuropeptide processing and nerve damage may be playing a role in the disease.

1.2 RESUME

L'une des causes de la lombalgie chronique est la maladie degenerative lombaire (MDL). Chez la plupart des gens, ce processus se développe normalement au cours du vieillissement, alors que chez d'autre gens, la MDL cause de la douleur chronique. Alors que les techniques d'imageries peuvent être utilisées pour détecter la dégénérescence des disques, il y a une faible correlation entre l'étendue de la dégénérescence et la douleur ressentie à l'évaluation médicale. Il est donc possible que des facteurs biochimiques soient impliqués dans le dévellopement et la maintenance de la douleur chronique, mais qu'ils soient indétectables par imagerie. Le but de cette étude est d'analyser le liquide céphalo-rachidien (LCR) de l'humain afin de mesurer les changements d'expression de proteins et ainsi, d'identifier les facteurs biochimiques impliqués dans la MDL. Des différences au niveau des protéines ont été observées entre le CSF de personnes asymptomatiques et de personnes souffrant de la MDL avec douleur. Marqueurs inflammatoires ont été altérés chez les patients présentant la MDL. Dans le cas des patients ayant la MDL avec douleur, il est possible que le métabolisme des neuropeptides et des dommages aux terminaisons nerveuses jouent un role dans la pathologie.

1.3 ACKNOWLEDGEMENTS

Since the start of my graduate studies, I've racked up a long list of people who have contributed to this thesis in some way or another. I would like to thank all of you who have helped and inspired me during my Masters study.

Laura, I am deeply indebted to you for your supervision, guidance, support, and inspiration. Without you this thesis would not have all been possible.

Lois, thank you for providing me with the materials required to perform this work.

Marcos, Patrick, Line and Sylvie – thank you all very much for the immense help with the technical side of proteomics.

Anne-Julie, Brian, James, Jason, Jon, Leigh, Lina, Magali, Maral, Peter, Serge, Stefania and Xiang – I couldn't wish for better people to work with. Everyday I enter and leave the lab with a big smile of my face because I have awesome friends like you.

Anna, Cynthia and Grace, I appreciate you for being very helpful with the practical side of things.

Mike W, you taught me some important life lessons. Thank you.

Betty, Ben and Clem – for all the battles we fought together.

Tim, you've been a great friend and an awesome roommate. Thanks for being there and helping me through some of the darker periods of my life.

And then there are all those who made life in Montreal a special and fun place: Alex, Angus, Bridget, Candy, Carlis, Cindy, Claudia, Emma, Gill, Isamu, Jen N, Jen L, Jess, Kanitha, Kun, Ljubica, Massey, Merica, Mervin, Mike C, Myra, Nan, Paul, Pooja, Wenyong.

Lastly, I thank my family for their continuous support and love. I owe you all so much.

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3.1 ABBREVIATIONS

ACN: acetonitrile

CHL1: neural cell adhesion molecule L1-like protein

CNS: central nervous system

CSF: cerebrospinal fluid

CT: computed tomography

DDD: degenerative disc disease

IDD: intervertebral disc degeneration

ELISA: enzyme linked immunosorbant assay

FA: formic acid

FGF: fibroblast growth factor

ICAT : isotope-coded affinity tags

IL: interleukin

iTRAQ: isobaric tags for relative and absolute quantitation

kD: kilodalton

MARS: multiple affinity removal system

MMP: matrix metalloproteinase

MMTS: methyl methanethiosulfonate

MRI: magnetic resonance imaging

MRM: multiple reaction monitoring

MS/MS: tandem mass spectrometry

mTRAQ: MRM tags for relative and absolute quantitation

NGF: nerve growth factor

NO: nitric oxide

NSAID: non-steroidal anti-inflammatory drug

PAGE: polyacrylamide gel electrophoresis

RIA: radioimmunoassay

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SERPING1: serine/cysteine proteinase inhibitor clade G member 1

TFA: trifluoroacetic acid

TGF: transforming growth factor

TPC: total protein concentration

TNF-α: tumor necrosis factor α

VAS: visual analogue scale

3.2 LIST OF FIGURES

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4 INTRODUCTION

The completion of the sequencing of the human genome in 2001 marked the advent of the post-genomic era. The human genome, totalling over 3 billion DNA base pairs, was originally thought by some to house over 100,000 genes. However, our genome is comprised of fewer genes than expected, with a modest 2% of it coding for proteins.[1] Following completion of the human genome project, it was determined that there are approximately 20,000 - 25,000 human genes.[2] Yet if we only have twice as many genes than the fruit fly, how can it be that our species is so much more complex?

Perhaps it is not our genes that make us complex, but instead the proteins. Some estimate that the number of human proteins is greater than 500,000.[3] This comparatively immense number of proteins is thought to be a result of alternative splicing and post-translational modifications. Thus the complexity of an organism is not due to its genome, but instead explained by its proteome, or the proteins that it expresses. An organism's genome remains constant, while its proteome is different from cell to cell. For example, a nerve cell may have the same genome as a muscle cell, and yet the two are able to perform greatly different functions because they express different proteins. An organism's proteome is also what allows it to interact with the environment. The development of an egg into a caterpillar, into a cocoon, into a butterfly, all takes place with the same genome, but vastly different proteomes. As it is the interplay of proteins which defines how the organism functions, it is not surprising that changes in protein expression can make the difference between disease and health, and that these differences are unlikely to be fully characterizable by gene expression analysis.

The huge growth in high-throughput technology has equipped the post-genomic life scientist with new tools for understanding the integrative biological system. Advances in mass spectrometry have revolutionized the way life scientists are able to examine protein expression. The following work summarizes how state of the art proteomics technology was used to investigate differences in protein expression in human cerebrospinal fluid, in order to gain a greater understanding of the mechanisms underlying chronic back pain.

4.1 DEGENERATIVE DISC DISEASE

In industrialized societies, back pain is one of the most common health conditions, with point prevalence rates previously reported in international studies from 12% to 35%, and lifetime prevalence rates from 49% to 80%.[4] Not only is it common, low back pain is the primary reason for persons under 45 years of age to limit their physical activities,[5,6] is the second most common symptomatic reason for physician visits,[7] and is the fifth most common reason for hospitalization.[8] Low back pain is thus extremely costly to society. For example, low back pain has been estimated to annually cost the United States 50 billion dollars,[9] the United Kingdom 10 billion British pounds,[4] and the Netherlands 4.4 billion US dollars.[10] As low back pain is closely associated with degenerative changes in the intervertebral discs,[11-15] understanding the relationship between degenerative disc disease and low back pain is paramount to solving this major health and socioeconomic problem.

Evolution has shaped the lumbar intervertebral discs to fulfill a variety of difficult requirements. The discs must be able to withstand compressive forces and distribute them across the torso, while at the same time allowing for a great degree of flexibility, movement, and stability. The discs separate the interlocking vertebral bodies that make up the spine, and occupy approximately a third of its height. Anatomically, the lumbar disc is composed of three main parts: the nucleus propulsus, the annulus fibrosus, and the vertebral endplates. The nucleus propulsus is the gelatinous core of the disc. It is composed mainly of water, collagen, and elastin fibres, all which allow the disc to perform its role as a spongy shock absorber, and thus is responsible for the ability of the discs to absorb and distribute excessive stresses and loads. Stability, on the other hand, is granted by the annulus fibrosus, the outer fibrous layer of the disc, which holds the nucleus propulsus in place, anchoring it to surrounding tissue. The annulus fibrosus is composed of 15-25 rings of collagen fibres. The fibres are aligned parallel to the rings and run approximately 60˚ to the vertical axis, with the fibres in adjacent rings alternating in orientation. The endplate is another unit composed of collagen. It is a thin horizontal layer of cartilage that separates the disc and the

vertebral body. See Figure 1 for a schematic diagram of the intervertebral disc and its surrounding structures.

Disc degeneration usually precedes degeneration of other musculoskeletal tissues. Surprisingly, disc degeneration can be earliest observed in the age group of 11 – 16 years,[16] and it has even been reported that about 20% of people in their teens have discs with mild degeneration.[17] With age, the nucleus propolsus degenerates by gradually losing water and becoming more fibrotic.[18] Macroscopically the annulus rings lose their organization by interdigitating and bifurcating,[19] while microscopically the collagen fibres of the annulus fibrosus undergo disorganization,[20] reducing tensile strength of the annulus. With age there is also an increased incidence of cell death within the disc. The cells of the disc begin to die and become necrotic or apoptotic.[21,22] In fact, evidence suggests that in adult discs, greater than 50% of cells are necrotic.[22] Another degenerative change is proliferation of nerve fibres and blood vessels.[23] A healthy disc has very few blood vessels, and the nerves are restricted to the outer rings of the annulus.[23]

Traditionally, disc degeneration is thought to result from an environmental injuries compounded with normal aging.[24,25] However this is no longer widely accepted as there is no clear effect of mechanical loading and disc degeneration,[26] and twin studies have shown that degenerative disc disease has a large genetic component to it. Heritability of lumbar degeneration has been reported to range from 52-77%,[27,28] and heritability of low back pain has been reported to range from 0-57%.[27,29-32] To explain the genetic influences, there have also been a number of gene polymorphisms and mutations identified that are associated with disc degeneration. Collagen I,[33,34] collagen IX,[35,36] collagen XI,[37] vitamin D receptor,[38] aggrecan,[39] cartilage intermediate layer protein,[40] IL-1,[41] and matrix metalloproteinase (MMP) -3,[42] are some examples of these identified genes. However, it is noteworthy that these gene polymorphisms may only have moderate contributions to the disease, as they only provide low odds ratio increases.[43] It is clear more research on the relationship between genetic factors, degenerative disc disease and low back pain needs to be performed.

4.2 PAIN IN DEGENERATIVE DISC DISEASE

There currently exist a number of hypotheses to explain the pain which can occur as a result of degenerative disc disease. These hypotheses range from nerve compression, abnormal nerve growth, release of pain-producing substances from the nucleus pulposus, to muscle control dysfunction. These hypotheses are not exclusive of one another and it is likely that, to an extent, they can all contribute to the development of back pain. Back pain is a complex and multifactorial problem, and thus it is unlikely that a single hypothesis can explain the vast diversity of clinical observations.

As the disc ages, it becomes more prone to structural failure. Annular tears become increasingly common after the age of 10 years.[16] Mechanical stresses and loads coupled with a weakened annulus fibrosus can result in disc herniation, a process where the contents of the nucleus propulsus migrate out of the disc. In experiments with cadaveric discs, disc herniation occurs when bending and compressive forces are applied to the disc, with either component exceeding physiological limits,[44,45] or with intense repetitive loading.[46,47] Structural failure of the disc can then produce pain by shifting vertebra and compressing adjacent nerve roots.[48] Compression of nerve roots elicits prolonged ectopic discharges in the nerve, producing pain.[49]

The damaged disc attempts to repair itself, and it is thought that this process may produce pain. Several animal models have demonstrated that the disc is unable to heal itself after gross damage.[50,51] After damage, local inflammation occurs, bringing macrophages to the damaged discs. The macrophages release growth factors and cytokines, such as FGF, TGF-β, IL-1, and TNF-α.[52] As the disc is mostly devoid of blood supply, cell death in the disc may occur as a result of a failure of nutrition, contributing to disc degeneration.[53] To facilitate the repair process, growth factors such as FGF promote vascularization of the disc in an attempt to bring oxygen and nutrients to the site of damage.[54] Other released growth factors, such as transforming growth factor (TGF) -α, also stimulate growth and proliferation of extracellular matrix producing cells, resulting in fibrosis.[52] The inflammatory cytokines released by the macrophages also attract

mast cells, which secrete nerve growth factor (NGF), promoting nerve growth into the disc.[55] In normal subjects, the nerves are restricted to the outer annulus fibrosus, while in low back pain subjects, aberrant nerve growth into the disc is observed.[56] This aberrant nerve growth is thought to contribute to the pathogenesis of chronic low back pain by exposing these nerve fibres to a pronociceptive inflammatory environment,[57] resulting in sensitization of nerve fibres to mechanical stimuli.[58]

The constituents of nucleus pulposus may also be a direct source of pain. Experiments where nucleus pulposus was applied to lumbar epidural space have demonstrated that the nucleus pulposus has the ability to induce ectopic discharge,[59,60] increased sodium channel density,[60] axonal edema and Schwann cell damage,[61] and behavioural manifestations such as mechanical hyperalgesia.[62] Possible biochemical factors in the nucleus pulposus which have been implicated in the sensitization of the nerve roots include phospholipase A_2,[63-66] tumor necrosis factor (TNF) -α,[67,68] nitric oxide (NO),[69] interleukin (IL) -1β,[70] IL-6,[71] IL-8,[57,70] MMPs,[72] and prostaglandin E2.[71] Together, these studies provide support for the hypothesis that degenerating intervertebral discs release pain producing substances.

Mechanical instability resulting from the degenerating disc may also result in pain.[73] Normally, when the spine receives a load, mechanoreceptors imbedded in the ligaments transmit signals to the neuromuscular control unit. The unit subsequently elicits a normal muscle response. The injured spine, however, may respond differently. Back pain patients are known to demonstrate delayed muscle responses,[74-76] and reduced balance and postural control.[77-80] Mechanical instability or injury to the ligaments and embedded mechanoreceptors may result in distorted signals to the neuromuscular control unit, which in turn elicits an improper motor response.[81] The improper motor response results in higher stresses and injury to tissue, and muscle fatigue. Over time these improper motor responses may lead to inflammation and chronic back pain.[81]

4.3 PROBLEMS WITH CURRENT DIAGNOSTICS FOR DISC DEGENERATION

While the occurrence of disc degeneration is greater in patients with low back pain than asymptomatic patients,[11,82,83] in many individuals, disc degeneration does not result in adverse pain symptoms. Degenerative abnormalities have been found in asymptomatic subjects, with magnetic resonance imaging demonstrating approximately 30% of adults having degenerative changes and without painful symptoms.[84,85] Because imaging methods rely on clinical correlation for usefulness, this severely limits the ability of imaging methods to predict low back pain. Without a method to screen and identify individuals who are at risk of developing symptomatic disc degeneration, decisions for pre-emptive and prophylactic interventions are not possible. Furthermore, the low correlation of degenerative changes of discs and low back pain, suggests that pain resulting from degenerative disc disease may be developed and maintained by biochemical factors, rather than by changes observable by imaging.

To confirm a link between a degenerated or herniated disc and low back pain, the gold standard diagnostic test called provocative discography is performed.[19] In this test, a contrast medium is injected into the disc, pressurizing it, and the patient's response to the injection is monitored. If the test is positive, the patient will feel an intense pain, suggesting that the disc in question is causing pain. This procedure is though to result in compression of nerve endings, or in addition, release of pain producing substances from the nucleus pulposus. A CT scan measuring contrast leakage is then performed to measure the integrity of the discs. The process does however carry numerous risks, such as infection,[86] neural injury,[87] and disc herniation.[88] The procedure is often used to identify discs which could be the source of low back pain for surgical spinal fusion, however the procedure is controversial because it has a high false positive rate in asymptomatic subjects,[89] and may be the reason for the large population of patients with failed back surgery syndrome, a condition where a patient has chronic low back pain and one or more back surgeries. About 20% of patients

undergoing spinal surgery will have failed back surgery syndrome,[90] and about 1 in 5 persons who have failed back surgery originally had surgery for degenerative disc disease.[91] Thus, there is a need for methods to better evaluate the source of pain.

4.4 THE POTENTIAL USE OF CEREBROSPINAL FLUID AS A VEHICLE FOR STUDYING DEGENERATIVE DISC DISEASE

The central nervous system (CNS) is bathed in cerebrospinal fluid (CSF). Thus, its close vicinity to the CNS makes it an excellent medium to study differential protein expression of diseases that have a neurological consequence. CSF is produced by the choroid plexus at a rate of about 0.3 ml/min.[92] It then passes through the interventricular formina, into the third ventricle, through the mesencephalic duct, into the forth ventricle, where it then pools in the subarachnoid space covering the cerebrum and spinal cord. The CSF then flows over the cerebral hemispheres to be finally absorbed by the intracranial venous sinuses.[93] The total volume of CSF at one time varies between 150-270 ml.[94] Physiologically, CSF is known to perform four major functions. First, the CSF provides an important protective role for the brain by providing physical support and shock protection in the form of buoyancy. Second, the CSF acts as a sink, providing a strong diffusion gradient for waste products to be removed from parenchymal cells.[93] Third, the CSF is a vehicle for distribution of neuroactive substances throughout the brain.[93] And finally, the CSF provides a medium for regulation of the brain extracellular environment, allows it to be regulated separately from the rest of the body.[93]

The concept of using CSF to monitor changes in the CNS is hardly new, as it has been used for diagnostic purposes for over 100 years.[93] With specific reference to degenerative disc disease, there have been a number of changes in CSF that have been characterized previously. For example, peripheral inflammation results in an increase in CSF levels of IL-1β.[95] As well, pain conditions have been reported to increase levels of cystatin C,[96,97] TNF-α,[98] nociceptin,[99] and NO metabolites[100] in CSF.

For the study of disc degeneration, CSF makes a particularly good medium because the spinal canal where CSF is obtained is in close proximity to the intervertebral discs. Interestingly, past experimental studies have demonstrated that total protein concentrations of CSF increase in patients with lumbar disc herniation,[48,101-106] possibly resulting from a breakdown of the blood-nerve barrier similar to that shown in nerve compression[107] and inflammation.[108] Other changes are also observable. For example, in an animal model of experimental nerve root injury the CSF of pigs with compressed S1 nerve roots exposed to nucleus pulposus, there were higher light-subunit neurofilament protein and nociceptin levels in the CSF compared to the sham group.[109] A similar study in humans also demonstrated that light-subunit neurofilament protein and S-100 protein were increased in the CSF of patients with disc herniation and sciatica compared to control subjects.[110]

Not only is CSF close in vicinity to the intervertebral discs and the CNS, it is also obtainable through a minimally invasive process. A lumbar puncture is a commonly performed technique where a needle is inserted at the L3/L4 or L4/L5 interspace, below the termination of the spinal cord.[111] After the needle is inserted into the spinal canal the CSF leaks out passively and can be collected. There are however, possible complications from the procedure. The most common complication of lumbar puncture is post dural puncture headache, occurring in up to 36.5% of procedures.[112] The headache usually begins after 48 hours post procedure, and may persist 1-2 days and in some cases even 2 weeks.[113] Other more rare complications include puncture of the internal vertebral venous plexus, nerve trauma, disc herniation, brain herniation,[111] and infection.[114] In a study with 1107 malleable needle lumbar punctures, the rate of venous plexus puncture has been estimated to be 2.2%.[115] Neurological injury was recorded in 24 cases in a study of 40640 spinal anesthetics, giving an injury rate of 0.06%.[116] Disc herniation is a more rare complication of lumbar puncture, with only one report in the literature.[117] Brain herniation is another serious complication which may occur after lumbar puncture, but its incidence is again rare.[111] A recent discussion of post dural puncture meningitis reviewed 179 reported cases.[114]

4.5 DIFFERENTIAL EXPRESSION PROTEOMICS

It is likely that there are many other unidentified genetic and environmental risk factors that contribute to degenerative disc disease and low back pain. In order to understand a complex phenomenon such as degenerative disc disease, it is important to understand functionally the widespread changes occurring in the body. This can be most readily accomplished with differential expression proteomics. Traditional methods, such as ELISA, or RIA, are inadequate because they only allow one to examine one protein at a time. Current technology in differential expression proteomics now provides the ability to identify and quantify hundreds of proteins simultaneously. As proteins are the final endpoints of the genetic blueprint, and are ever changing to the influence of the genome and the environment, understanding how proteins interact on a systems level holds great potential for understanding disease. By identifying biomarkers – proteins associated with degenerative disc disease or low back pain – we would not only understand more about the disease process, but we may be able to uncover new targets for pharmacological modulation and find biomarkers that identify the patients most at risk for degenerative disc disease, thus permiting prophylactic or preventative treatments. In addition, there may be biomarkers that predict whether or not a patient would benefit from surgical intervention. Such biomarkers would greatly reduce the incidence of failed back surgery syndrome.

4.6 TECHNICAL CHALLENGES OF CEREBROSPINAL FLUID PROTEOMIC STUDIES

Recent advances in mass spectrometry have revolutionized the study of proteins by providing the possibility of high throughput identification and quantification of proteins. The process begins with sampling of the proteins from the biological source. After purification of the proteins to remove contaminants, the protein sample undergoes enzymatic digestion to produce small peptides. The peptides are further fractionated on a liquid chromatography column prior to mass spectrometry. The peptides are ionized and the first mass spectrometer determines

the mass of peptides that co-elute from the column. Next, one of these peptides is selected to enter an argon gas field that excites the peptide through gaseous collisions, fragmenting the peptide at peptide bonds. The masses of the fragments are measured, and the mass spectra obtained here can be used to determine the amino acid sequence of that peptide. This information is then used to identify the parent protein from which that smaller peptide was derived from a protein database. Figure 2 shows this process in diagrammatic form.

One of the challenges of differential expression proteomics lies in sample preparation. CSF contains thousands of proteins with concentrations ranging over 10 orders of magnitude.[118] Cerebrospinal fluid CSF is 67% albumin, and in fact, the top 10 CSF proteins make up about 99% of the total protein content of CSF.[119] To further complicate matters, concentration of total protein in CSF is quite dilute and can fluctuate greatly. Levels between 150 and 500 mg/l are considered normal.[93] Another difficulty in analyzing CSF with mass spectrometry is the high salt concentration of CSF. The salt concentrations are similar to those seen in blood, while the total protein in CSF is about 200 times less than that in blood plasma.[93] Biological salts interfere with the mass spectrometry process, and must be removed prior to analysis. Thus, the identification and quantification of proteins present in CSF in lower abundances is a significant challenge.

4.7 SAMPLE PREPARATION TECHNIQUES FOR CEREBROSPINAL FLUID PROTEOMIC STUDIES

There are a multitude of techniques available to isolate proteins and separate them from salts and other interfering substances.[120] Ultrafiltration is a common method where a filter with a molecular mass cut off acts as a sieve and permits salts and impurities to pass through the filter, but not the larger proteins. Ultrafiltration filters are commonly built to fit in centrifuges so that the samples are gravity fed through the filters. Another similar technique, called dialysis, can be used to separate salts from protein. Here, a semi-permeable membrane is placed between a sample and a large volume of dialysate. Small compounds like salts can permeate the membrane and exit the sample through diffusion, but the

larger proteins are unable to do so. Protein precipitation is another common technique, whereby organic solvents such as acetone are used to precipitate proteins. In addition to salts, protein precipitation allows the removal of contaminants such as surfactants and lipids that remain in the supernatant. The protein pellet is then reconstituted in a suitable buffer free of contaminants. Another technique used to remove salts is size exclusion chromatography, where salts and small contaminants are trapped in a polyacrylamide matrix, while larger proteins are too big to enter the matrix and flow freely out of the column.

It is assumed that most potential protein biomarkers exist at concentrations lower than the mg/L level in CSF. As the mass spectrometer selects the most abundant peptides for sequencing, methods to remove highly abundant proteins like albumin are required. Methods such as ultrafiltration can also be used to remove highly abundant proteins. For example, abundant proteins such as albumin (66 kD), transferrin (77 kD), and IgG (150 kD), can be depleted by selecting an ultrafiltration unit with a cutoff of 50 kD or less. This has been performed previously with CSF with mixed results, one study identifying 148 proteins,[121] and another identifying only 46.[118] Another popular method for abundant protein removal is immunodepletion, where antibodies are used to capture abundant proteins. So far commercial immunodepletion columns are built only for abundant protein depletion of serum proteins, but there is significant overlap in abundant protein species to allow for the adaptation of these columns for use with CSF. In one study, two protein depletion cartridges, the multiple affinity removal system (MARS) cartridge (Agilent Technologies) and the ProteoSeek cartridge (Pierce Biotechnology), were tested for use with CSF. In this case, 171 and 163 proteins were identified respectively.[118] Solvent depletion is also a viable method to deplete abundant proteins. One study used sequential acetonitrile precipitation to remove abundant proteins. Acetonitrile precipitates larger proteins before smaller proteins, and thus, highly abundant large proteins can be separated from the smaller proteins by this method. With this method, more than 300 proteins were identified.[122]

Fractionation of the sample – whether at the protein level or the peptide level, helps to further increase the number of identifiable proteins by simplifying the analyte at the mass spectrometer. At the protein level, there are a number of ways to fractionate proteins. SDS polyacrylamide gel-electrophoresis permits proteins to be separated by size. Following electrophoresis, proteins bands can be cut out of the gel for analysis. Proteins can also be separated by isoelectric point in this manner, and the techniques can be combined with SDS-PAGE for further fractionation by 2D gel electrophoresis. Alternatively, separation by isoelectric point can also be performed with in solution phase isoeletric focusing. There is essentially no limit to the variety of techniques that can be used to fractionate proteins for proteomics. Ion-exchange chromatography can be used to separate proteins according to their charge. Size-exclusion chromatography can be used to separate proteins by size. Hydrophobic interaction chromatography that can be used to separate proteins by hydrophobicity. These techniques can be chained together to produce separation in 2 or more dimensions. The ideal sample preparation step, however, will separate samples with few manipulations, as the more manipulations – the greater the variability and potential for sample loss.

Fractionation at the peptide level is commonly done to prevent all the peptides from arriving at the mass spectrometer at once. This gives the mass spectrometer the temporal resolution to analyze the peptides. This is most commonly performed with hydrophobic interaction chromatography. In this process, peptides or proteins are passed through a stationary solid phase. For the purposes of proteomics, this stationary phase is usually a resin made up of a hydrophilic compound such as octadecyl siloxane (C18). In this case, salts and small ionic contaminants elute from the column while the more hydrophilic peptide and proteins are retained. This process can be used to remove salts or to separate peptides. It is also common for peptides to be fractionated on a second dimension before the C18 fractionation. In proteomic experiments this column is usually a strong cation exchange column, which separates peptides according to their degree of positive charge.

In differential expression proteomics, quantification is performed either with or without an isotope label. Label free quantitation is based on the observation that protein abundance correlates with the number of spectra sampled.[123] This is because abundant proteins will have a greater amount of unique peptides identified, and each unique peptide will have a larger chromatogram. The end result is that the more abundant the protein, the more spectra there will be for it to be sampled. Past experiments have shown that spectral counting methods can give relative abundances over 2 orders of magnitude.[124] Spectral counting has been found to be sensitive enough to detect changes as low as 2.5 fold, however, this is less sensitive than protein ratios achievable with chemical labels, which can determine ratios significant to ~1.5 fold up to 100 fold.[125] On the other hand, the drawbacks of isotope labels are that they are expensive, can only be used for pairwise comparisons between samples, and cannot be used for retrospective comparisons.[125]

Isotope labelling began with the development of ICAT, a label that reacts with cysteine residues.[126] Proteins from different sources are given a different isotopic label – either a light label (^{12}C) or a heavy label (^{13}C). Under reducing conditions, the labels are reacted to the sulfhydryl moieties of the cysteine residues of the protein, forming covalent bonds. The samples are then pooled, and enzymatically digested to create small peptide fragments. Then, when the mass of the peptide is determined by mass spectrometry, the light and heavy peptides will differ by 9 mass units. The ratio between the heavy and light peptides gives the relative abundance of the peptide species.

A newer isotopic label has been created which reacts with amino groups of peptides called iTRAQ (isobaric tags for relative and absolute quantitation). In addition to its peptide reactive group, the label contains a cleavable reporter region, and a balance which ensures that the labels are isobaric, meaning they have the same mass. This is more favourable to ICAT, because not all peptides have cysteine amino acids available for labelling, but all have an N-terminal amino group. In addition, as lysine has a free amino group, iTRAQ will also react with it. Because this modification of lysine will prevent trypsin cleavage, unlike

ICAT, iTRAQ is commonly applied to peptide fragments after they have been digested. Originally, iTRAQ was formulated with 4 different reporter masses of 114, 115, 116 and 117 daltons. However, it currently is produced with 8 different masses, thus allowing up to 8 samples to be compared to one another, a significant advantage compared to the 2 masses that ICAT affords. Another difference is that quantitation occurs at the tandem mass spectrometry (MS/MS) stage where the iTRAQ labels are fragmented into reporter ions. This is possible because the iTRAQ labels are isobaric, so at the MS stage the peptides are all the same mass. ICAT labels are not isobaric, and thus they complicate the interpretation of the MS/MS spectra.

Differential expression proteomics permits the quantitation of many different proteins at once in a high-throughput manner. However, there are a number of technique-specific drawbacks to this method. First, the abundances determined are relative, whereas with traditional methods like ELISA and RIA, the abundances determined can be established absolutely. The technique is also insensitive to loss of function mutations. For example, whether or not a protein will bind to its receptor cannot be determined from differential expression proteomics. In this case, information at the genetic or the functional level would need to be gathered. Another limiting factor to proteomic experiments is sensitivity. Traditional methods are not as susceptible to interfering substances and abundant proteins. Perhaps the greatest drawback about proteomics experiments is that there is a random component to the ionization and MS/MS selection process. Thus, the reproducibility of experiments can suffer, in particular when it comes to proteins of low abundance.[127]

4.8 OVERVIEW OF THE CURRENT STUDY

Degenerative disc disease is a condition resulting from the deterioration of the intervertebral discs. The process can be facilitated by acute or repeated injury, or can develop from the aging process. It is a leading cause for chronic and debilitating low back pain. In the current study, our objectives were to search for biomarkers – proteins that are associated with disc degeneration or low back

pain – in CSF. The discovery of biochemical markers in CSF with predictive value for the treatment of low back pain would not only be of great clinical use, but it could also be important for understanding the pathophysiology behind disc degeneration and low back pain. In addition, biomarkers to prevent failed back surgery syndrome would prevent the more than 80,000 failed back surgeries per year.[91]

Two approaches were taken towards the analysis of CSF. We began first by evaluating the feasibility of quantitating neuropeptide expression in CSF with mass spectrometry techonology. Next we moved on to study protein expression in CSF, and utilized two depletion techniques to remove abundant proteins. With these methods, we were able to observe changes in protein expression of CSF from subjects with degenerative disc disease and low back pain, and identify several putative biomarkers.

5 METHODS – EXPERIMENTAL SAMPLES

Three patient groups were studied: pain-free subjects without disc degeneration, pain-free subjects with disc degeneration, and low back pain subjects with disc degeneration. Questionnaires, physical examinations and CSF collection were performed at the University of Minnesota General Clinical Research Facility. Lumbar MRIs were collected at the Fairview University Medical Center.

5.1 SAMPLE SELECTION

For the pain-free groups with or without disc degeneration, 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 low back pain over the last three months. Volunteers were excluded if they use 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 are using antidepressants and have not been on a steady dose for at least 2 months.

Subjects were evaluated by lumbar MRI by a blinded observer to determine if asymptomatic disc degeneration was present. Subjects with MRIs scoring ≤ 3 on the Thompson scale,[128] were placed in the without disc degeneration group. Those scoring 4 or 5 on the Thompson scale,[128] were placed in the with disc degeneration group.

Patients' age 21-65 years with chronic low back pain due to diagnosed degenerative disc disease are recruited for the low back pain group with degenerative disc disease. The MRIs of these patients was evaluated by a blind observer. Only those scoring 4 or 5 on the Thompson scale,[128] with a minimum of 6 months of severe pain, and selected for disc removal and spinal fusion were included in the study. Patients were excluded unless they belonged to one of the following three medication regiments: non-steroidal anti-inflammatory drug (NSAID) and opioid, NSAID and steroid, opioid and steroid.

General exclusion criteria for any subject included complicating medical factors such as previous spine surgery, pregnancy, lactation, meningitis, spondylolisthesis, scoliosis, osteoporosis, neuropathies, and neurological conditions (such as psychosis, dementia, Parkinsons, etc). Subjects are asked to refrain from strenuous exercise 3 days before the pain assessment and CSF collection.

5.2 DATA COLLECTION

As part of a comprehensive medical history questionnaire, a pain medication inquiry was performed. This was performed in the groups without chronic pain to ensure that analgesic medication would not alter pain scores or affect the levels of nociceptive mediators in the CSF. In the group with chronic pain, analgesic use was not withheld due to ethical reasons, but data was collected for analysis as a possible co-variant.

All subjects were assessed for perceived pain using a visual analogue scale out of 100. Pain free subjects were included in the study if they reported scores of ≤ 10, and low back pain subjects were included if they reported scores ≥ 25. Subjects also completed a short form of the McGill pain questionnaire.[129,130] The

McGill pain questionnaire allows statistical analysis to be performed on the qualitative aspect of pain. Next, the Oswestry low back disability index version 2.0[131] was used to evaluate how chronic low back pain affected subjects' perceived ability to perform daily activities.

Physical impairment and functional performance were measured in each of the subjects. A physical impairment index, consisting of total flexion, total extension, average lateral flexion, straight leg raise, bilateral active SLR, sit-up and spinal tenderness was determined by a physical therapist.[132] Functional impairment was assessed as described previously using the following methods: 50 ft speed walk, 5 minute walk, timed up-and-go, timed sit-to-stand, timed repeated trunk flexion, and loaded reach.[133]

5.3 COLLECTION OF CEREBROSPINAL FLUID

CSF was collected with a 25 gauge Whitacre spinal needle under i.v. sedation with midazolam. The needle was introduced to the spinal canal according to standard practice. 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. Collected CSF was then chilled on wet ice (0˚C). CSF was then centrifuged at 250 g for 10 minutes, and the supernatant aliquoted into previously labeled cryovials at 100-500 µl and flash frozen in liquid nitrogen. Samples were transported in a liquid nitrogen container until storage at -140˚C at the University of Minnesota. Samples were then transported on dry ice to McGill University where they were stored at -80˚C.

Following CSF collection, each subject was then 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 are given to the subjects, and they are released to a friend or family member who escorted them home and stayed with them overnight.

5.4 ETHICAL CONSIDERATIONS

Scientific experimentation involving humans plays an important role in understanding our own bodies and minds. It is by studying ourselves that we can move closer to finding ways to relieve the disease and distress that afflict us. Numerous past medical breakthroughs, of which we owe our ability to treat or prevent what was in the past considered fatal or disabilitating, have been founded on human research. Yet when performing human research, it is paramount to keep in mind that use of human subjects in research is a special privilege – with special ethical responsibilities.

Human research subjects are in a vulnerable position, and appropriate measures have to be taken to protect their rights. On one hand, human subjects have the potential to provide important information that would be of benefit to humankind as a whole. On the other hand, they provide these benefits at a risk to themselves. As a result of these conflicting values, regulatory bodies have implemented systems of oversight and review to protect both human subjects and the advancement of medical science, from legal, practical, and ethical standpoints.

To take part in an experiment, human subjects must volunteer themselves. To ensure volunteers can make informed decisions regarding participation, potential subjects were informed of this study's goals, why the research is being done, and what is expected of them. Importantly, subjects were informed that there are no direct benefits to partaking in this study, and that they are not required to participate and may quit the study any time with no explanation required with no penalty. They are also warned of the possible risks of partaking in the study. In particular, the risks of CSF withdrawal are disclosed, such as the more common postdural puncture headache, and the very rare possibility of neurological injury, spinal hematoma, infection, and brain injury. They are also told of the side effects of the medication midazolam, such as allergic reaction, prolonged drowsiness, breathing problems and confusion.

Furthermore, subjects were also compensated $100 to partake in the study. This amount was chosen to reimburse subjects for their time. This small compensation ensures that subjects are not participating for monetary gain. In fact,

a number of subjects traveled far distances just so they could partake in this study, and explained that they hoped their participation would be helpful to prevent others from having to experience the debilitating pain they experience.

Because medical records, questionnaires, functional tests, and the results of experiments may contain sensitive information, patient confidentiality is important to maintain. To ensure sensitive information is not distributed, each specimen is coded without any confidential information concerning the patient. The code is only accessible by the principal and co-investigators, and subjects are assured that no information that can identify them will be present in any publications or presentations.

6 METHODS – PROTEOMIC ANALYSIS

Several proteomic experiments were attempted. First, the feasibility of analyzing CSF by focusing on peptides was evaluated by labeling samples of known peptide "standards", for analysis in parallel with peptides isolated from commercial CSF by ultrafiltration. Second, the feasibility of analyzing CSF with the aide of protein standards was evaluated using a strategy in which a mixture of protein standards was labeled with iTRAQ for detection and quantitation. Next, CSF from pooled experimental subjects was analyzed. Immunodepletion and acetonitrile precipitation was used to remove the abundant proteins from the pooled CSF. See Figure 3 for a flow chart detailing an overview of the sample preparation performed on the CSF obtained from the experimental subjects.

6.1 PEPTIDE STANDARDS

Human synthetic peptides were obtained commercially. Substance P, neuropeptide Y, bradykinin and dynorphin A 1-17 were purchased from Anaspec Incorporated. Leucine-enkephalin, methionine-enkephalin, β-endorphin and oxytocin were purchased from Phoenix Pharmaceuticals. 10 µg each of substance P, neuropeptide Y, bradkykinin, dynorphin 1-17, leucine-enkephalin, methionine-enkephalin, β-endorphin and oxytocin were combined from frozen aliquots kept at

-80˚C. The total pooled 80 μg of peptide was then lyophilized on a centrifugal evaporator.

6.2 PROTEIN STANDARDS

Human recombinant and purified proteins were purchased commercially. IL-1β, IL-10, and TNF-α were obtained from Biovision Incorporated. NGF was obtained from R&D Systems. Purified SPARC protein was obtained from Kamiya Biomedical Company. Purified transferrin was obtained from Rockland Immunochemicals. 2 μg IL-1β, 3 μg IL-10, 2.5 μg TNF-α, 20 μg NGF, 15 μg SPARC and 20 μg transferrin was combined from frozen aliquots kept at -80˚C. The total pooled 62.5 μg of protein was then lyophilized on a centrifugal evaporator.

6.3 PEPTIDE ISOLATION FROM COMMERCIAL CSF

Commercial CSF was obtained from Biological Specialty Corporation. Microcon 10 kD nominal molecular weight cutoff ultrafiltration units were obtained from Millepore. 500 μl of commercial CSF was placed in a Microcon 10 kD nominal molecular weight cutoff ultrafiltration unit. Samples were centrifuged at 14,000 G for 60 minutes at 4˚C. 345 μl of ultrafiltrate was evaporated with a centrifugal evaporation unit.

6.4 POOLING OF EXPERIMENTAL CSF

Samples were pooled for proteomic analysis. Four groups were pooled: 1) young healthy controls, 2) age controlled subjects without degenerative disc disease or low back pain, 3) subjects with degenerative disc disease but without low back pain, 4) subjects with both degenerative disc disease and low back pain. Pooling began with the thawing of 500 μl of CSF from each subject. 15 μl per subject was removed from the aliquot for Bradford protein determination (Pierce Biotechnology) according to kit protocol. The rest of the CSF was pooled by mixing all the CSF belonging to the group into a single vessel, followed by vortexing. Then the pool of CSF was aliquoted into 500 μl aliquots and refrozen

at -80˚C. Subject information is shown in Table 1. A diagram showing the sample pooling process is shown in Figure 4.

6.5 SDS-PAGE GEL ELECTROPHORESIS

Gel electrophoresis was performed with BioRad 8 – 16% polyacrylamide precast Ready Gels. Gels were unpacked and loaded in the electrophoresis chamber. The chamber was loaded with SDS-PAGE running buffer, and samples loaded in the gel wells. Gel electrophoresis was performed at 180 V.

Following completion of SDS-PAGE, gels were then silver stained for visualization. Gels were first fixed in fixative buffer overnight, followed by extensive washing with 30% ethanol in water. Gels were then reduced with sodium thiosulfate, and then stained with silver nitrate. The gels were developed with sodium carbonate and stored in 5% acetic acid until they were scanned with a gel reader.

6.6 IMMUNODEPLETION OF CSF ABUNDANT PROTEINS

Each pooled CSF sample was depleted twice by the Proteoprep 20 column, as a single depletion step may not remove all abundant proteins. To begin, 500 µl of pooled CSF from each group was thawed on ice. Each sample was filtered at 2000 g for 60 seconds through a Corning Spin-X 0.22 µm centrifuge tube to remove any particulates. The ProteoPrep 20 plasma immunodepletion column was equilibrated and readied for depletion according to manufacturer's protocol.

The ProteoPrep 20 removes the top 20 abundant proteins in plasma. As there is currently no depletion column designed specifically for CSF, the Proteoprep 20 immunodepletion column was adapted for use with CSF. The ProteoPrep 20 immunodepletion column removes albumin, IgG, transferring, fibrinogen, IgA, alpha-2-macroglobin, IgM, alpha-1-antitrypsin, complement C3, haptoglobin, apolipoprotein A1, apolipoprotein A2, apolipoprotein B, acid-1-glycoprotein, ceruloplasmin, compliment C4, complement C1q, IgD, prealbumin, and plasminogen.

CSF immunodepletion was performed in 100 µl steps. The order of CSF immunodepletion was randomized. 100 µl of filtered CSF was added to the top of the immunodepletion column, and the CSF was incubated for 10 minutes at room temperature. The column was spun at 1000 g for 30 seconds and the flow through was saved. The column was washed with 100 µl equilibrium buffer, and the column was again centrifuged and the flow through pooled. The wash step was performed once more. The flow through was kept chilled on ice during the procedure. Bound proteins were then eluted with elution buffer, and the column was re-equilibrated according to manufacturer's protocol. The next 100 µl CSF sample was loaded onto the column, and the process repeated.

The flow-through of the CSF was concentrated on a centrifugal evaporation unit until the volume was approximately 300 µl. The column was equilibrated and readied for use. 100 µl of flow-through was placed on the column and incubated at room temperature for 10 minutes. The column was centrifuged and the process was repeated twice more until the entire flow-through passed through the column once more. The column was then washed twice with equilibrium buffer. The second flow through was stored at 4˚C until further analysis. The bound proteins were then eluted and the process repeated for each sample.

The second flow through was then desalted on a Vivaspin 500 5 kD nominal molecular weight cutoff ultrafiltration unit (Sartorius Stedim). The sample was approximately 500 µl after the second flow through. The sample was filtered until < 50 µl remained. 500 µl MilliQ H_2O was added to the sample and it was filtered again. This was repeated twice to remove and exchange the equilibrium buffer with water. Protein quantification of the second flow through with the Bradford protein determination method (Pierce Biotechnology) measured slightly more than 10 µg of total protein per pool of CSF. 10 µg of depleted CSF protein from each group was transferred to a new microcentrifuge tube for digestion and iTRAQ labeling detailed below.

6.7 PRECIPITATION OF CSF ABUNDANT PROTEINS

1500 µl of pooled CSF from each group was thawed on ice. Each sample was dialyzed for 24 hours against MilliQ H_2O using an Ettan Mini-dialysis unit to remove biological salts. The pooled CSF was then separated into two 700 µl aliquots and placed into 2 ml microcentrifuge tubes. 1050 µl, or 1.5X the volume of CSF, was added to each 700 µl unit of CSF. The microcentrifuge tubes were vortexed for 10 seconds and incubated for 1 hour at room temperature. The samples were then centrifuged at 15,000g for 10 minutes. The supernatant was transferred to another vessel, and the samples were evaporated in a centrifugal evaporation unit to remove the acetonitrile. The lyophilized acetonitrile soluble protein was reconstituted in H_2O for protein determination. Protein quantification of the second flow through with the Bradford protein determination method (Pierce Biotechnology) measured approximately 10 µg of total protein per pool of CSF. 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 to remove impurities, and in the other run, peptides were not ziptipped. The results of these two runs were combined in the data analysis phase.

6.8 TRYPSIN DIGESTION

Sequencing grade modified trypsin was purchased from Promega Corporation. In short, lyophilized peptides or proteins were reconstituted in 20 µl of 0.5 M triethylammonium bicarbonate pH 8.5 and 2 µl of 50 mM TCEP. All samples with the exception of the peptide standards had 1 µl of 2% SDS added. Samples were incubated for 1 hour at 60˚C to denature the sample and reduce any cysteine bonds. 1 µl of 200 mM methyl methanethiosulfonate (MMTS) was then added to samples and tubes were incubated for 10 minutes at room temperature to block cysteine residues. Trypsin was added to the sample from a 0.1 µg/µl stock aliquot in 3% acetic acid. Peptide standards, protein standards, CSF peptides, individual pooled immunodepleted CSF samples and pooled acetonitrile soluble

CSF samples had 0.8, 1, 1, 0.25, and 0.24 µg of trypsin added, respectively. The sample was incubated overnight at 37˚C.

6.9 iTRAQ LABELING

iTRAQ reagents and related chemicals were purchased from Applied Biosystems. For the peptide standards, the standard was split into 4 tubes of 6 µl each. iTRAQ reagents were reconstituted with 70 µl of ethanol. Next, half of the 114, 115, 116, and 117 iTRAQ reagent was added to each tube separately. The tubes were incubated for 1 hour to complete the labeling process. Standards A, B, C and D were then made according to Table 2, by combining the appropriate amount of peptide with each label.

For the protein standards, the standard was split into 4 tubes of 5 µl each. iTRAQ reagents 114, 115, 116 and 117 were reconstituted with 70 µl ethanol and the entire iTRAQ reagent was added to the four tubes separately, and incubated for 1 hour to complete the labeling process. Standards E, F, G, H and I were then made according to Table 3, by combining the appropriate amount of labeled digested protein.

For both the immunodepleted pooled CSF samples, iTRAQ reagents were reconstituted with 70 µl of ethanol. The 114 label was combined with the pooled CSF from age controlled subjects without disc degeneration. The 115 label was combined with the pooled CSF from subjects with disc degeneration but without low back pain. The 116 label was combined with the pooled CSF from subjects with both disc degeneration and low back pain. The 117 label was combined with the pooled CSF from young healthy controls without disc degeneration or low back pain. The entire iTRAQ reagent was transferred to the pooled CSF samples. Samples were then incubated for 1 hour at room temperature to complete the labeling process.

6.10 1D CHROMATOGRAPHY

Samples were analyzed at the Genome Quebec Proteomics Platform. The sample was reconstituted in 10%ACN:0.1%TFA. The sample was diluted with 30

µl of 3%ACN:0.1%FA, and concentrated and desalted with a SCX Zip-Tip column (Millepore). The Zip-Tip column was activated with acetonitrile, and then washed with water. The sample was loaded on the Zip-Tip to bind the peptides. The Zip-Tip is then washed with 0.5% FA to wash the peptides. After removing the wash solution, the Zip-Tip is filled with acetonitrile to extract the peptides. Extracted peptides were loaded on a 1100 series nanoHPLC system using a Biobasic C18 (10 x 0.075 mm) integrafrit 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.

6.11 2D CHROMATOGRAPHY

Samples were analyzed at the Genome Quebec Proteomics Platform. The sample was reconstituted in 10%ACN:0.1%TFA. Sample was diluted with 30 µl of 3%ACN:0.1%FA. A fraction of the sample was injected onto a Zorbax Bio-SCXII 50x0.8mm strong cation exchange column (Agilent). Elution from SCX column was done by stepwise 40 µl 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 (5x0.3 mm) at 15 µl/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 x 0.075 mm) integrafrit 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.

6.12 MASS SPECTROMETRY

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 and 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.

6.13 DATABASE SEARCH

Peptide standards and CSF peptide samples were searched on the SwePep human peptide database obtained in FASTA format on September 26, 2007. The SwePep database is a database created to identify endogenous peptides by mass spectrometry.[134] Protein standards, immunodepleted CSF and acetonitrile soluble CSF was searched using a UniProt human protein database obtained on March 2008 containing 71371 sequences. UniProt is a comprehensive database on protein sequences that is expertly curated and updated every three weeks.[135] Spectral processing included peak smoothing and centroiding without de-isotoping and peak picking for peaklist generation was done with 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).

6.14 STATISTICAL ANALYSIS

iTRAQ ratios were obtained with ProQuant ver1.4 (Applied Biosystems). iTRAQ ratios were converted from absolute ratios to $\log_{10}$ space for statistical manipulations. One sample t-tests were performed using GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/).

7 RESULTS

7.1 PEPTIDE STANDARDS

Peptide standards created from synthetic peptides were used to determine the accuracy iTRAQ to theoretical values, and to explore the possibility of using a standard as a control group for absolute quantification of particularly interesting peptides. Standard peptide mixtures were created as according to Table 2.

The closeness of the experimentally obtained values to theoretical ratios is shown graphically in Figure 5A. While there was variation observed between the values of experimentally obtained ratios and theoretical ratios, the direction and relative magnitude of the ratios agreed with experimental data. Furthermore, the experimental values were not significantly different from the theoretical values, as shown in Figure 5B.

In terms of which peptides were observable in the peptide standards, only 6 out of the 8 peptides in the standard were found. Bradykinin, dynorphin A, neuropeptide Y, β-endorphin, substance P, and oxytocin were observed by mass spectrometry. Example spectra of observed peptides are shown in Figure 6. Interestingly, only the smallest peptides, leucine- and methionine-enkephalin, were not observable by mass spectrometry.

7.2 CSF ULTRAFILTRATION

With a cutoff of 95% confidence, 10 kD cutoff ultrafiltration of commercial cerebrospinal fluid yielded four peptides observable by mass spectrometry. They were peptide fragments of secretogranin-1, secretogranin-5, somatostatin and VGF nerve growth factor inducible. Observed spectra are

displayed in Figure 7. All of these neuropeptides are known to be in CSF. VGF nerve factor inducible and secretogranin-1 has been previously identified using similar proteomic methods in CSF.[120] Somatostatin has been shown to be present in CSF through radioimmunological methods.[136] Secretogranin-5 was also previously identified in CSF using proteomic methods.[137]

Secretogranin-1, also known as chromogranin B, is widely distributed in neuroendocrine cells.[138] Intracellularly, secretogranin-1 is proposed to play roles in the biogenesis of secretory vesicles.[139] Secretogranin-1 also has a number of extracellular effects. There have been a number of peptides derived from secretogranin-1 that have been identified. One is called secretolytin, a peptide that exhibits antibacterial activity.[140] Other peptides with uncertain function include $GAWK_{440-513}$,[141] BAM-1745,[142] SR-17,[143] HQ-34,[143] and PE-11.[144]

Secretogranin-5 is also involved with the secretory machinery of neuroendocrhine cells. Secretogranin-5, also called 7B2, binds and activates the enzyme prohormone convertase 2 which is involved with enzymatic maturation of peptide hormones and neuropeptides.[145]

Various VGF peptides have been shown to produce biological effects. TLQP-21, a VGF derived peptide, protected neurons from serum deprivation and potassium induced death.[146] Also, VGF is known to be regulated by exercise, and administration of a VGF-derived peptide to mice produced an antidepressant response.[147]

Somatostatin is produced throughout the central nervous system, and in many peripheral organs.[148] It produces analgesia when administered to rodents[149] and humans.[150,151] Its short half life, however, prevents its use therapeutically. Instead, octreotide, a stable analogue of somatostatin, is used clinically to produce analgesia in patients with a variety of chronic pain conditions.[152]

7.3 PROTEIN STANDARDS

Protein standards created from recombinant or purified proteins were used to determine the accuracy iTRAQ to theoretical values and to explore the possibility of using a standard as a control group for absolute quantification of

particularly interesting proteins. Standard peptide mixtures were created as according to Table 3.

The closeness of the experimentally obtained values to theoretical ratios is shown graphically in Figure 8A. While there was varience observed between the values of experimentally obtained ratios and theoretical ratios, the direction and relative magnitude of the ratios generally agreed with experimental data. Furthermore, experimental values were not significantly different from theoretical values, as seen in Figure 8B. Compared to the peptide standards, on average the protein standards had half the variation of the peptide standards.

In terms of which proteins of the standard were observable, all 6 proteins in the standard were found using the Mascot search engine. However, with the Interrogator search engine that is used for iTRAQ quantification, only 5 out of the 6 proteins in the standard were found. NGF was not identified. Example Mascot spectra of observed peptides from standard proteins are shown in Figure 9.

7.4 PROFILE OF POOLED CSF

According to 1D gel electrophoresis shown in Figure 10, the profiles of the different experimental groups were remarkably similar. When comparing pooled CSF from persons without degenerative disc disease or pain, from persons with degenerative disease with no pain, and from persons with degenerative disc disease and pain, the most striking difference was the presence of a band under 50 kD in the pool from persons without degenerative disc disease or pain. Other than this difference, the 1D profiles of the CSF looked essentially the same. However, when compared to commercial CSF, numerous differences were observable in the 1D profile. This is likely due to the complex and undefined nature of the pooled commercial sample.

7.5 CSF IMMUNODEPLETION FOLLOWED BY 1D LC MS/MS

The ProteoPrep 20 immunodepletion successfully removed abundant proteins as shown by gel electrophoresis in Figure 11. After immunodepletion, many protein bands were no longer visible, and many protein bands increased in

intensity, suggestive that high abundant proteins were removed, resulting 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 kD. The second depletion removed a greater amount of albumin among other proteins.

A total of 83 proteins were observed. A list of the observed proteins and ratio differences is given in Table 4. At least 60 of the identified proteins have been previously identified in cerebrospinal fluid using proteomic techniques.[153-157] A number of proteins in this study have not been reported previously in cerebrospinal fluid using proteomic techniques. These include putative uncharacterized protein DKFZp781G125 (Q68CP7), limbic system-associated membrane protein precursor (Q13449), uncharacterized protein C2orf55 (Q6NV74), and cDNA FLJ40018 fis (Q8N849). Example spectra of immunodepleted CSF proteins are shown in Figure 12.

7.6 CSF ACETONITRILE PRECIPITATION

According to the silver stained 1D gel electrophoresis of the acetonitrile precipitated CSF, acetonitrile precipitation was successful in separating high molecular weight proteins from low molecular weight proteins (Figure 13). Notably, the precipitation was able to remove albumin, the most abundant CSF protein, from the low molecular weight fraction. The low molecular weight fraction consisted of proteins of 35 kD or smaller. Interestingly, the trypsin digestion was not complete, even when large amounts of trypsin were mixed with the samples for digestion.

The number of proteins observed totaled 52 proteins. A list of the observed proteins and ratio differences is given in Table 5. At least 36 of the identified proteins have been previously identified in cerebrospinal fluid using proteomic techniques.[153-157] A number of proteins in this study have not been reported previously in cerebrospinal fluid using proteomic techniques. These include zinc finger protein 512B (Q96KM6), prosaposin (Q53FJ5), PRAME family member 1 (O95521), PP11517 (Q71RD3), cDNA FLJ40039 fis (Q8N1L7),

N_2-N_2-dimethylguanosine tRNA methyltransferase (Q9NXH9), uncharacterized protein ZNF579 (A8MUW7), uncharacterized protein NPTXR (A6NDI5), tumor necrosis factor receptor superfamily member 21 (O75509), and others. Example spectra of acetonitrile precipitation CSF proteins are shown in Figure 14.

8 FIGURES AND TABLES

8.1 TABLE 1. SUBJECT STATISTICS

Pool X: Young healthy controls (without disc degeneration or low back pain)

Sample ID	*Age*	*Sex*	***VAS***
1026	27	M	0
1027	27	M	0
1128	26	F	3
1130	26	F	6
1132	25	F	0
1214	27	M	1
Average ± SEM	26.3 ± 0.8	3 M : 3 F	1.7 ± 1.0

Pool A: Age controlled subjects without disc degeneration or low back pain

Sample ID	*Age*	*Sex*	*VAS*	***TPC (µg/ml)***
1078 (2657)	41	F	0	255
1140	33	M	0	219
1170	44	M	0	338
1216	43	M	2	309
1219	43	F	0	228
1223	39	M	0	238
Average ± SEM	40.5 ± 4.1	4 M : 2 F	0.3 ± 0.3	264.5 ± 48.1

Pool B: Subjects with disc degeneration, but without low back pain

Sample ID	*Age*	*Sex*	*VAS*	***TPC (µg/ml)***
1028 (1615)	50	M	0	332
1137	50	M	0	317
1183	49	M	0	479
1201	49	F	0	301
1204	42	M	0	245
1209	59	F	0	218
1218	25	M	3	178
1240	43	M	0	239
Average ± SEM	45.9 ± 9.9	6 M : 2 F	0.4 ± 0.4	288.6 ± 93.1

Pool C: Subjects with both disc degeneration and low back pain

Sample ID	*Age*	*Sex*	*VAS*	***TPC (µg/ml)***
1022 (2328)	50	M	54	310
1106	43	F	81	266
1129	47	M	37	208
1187	36	F	88	374
1193	53	M	51	241
1194	37	F	55	232
1212	28	M	98	277
1173	36	M	65	307
Average ± SEM	41.3 ± 8.4	5 M : 3 F	66.1 ± 8.5	276.9 ± 52.9

8.2 TABLE 2. RATIOS OF STANDARD PEPTIDES

	Volume of equimolar labeled peptide (µl)				
Standard:	114	115	116	117	Total
A	2	6	10	14	32
B	14	2	6	10	32
C	10	14	2	6	32
D	6	10	14	2	32

Peptide standards A, B, C and D were created to test the accuracy of iTRAQ labels. Equivalent amounts of peptide were labeled, and different amount of these peptides were combined to create standards of different ratios.

8.3 TABLE 3. RATIOS OF STANDARD PROTEINS

	Volume of equimolar labeled protein (µl)				
Standard:	114	115	116	117	Total
E	10	20	30	40	100
F	20	10	5	1	36
G	8	2	12	6	28
H	30	15	10	5	60
I	32	53	43	48	176

Protein standards E, F, G, H, and I were created to test the accuracy of iTRAQ labels. Equivalent amounts of peptide were labeled, and different amount of these peptides were combined to create standards of different ratios.

8.4 TABLE 4. LIST OF OBSERVED PROTEINS IN IMMUNODEPLETED CSF

N	Unused ProtSc	Accession	Protein Name	Ratio DDD:Control	Ratio Painful DDD: control
1	24.38	P02649	Apolipoprotein E precursor	1 P=1	1.0702 P=0.0002
2	12.36	P02790	Hemopexin precursor	1.0763 P= 0.1832	1.1213 P=0.0407
3	10.12	P41222	Prostaglandin-H2 D-isomerase precursor	0.8572 P<0.0001	0.8703 P<0.0001
4	9.53	P01019	Angiotensinogen precursor	0.9885 P=0.7545	0.9513 P=0.2785
5	8.4	Q14515	SPARC-like protein 1 precursor	1.0177 P=0.6745	1.0323 P=0.5143
6	8.21	P01034	Cystatin-C precursor	1.0773 P=0.0002	1.1304 P<0.0001
7	7.92	P01011	Alpha-1-antichymotrypsin precursor	1.1789 P=0.0002	1.2712 P<0.0001
8	7.45	P05090	Apolipoprotein D precursor	1.0828 P=0.3151	1.1432 P=0.0319
9	7.35	Q12860	Contactin-1 precursor	1.0084 P=0.937	0.9936 P=0.9396
10	7.02	O00533	Neural cell adhesion molecule L1-like protein precursor	1.1348 P=0.035	1.1421 P=0.0566
11	6.4	P10909	Clusterin precursor	0.9976 P=0.9691	1.1071 P=0.0885
12	6.34	A8K1K1	cDNA FLJ76342, highly similar to Homo sapiens carnosine	0.9815 P=0.7491	1.0529 P=0.6598
13	6.32	Q9UBP4	Dickkopf-related protein 3 precursor	1.0569 P=0.4224	1.0581 P=0.3062
14	6.01	Q5T4F8	Cartilage acidic protein 1	0.9402 P=0.4825	1.0452 P=0.2486
15	5.81	P05067	Amyloid beta A4 protein precursor	1.1926 P=0.0588	1.1769 P=0.1105
16	5.78	P51693	Amyloid-like protein 1 precursor	1.1381 P=0.0175	1.1675 P=0.002
17	5.54	Q9UHG2	ProSAAS precursor	1.0832 P=0.0624	1.1687 P<0.0001
18	5.49	P06727	Apolipoprotein A-IV precursor	1.0415 P=0.54	1.1618 P=0.0231
19	5.19	P02765	Alpha-2-HS-glycoprotein precursor	0.943 P=0.6035	0.9409 P=0.4026
20	5.09	P02768	Serum albumin precursor	0.8212 P=0.0356	0.9607 P=0.6811
21	4.84	Q6H301	Kallikrein 6	1.0228 P=0.5505	1.0613 P=0.2335

22	4.58	P04217	Alpha-1B-glycoprotein precursor	0.95 P=0.1438	1.0188 P=0.5349
23	4.57	P00441	Superoxide dismutase	0.7843 P=0.0002	0.7785 P=0.0692
24	4.26	Q5UGI6	Serine/cysteine proteinase inhibitor clade G member 1 splice variant 2	0.8979 P=0.0461	0.9627 P=0.441
25	4.15	A8K021	cDNA FLJ78130, highly similar to Homo sapiens chromogranin B	1.0627 P=0.8014	1.0964 P=0.688
26	4.01	A2A2E1	Signal-regulatory protein alpha	1.011 P=0.8809	0.9461 P=0.3742
27	4	P13987	CD59 glycoprotein precursor	1.0008 P=0.9921	1.0392 P=0.6463
28	3.99	Q5T0I2	Gelsolin	1.1603 P=0.0334	1.1719 P=0.0075
29	3.92	Q6GTG1	Group-specific component	0.9573 P=0.4763	0.9831 P=0.6562
30	3.77	P08603	Complement factor H precursor	1.0867 P=0.4611	1.0936 P=0.3941
31	3.65	O43505	N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase	0.9976 P=0.9402	1.0593 P=0.1452
32	3.4	P10645	Chromogranin-A precursor	1.4407 P=0.0011	1.5071 P=0.0009
33	3.05	P08294	Extracellular superoxide dismutase [Cu-Zn] precursor	0.8923 P=0.0063	0.9291 P=0.1123
34	2.8	P01008	Antithrombin-III precursor	0.9639 P=0.5247	0.9591 P=0.3808
35	2.75	O94985	Calsyntenin-1 precursor	0.9631 P=0.0318	1.0105 P=0.6929
36	2.4	Q5JP67	B-factor, properdin	1.0023 P=0.9802	1.0467 P=0.6732
37	2.29	P30086	Phosphatidylethanolamine-binding protein 1	1.0797 P=0.2547	1.1166 P=0.3324
38	2.18	P02749	Beta-2-glycoprotein 1 precursor	1.0365 P=0.4418	1.0709 P=0.5667
39	2.14	P02760	AMBP protein precursor	0.9876 P=0.8527	1.0013 P=0.9836
40	2.14	P02452	Collagen alpha-1(I) chain precursor	0.9643 P=0.9211	1.0133 P=0.9734
41	2.09	Q96PQ0	VPS10 domain-containing receptor SorCS2 precursor	1.3647 P=0.3562	1.0401 P=0.8047
42	2.05	P04004	Vitronectin precursor	1.0634 P=0.613	1.2275 P=0.1875
43	2.04	P61769	Beta-2-microglobulin precursor	1.1788 P=0.091	1.2448 P=0.0499
44	2.04	Q68CP7	Putative uncharacterized protein DKFZp781G125	0.7256 P=0.127	1.3191 P=0.3898
45	2.03	Q2UY09	Collagen alpha-1(XXVIII) chain precursor	1.4578 P=0.1377	0.9548 P=0.8701
46	2.02	Q96IZ7	Arginine/serine-rich coiled-coil protein 1	1.0084 P=0.9923	1.1675

47	2.02	P10451	Osteopontin precursor	1.6927 P=0.1357	2.0399 P=0.1356
48	2.01	Q92831	Histone acetyltransferase PCAF	1.0451 P=0.8686	0.9234 P=0.7118
49	2.01	Q7Z532	Osteoglycin OG	0.9597 P=0.3824	0.9036 P=0.5372
50	2.01	P02750	Leucine-rich alpha-2-glycoprotein precursor	0.8592 P=0.2855	0.9456 P=0.1405
51	2	Q8TDB2	Transthyretin amyloidosis variant D38V	0.9512 P=0.5949	1.0393 P=0.1804
52	2	Q9UDW8	WUGSC:H_DJ0747G18.3 protein	1.9698 P=0.1009	1.2768 P=0.5496
53	2	P55290	Cadherin-13 precursor	0.8877	0.9715
54	2	P05408	Neuroendocrine protein 7B2 precursor	0.8859	0.9526
55	2	Q13449	Limbic system-associated membrane protein precursor	0.9327	0.8852
56	2	P07339	Cathepsin D precursor	0.7672	0.9981
57	1.98	Q59F90	Brevican isoform 1 variant	0.9239 P=0.4215	0.9146 P=0.3759
58	1.73	A4D0S3	Neuronal cell adhesion molecule	0.9873 P=0.7744	1.0071 P=0.8429
59	1.71	A8UHA1	Autotaxin isoform gamma	0.8591 P=0.0874	0.8512 P=0.1459
60	1.7	Q96SL8	Flt3-interacting zinc finger protein 1	1.1097	1.3086
61	1.62	P13592	Neural cell adhesion molecule 1, 120 kDa isoform precursor	0.9218 P=0.5822	0.91 P=0.3854
62	1.53	A3KFI4	Neuroblastoma, suppression of tumorigenicity 1	0.9751 P=0.855	1.0784 P=0.5121
63	1.49	P36955	Pigment epithelium-derived factor precursor	0.766 P=0.4572	0.7445 P=0.2127
64	1.44	A7E2D6	NAV2 protein	0.896 P=0.2085	0.9654 P=0.5932
65	1.43	Q5SVQ8	Zinc finger and BTB domain-containing protein 41	0.8843 P=0.7511	0.935 P=0.853
66	1.42	Q8N392	Rho GTPase-activating protein 18	1.4147 P=0.3004	1.3521 P=0.3598
67	1.41	Q6NV74	Uncharacterized protein C2orf55	0.9036 P=0.0147	1.0972 P=0.2396
68	1.4	P02751	Fibronectin precursor	1.135 P=0.3472	1.3152 P=0.0143
69	1.4	Q8WXD2	Secretogranin-3 precursor	0.932	1.2176
70	1.4	Q8N849	cDNA FLJ40018 fis, clone STOMA2006398	1.1281 P=0.4983	1.0785 P=0.8243
71	1.35	Q99460	26S proteasome non-ATPase regulatory subunit 1	0.8681 P=0.5421	0.8276 P=0.1927

72	1.31	Q59GK9	Ribosomal protein L21 variant	1.0652 P=0.493	1.7488 P=0.2932
73	1.19	P01042	Kininogen-1 precursor	0.9257 P=0.5767	0.9575 P=0.7582
74	1.13	A8K006	cDNA FLJ76199, highly similar to Homo sapiens phospholipid transfer	1.7352 P=0.2997	1.458 P=0.1554
75	1.03	A6NNA2	Uncharacterized protein ENSP00000373454	0.9656 P=0.7015	1.0973 P=0.5435
76	1.03	O15085	Rho guanine nucleotide exchange factor 11	0.8561 P=0.0678	0.8526 P=0.2209
77	1.02	Q59EN5	Prosaposin variant	0.875 P=0.2736	0.9078 P=0.3542
78	1.01	Q8IUE0	Homeobox protein TGIF2LY	1.035 P=0.925	1.0599 P=0.5279
79	1.01	Q17R45	Calcium channel, voltage-dependent, alpha 2/delta subunit 1	0.9327 P=0.8181	1.0071 P=0.9431
80	1.01	P41162	ETS translocation variant 3	0.9612 P=0.8219	0.5515 P=0.2155
81	1.01	Q6ZRH9	Uncharacterized protein FLJ46347	1.0273 P=0.9247	1.2469 P=0.446
82	1	Q5T7W7	Uncharacterized protein C9orf97	2.2274	1.5423
83	1	A1L305	DLEC1 protein	1.0861 P=0.4031	0.9769 P=0.9103

Ratio DDD:control compares the relative amount of CSF protein of those with asymptomatic degenerative disc disease to healthy controls. Ratio painful DDD:control compares the relative amount of CSF protein of those with degenerative disc disease and low back pain, to healthy controls. P-value denotes the probability that the ratio was different from 1, as determined by t-test.

Legend:

Red	Protein was significantly increased. P-value of 0.001 or lower.
Fushia	Protein was significantly increased. P-value of 0.01 or lower.
Light pink	Protein was significantly increased. P-value of 0.05 or lower.
White	Protein was not significantly changed. P-value was greater than 0.05.
Light blue	Protein was significantly decreased. P-value of 0.05 or lower.
Blue	Protein was significantly decreased. P-value of 0.01 or lower.
Dark blue	Protein was significantly decreased. P-value of 0.001 or lower.

8.5 TABLE 5. LIST OF OBSERVED PROTEINS IN ACETONITRILE PRECIPITATED CSF

N	ProtScore	Accession	Protein Name	Ratio DDD:control	Ratio Painful DDD:control
1	14	P01034	Cystatin-C precursor	1.1839 P=0.0001	1.205 P<0
2	12	P02768	Serum albumin precursor	0.8091 P<0	0.9024 P<0
3	9.64	P41222	Prostaglandin-H2 D-isomerase precursor	0.919 P=0.0265	0.9569 P=0.383
4	9.58	Q9UHG2	ProSAAS precursor	1.0636 P=0.2777	1.1491 P=0.0057
5	8.13	P05090	Apolipoprotein D precursor	0.9217 P=0.1486	0.989 P=0.7917
6	7.62	A8K021	Chromogranin B	0.9844 P=0.8123	0.9735 P=0.836
7	6.98	Q68CK4	Leucine-rich alpha-2-glycoprotein	1.0377 P=0.7491	1.0562 P=0.5787
8	4.13	Q9UDW8	WUGSC:H_DJ0747G18.3 protein	0.8348 P=0.0781	1.1025 P=0.7335
9	4.02	Q9Y355	Apolipoprotein A1	0.6534 P=0.0266	1.0286 P=0.8157
10	4.01	Q5T539	Orosomucoid 1	0.4907 P=0.002	0.8394 P=0.2591
11	4.01	Q8IWU5	Extracellular sulfatase Sulf-2 precursor	1.3042 P=0.3928	1.187 P=0.3355
12	3.02	P02652	Apolipoprotein A-II precursor	0.8453 P=0.0584	1.0282 P=0.6387
13	2.59	P02649	Apolipoprotein E precursor	1.0528 P=0.2472	0.9955 P=0.8904
14	2.52	P01344	Insulin-like growth factor II precursor	0.9917 P=0.9303	1.228 P=0.0138
15	2.4	Q6FGG5	SCRG1 protein	1.0534 P=0.610	1.1252 P=0.451
16	2.36	P61769	Beta-2-microglobulin precursor	1.0194 P=0.8702	1.0631 P=0.3324
17	2.32	P51693	Amyloid-like protein 1 precursor	1.1454 P=0.4333	1.2547 P=0.2073
18	2.29	Q14525	Keratin, type I cuticular Ha3-II	0.5977 P=0.2242	1.0797 P=0.7059
19	2.18	Q2HXI4	Protein tyrosine phosphatase receptor type D	1.1549 P=0.6974	0.2561 P=0.2847
20	2.13	P02765	Alpha-2-HS-glycoprotein precursor	0.6661 P=0.0348	0.7542 P=0.2476
21	2.11	Q96KM6	Zinc finger protein 512B	0.7011 P=0.4989	0.684 P=0.3728
22	2.1	Q549C7	Transthyretin precursor	1.278 P=0.0869	1.151 P=0.1872

				0.9361	1.1737
23	2.05	P10451	Osteopontin precursor		
				0.8824	0.8871
24	2.03	P58417	Neurexophilin-1 precursor		
			Rho guanine nucleotide exchange	0.7959	0.027
25	2.02	O15085	factor 11		
				1.0862	1.2929
26	2.01	Q53FJ5	Prosaposin	P=0.1954	P=0.034
				0.937	1.0465
27	2.01	A3KPE2	Apolipoprotein C-III	P=0.7609	P=0.8271
				1.0581	1.3699
28	2.01	O95521	PRAME family member 1		
				1.0238	1.0894
29	2	Q6FHD0	SGNE1 protein	P=0.3982	P=0.5568
				1.1455	0.9886
30	2	Q6FHL8	CTGF protein	P=0.8487	P=0.9863
				0.9231	0.9934
31	2	Q6JIA6	Kallikrein 6 variant 4	P=0.095	P=0.8666
				1.2724	1.1623
32	2.65	Q8WZ42	Titin	P=0.2813	P=0.4656
				0.9206	0.1505
33	1.46	Q8WXI7	Mucin-16	P=0.8944	P=0.2451
				1.6232	1.243
34	1.42	Q06481	Amyloid-like protein 2 precursor	P=0.2568	P=0.0391
				1.1672	
35	1.41	A8K2W6	phospholipase A2, group VII		
				1.2646	
36	1.41	Q71RD3	PP11517		
				0.7844	0.9388
37	1.42	P05067	Amyloid beta A4 protein precursor		
				0.9841	1.053
38	1.4	A6NCP9	Uncharacterized protein RBP4		
			cDNA FLJ40039 fis, clone	0.9579	1.0274
39	1.4	Q8N1L7	SYNOV2000397		
			N(2),N(2)-dimethylguanosine tRNA	1.3695	1.3821
40	1.31	Q9NXH9	methyltransferase	P=0.0084	P=0.2816
				1.5881	
41	1.24	A8MUW7	Uncharacterized protein ZNF579		
			DNA-directed RNA polymerase,	2.0874	0.9126
42	1.23	O00411	mitochondrial precursor		
			Xin actin-binding repeat-containing	1.3216	1.563
43	1.2	A4UGR9	protein 2		
			ATP-binding cassette sub-family A	2.5972	
44	1.08	Q8IZY2	member 7		
				0.9511	0.9267
45	1.07	Q5U5U6	Ubiquitin B	P=0.6288	P=0.4121
				0.4257	0.4602
46	1.07	A8K052	alpha-1-B glycoprotein	P=0.2118	P=0.1189
				1.1893	1.0905
47	1.06	O94910	Latrophilin-1 precursor		

48	1.06	Q9H2U1	Probable ATP-dependent RNA helicase DHX36	1.2231	1.1272
49	1.05	A6NDI5	Uncharacterized protein NPTXR	0.8055 P=0.1826	0.826 P=0.2771
50	1.03	Q92830	General control of amino acid synthesis protein 5-like 2	1.2444 P=0.6139	1.165 P=0.5872
51	1.02	Q96FC9	Probable ATP-dependent RNA helicase DDX11	0.6982 P=0.5268	1.1212 P=0.3255
52	1.01	O75509	Tumor necrosis factor receptor superfamily member 21 precursor	1.0512	1.1628

Ratio DDD:control compares the relative amount of CSF protein of those with asymptomatic degenerative disc disease to healthy controls. Ratio painful DDD:control compares the relative amount of CSF protein of those with degenerative disc disease and low back pain, to healthy controls. P-value denotes the probability that the ratio was different from 1, as determined by t-test.

Legend:

Red	Protein was significantly increased. P-value of 0.001 or lower.
Fushia	Protein was significantly increased. P-value of 0.01 or lower.
Light pink	Protein was significantly increased. P-value of 0.05 or lower.
White	Protein was not significantly changed. P-value was greater than 0.05.
Light blue	Protein was significantly decreased. P-value of 0.05 or lower.
Blue	Protein was significantly decreased. P-value of 0.01 or lower.
Dark blue	Protein was significantly decreased. P-value of 0.001 or lower.

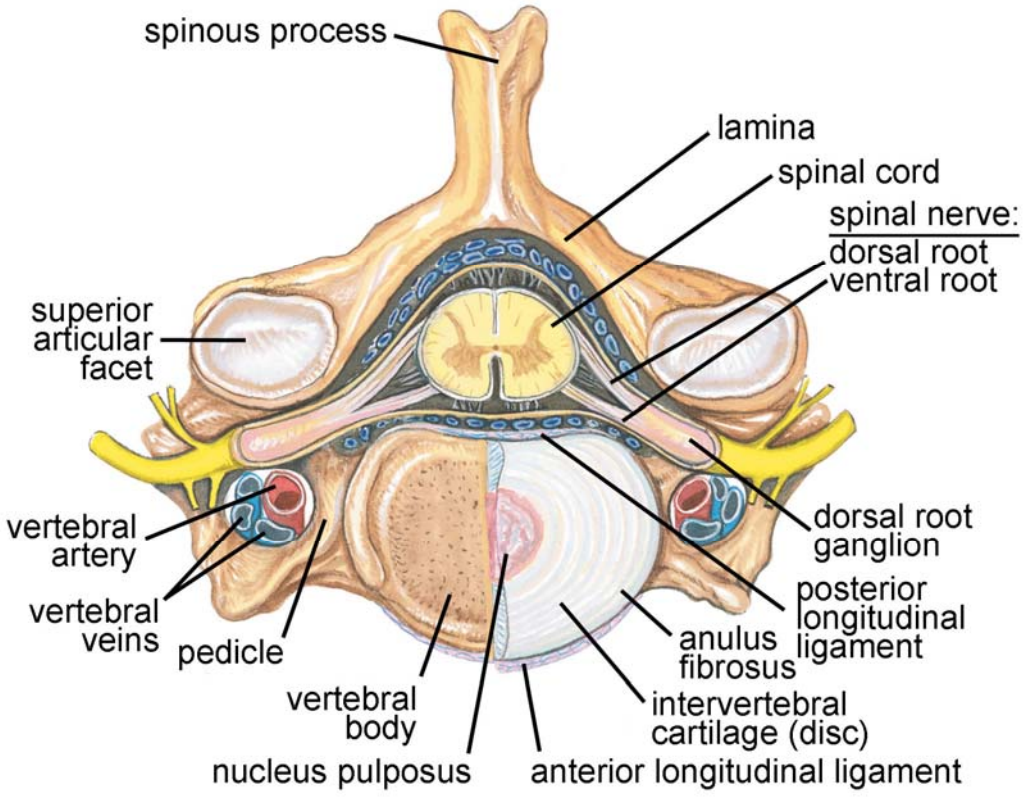
8.6 TABLE 6. SUMMARY OF DIFFERENTIALLY EXPRESSED PROTEINS IN IMMUNODEPLETED AND PRECIPITATED CSF

		ProteoPrep 20		*Immunodepletion*	
Protein Name	Possible functions	DDD	Painful DDD	DDD	Painful DDD
Apolipoprotein A-IV	Nerve damage and repair	-	↑	-	-
Apolipoprotein D	Nerve damage and repair	-	↑	-	-
Apoplipoprotein E	Nerve damage and repair	-	↑	-	-
Hemopexin	Nerve damage and repair	-	↑	ND	ND
Neural cell adhesion molecule L1-like protein	Nerve damage and repair	↑	-	ND	ND
Insulin-like growth factor II	Nerve damage and repair	ND	ND	-	↑
Amyloid-like protein 2	Nerve damage and repair	ND	ND	-	↑
Prosaposin	Nerve damage and repair	ND	ND	-	↑
ProSAAS precursor	Neuropeptide processing	-	↑	-	↑
Prostaglanin H2 D-isomerase	Inflammation	↓	↓	↓	-
α-1-antichymotrypsin precursor	Inflammation	↑	↑	ND	ND
Orosomucoid	Inflammation	ND	ND	↓	-
Gelsolin	Inflammation	↑	↑	ND	ND
Chromogranin A	Inflammation	↑	↑	ND	ND
β-2-microglobulin	Inflammation	-	↑	-	-
α-2-HS-glycoprotein	Inflammation	-	-	↓	-
Serine/cysteine proteinase inhibitor clade G member 1 splice variant 2	Inflammation	↓	-	ND	ND
Superoxide dismutase	Oxidative stress	↓	-	ND	ND

Amyloid-like protein 1 precursor	Oxidative stress	↑	↑	-	-
Cystatin C	Tissue remodeling	↑	↑	↑	↑
Fibronectin	Tissue remodeling	-	↑	ND	ND
Calsyntenin-1	Unknown	↓	-	ND	ND
N_2,N_2-dimethylguanosine tRNA methyltransferase	Unknown	ND	ND	↓	-
Uncharacterized protein C2orf55	Unknown	↓	-	ND	ND

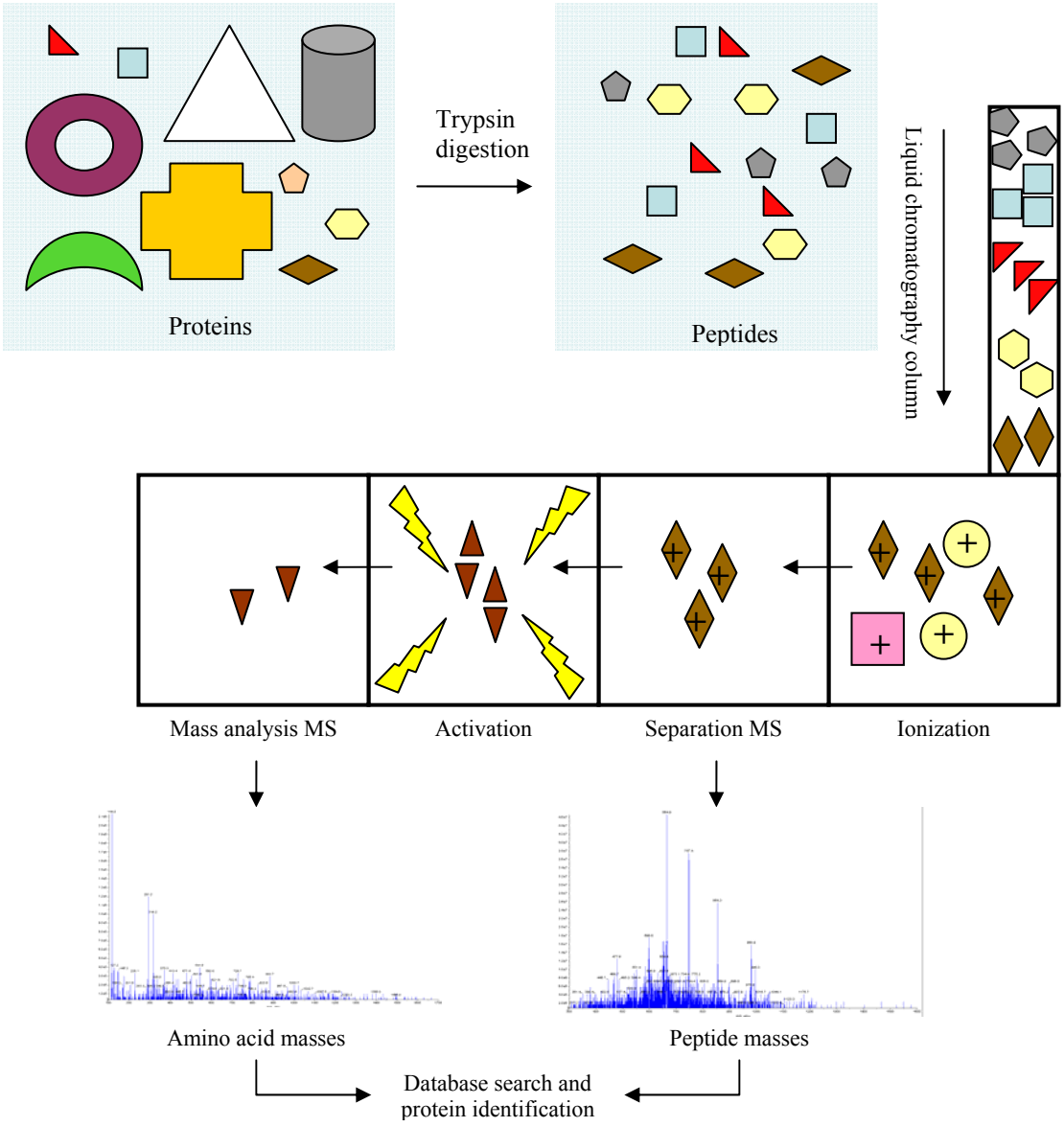
This table summarizes proteomic differences observed in both the proteoprep 20 immunodepletion experiment and the acetonitrile precipitation experiment. Proteins which were not differentially expressed are not shown. The columns 'DDD' denote the direction (increase, decrease, no change, or not detected) of asymptomatic degenerative disc disease CSF protein levels when compared to the control group. Similarly, the columns 'painful DDD' denote the direction (increase, decrease, no change, or not detected) of painful degenerative disc disease CSF protein levels when compared to the control group.

8.7 FIGURE 1. SCHEMATIC OF THE INTERVERTEBRAL DISC AND SURROUNDING STRUCTURES



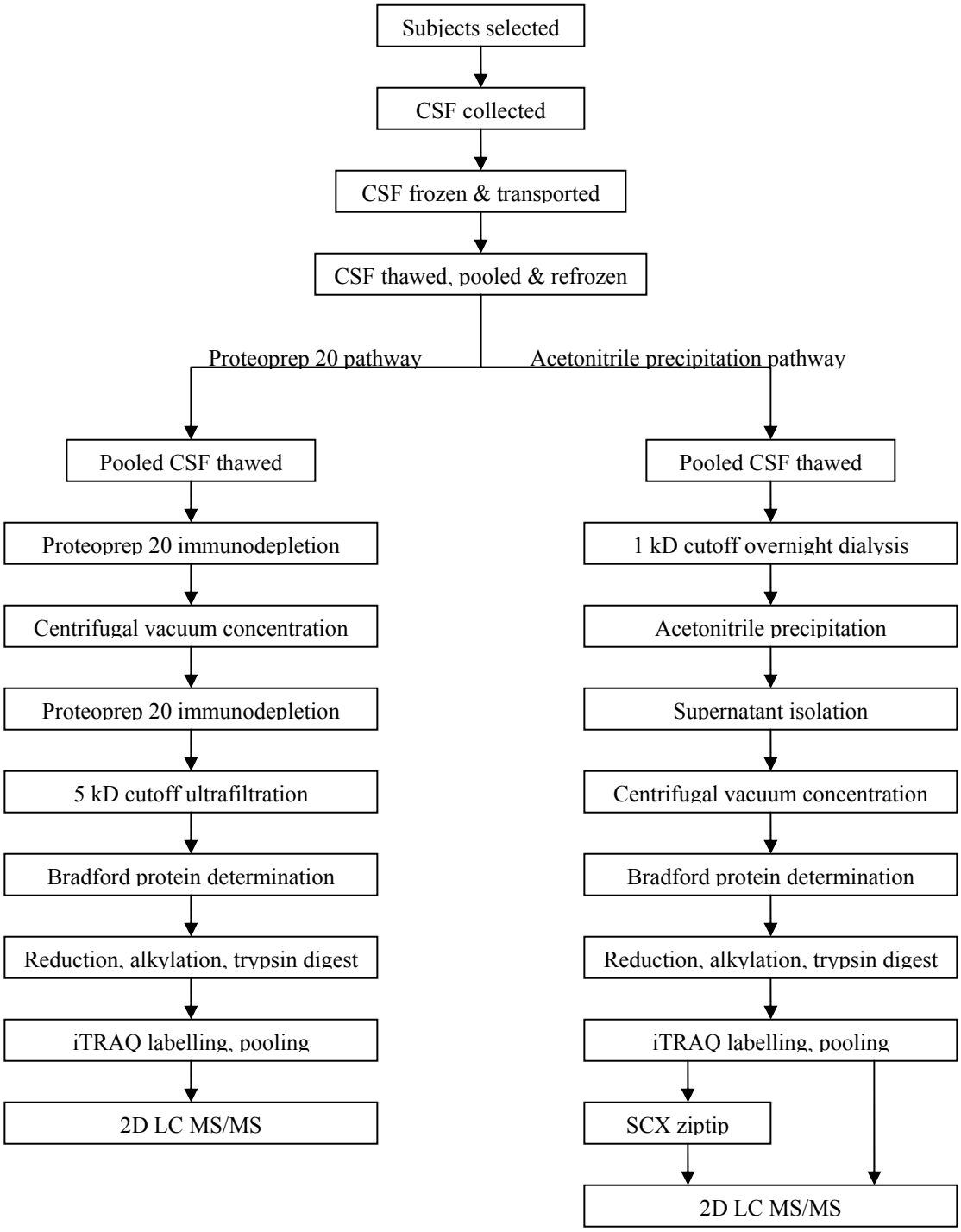
The intervertebral disc consists of the gelatonous nucleus pulposus, and the fibrous annulus fibrosus. In this figure it has been bisected to show the underlying vertebral body. Adapted from http://www.t-hesselberg.dk

8.8 FIGURE 2. IDENTIFICATION OF PROTEINS BY TANDEM MASS SPECTROMETRY

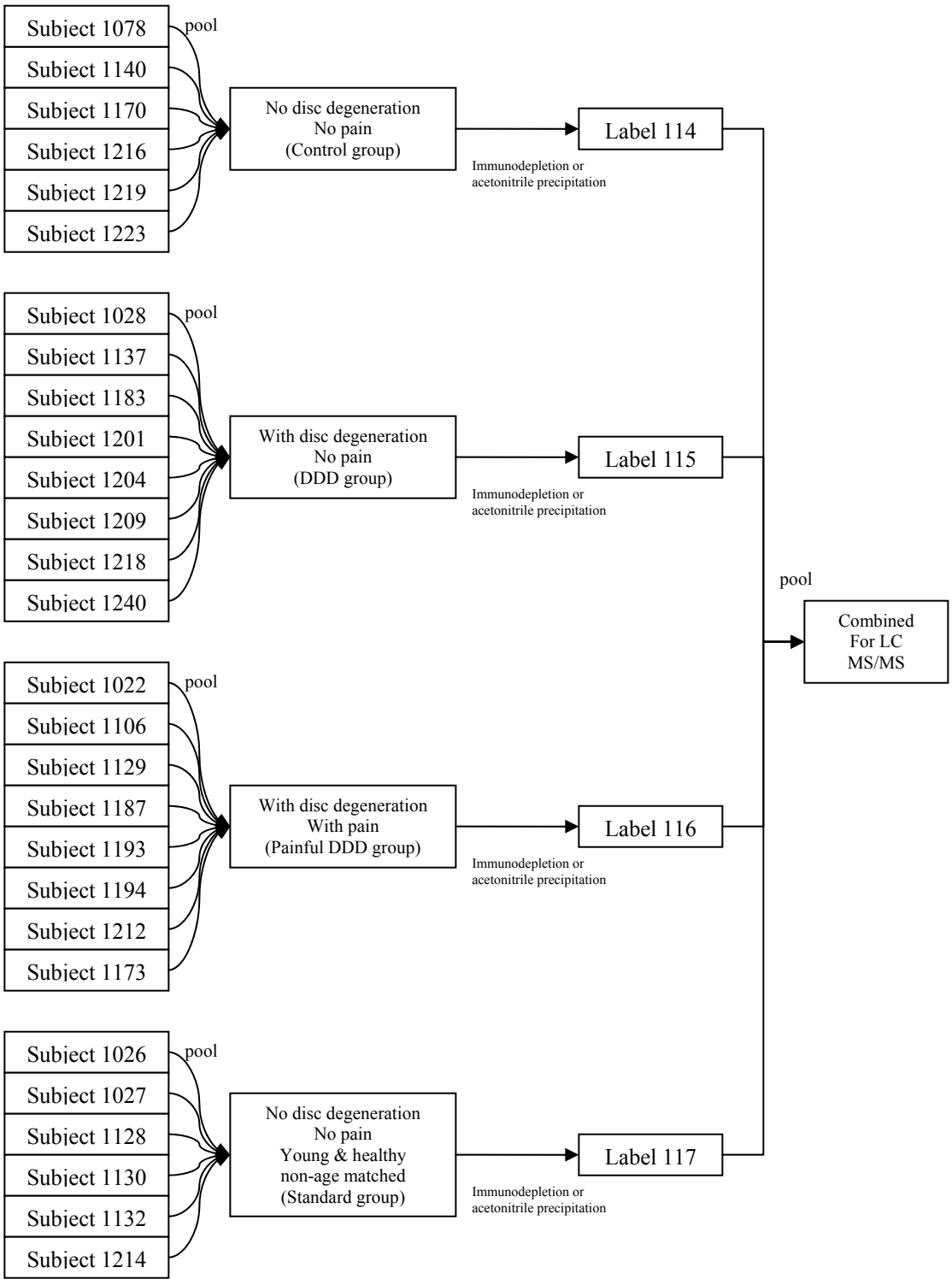


Proteins are digested into peptide fragments. The peptide fragments are then separated by liquid chromatography. Peptides are then ionized and the mass of the peptide determined. Next the peptides are fragmented and the mass of the fragments are obtained. Using algorithms, a search engine and a protein database, the combination of peptide fragments and the intact peptide masses is used to identify the protein.

8.9 FIGURE 3. WORKFLOW OF 2D LC MS/MS EXPERIMENTS

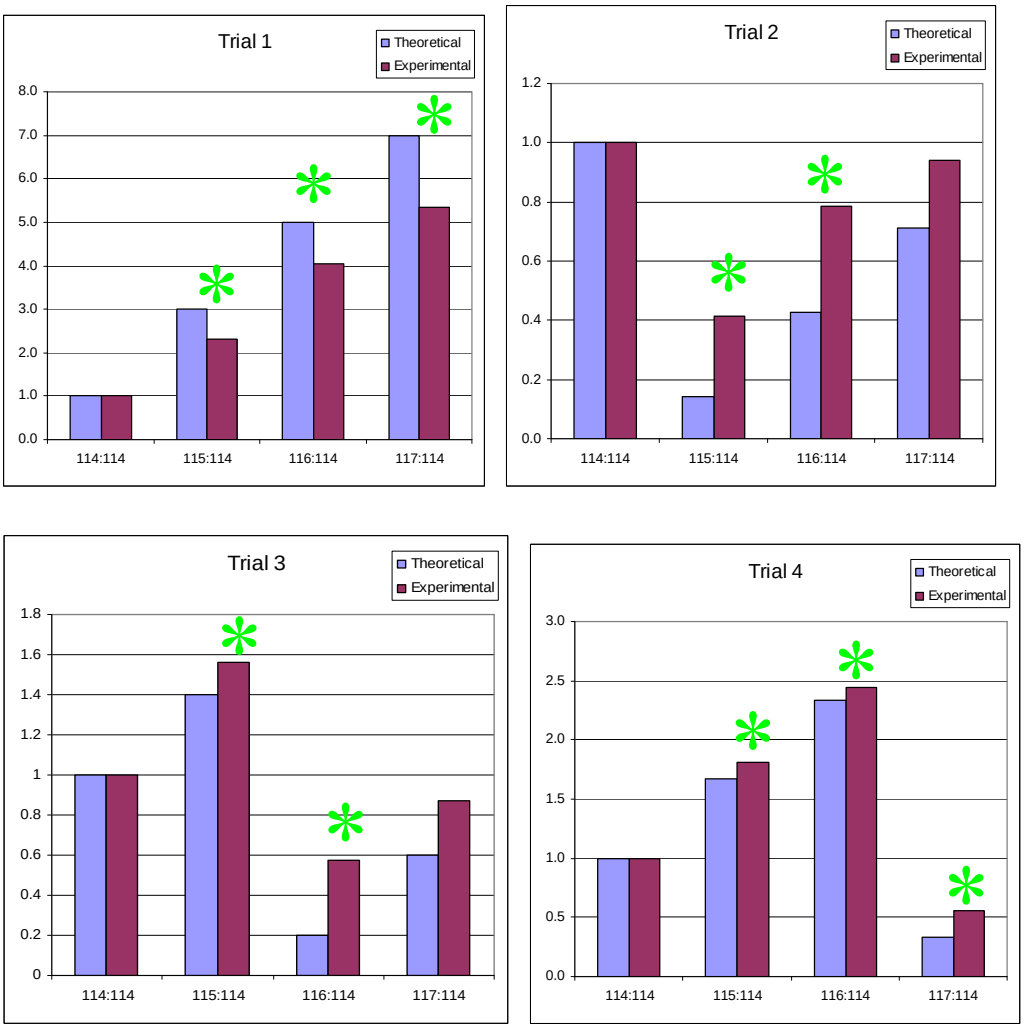


8.10 FIGURE 4. POOLING OF SAMPLES



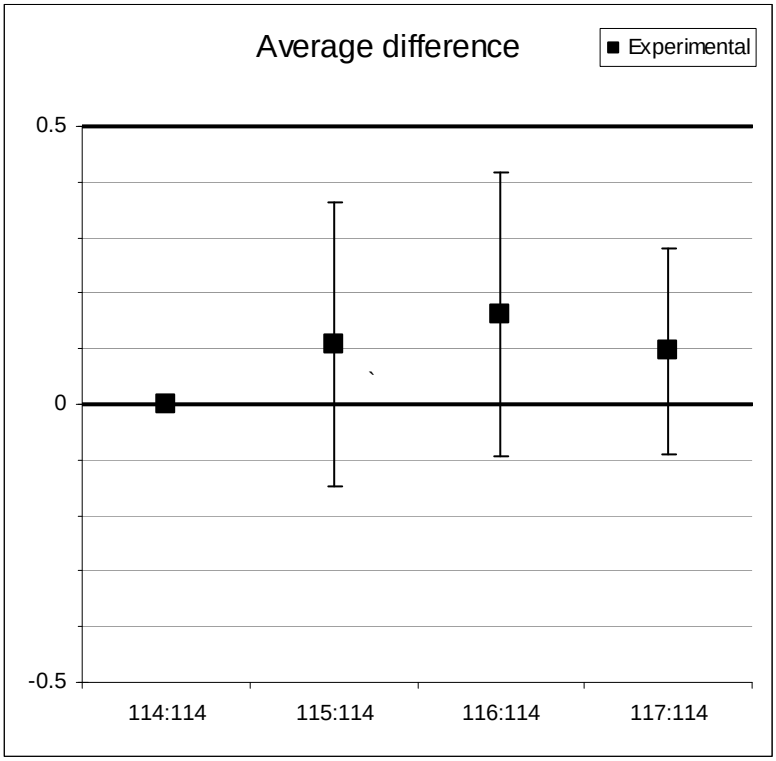
8.11 FIGURE 5. iTRAQ RATIOS FOR STANDARD PEPTIDE MIX

A



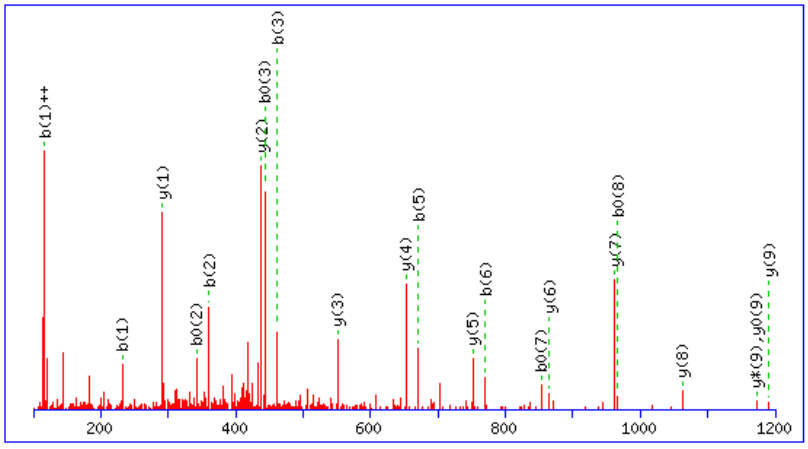
The average experimentally obtained ratios were plotted with theoretical ratios. All ratios were normalized to label 114, which was designated as a ratio of 1. * denotes that peptides in this group were significantly different from 114:114, or a ratio of 1.

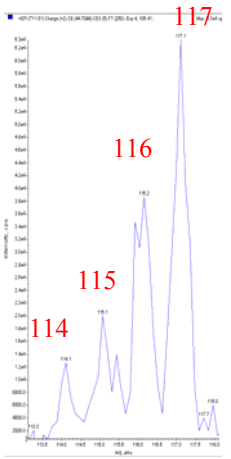
B



The average deviation from expected values (± SD) of the peptide ratios when normalized to label 114 was plotted in logarithmic$_{10}$ space.

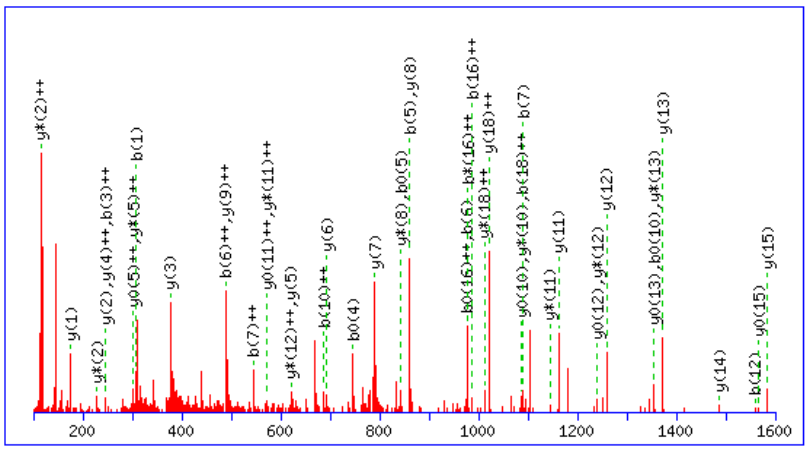
8.12 FIGURE 6. EXAMPLE SPECTRA OF iTRAQ LABELED STANDARD PEPTIDES

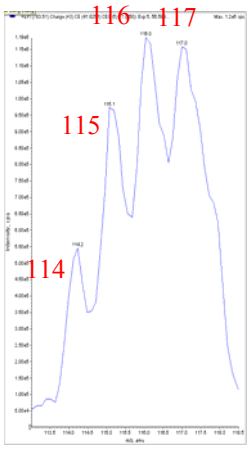




Peptide: SQTPLVTLFK
Name: β-endorphin (AA #10 - 19)
Mascot ion score: 79
Source: 1_120308SpikePeptidesDigiTRAQ

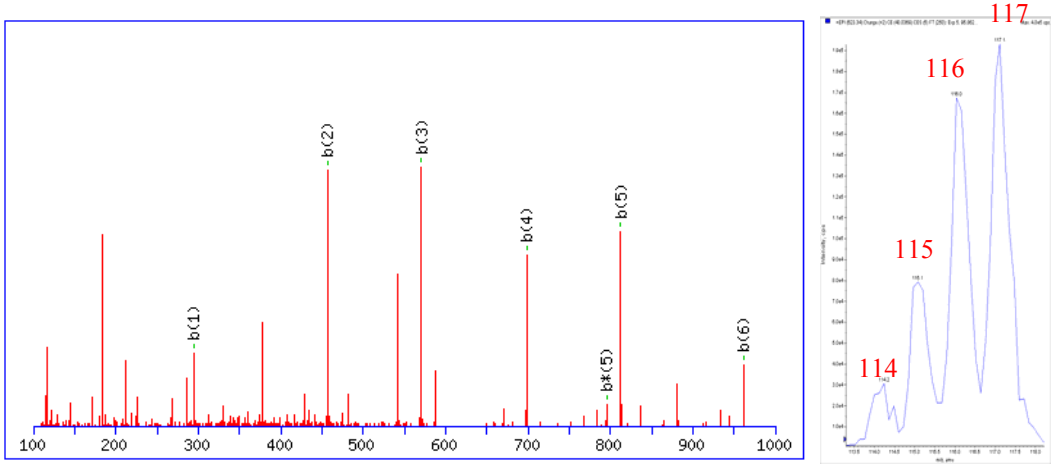
Theoretical ratio: 1 / 3 / 5 / 7
Experimental ratio: 1 / 2.3 / 4.2 / 5.9





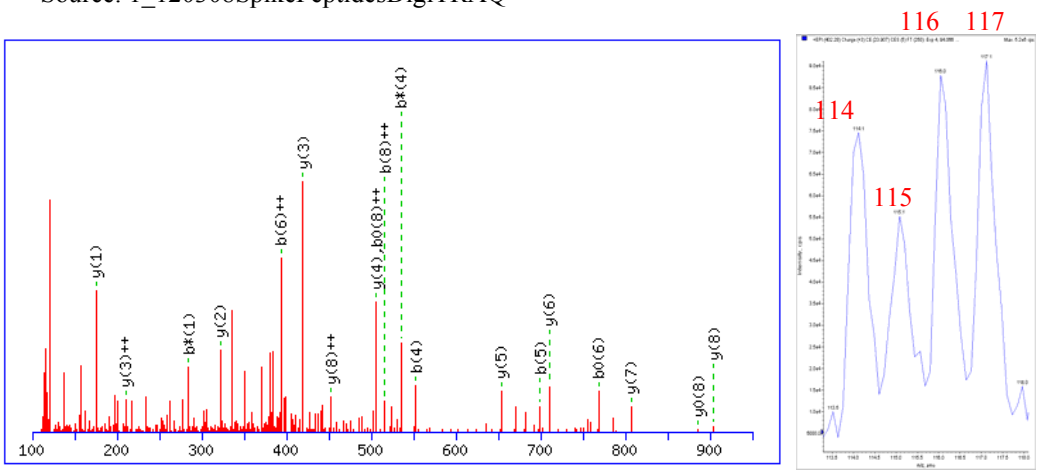
Peptide: YPSKPDNPGEDAPAEDMAR
Name : Neuropeptide Y (AA #1 - 19)
Mascot ion score : 55
Source: 1_120308SpikePeptidesDigiTRAQ

Theoretical ratio: 1 / 3 / 5 / 7
Experimental ratio: 1 / 2.2 / 3.6 / 5.1



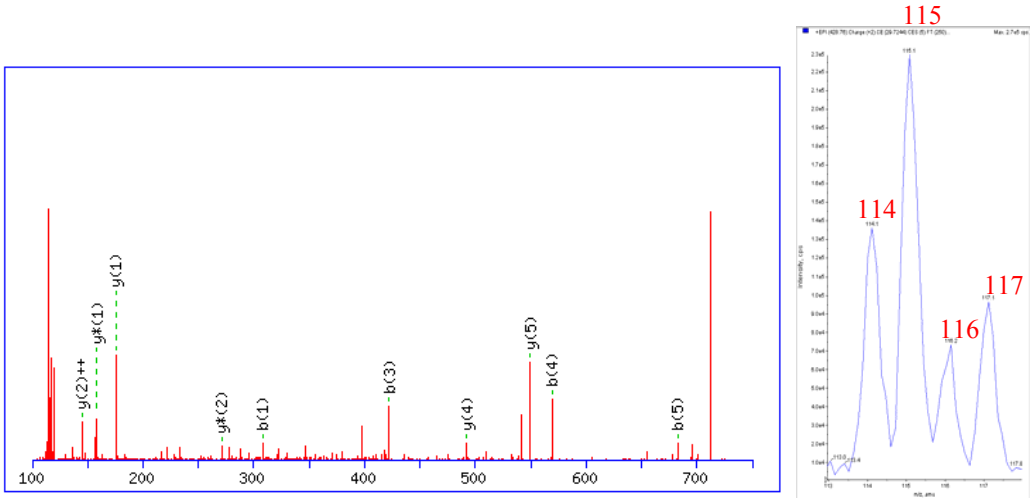
Peptide: CYIQNCPLG
Name: Oxytocin (AA #1 - 9)
Mascot ion score: 49
Source: 1_120308SpikePeptidesDigiTRAQ

Theoretical ratio: 1 / 3 / 5 / 7
Experimental ratio: 1 / 2.6 / 4.2 / 5.6



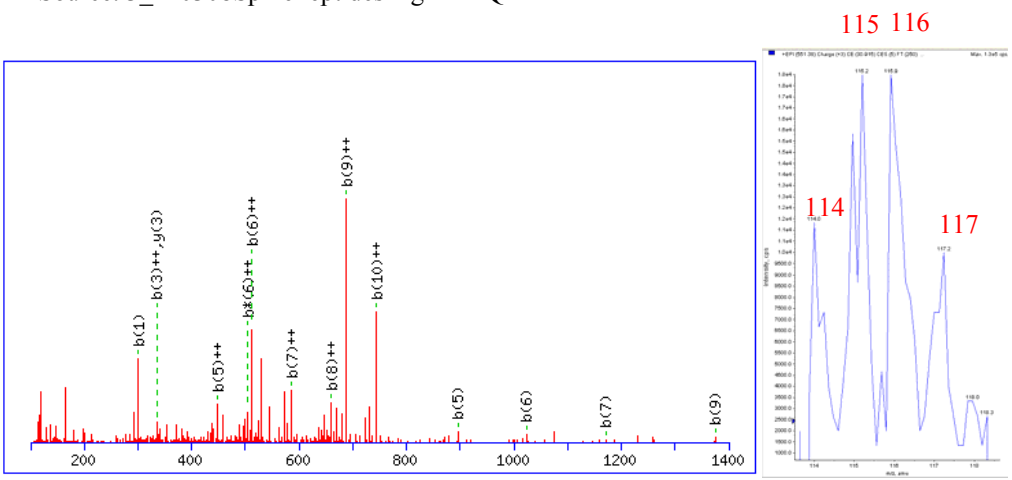
Peptide: RPPGFSPFR
Name: Bradykinin (AA #2 - 9)
Mascot ion score: 69
Source: 2_120308SpikePeptidesDigiTRAQ

Theoretical ratio: 1 / 0.14 / 0.33 / 0.20
Experimental ratio: 1 / 0.56 / 1.03 / 1.15



Peptide: YGGFLR
Name: Dynorphin A (AA #1-6)
Mascot ion score: 26
Source: 3_120308SpikePeptidesDigiTRAQ

Theoretical ratio: 1 / 3.40 / 0.20 / 0.60
Experimental ratio: 1 / 1.57 / 0.46 / 0.78

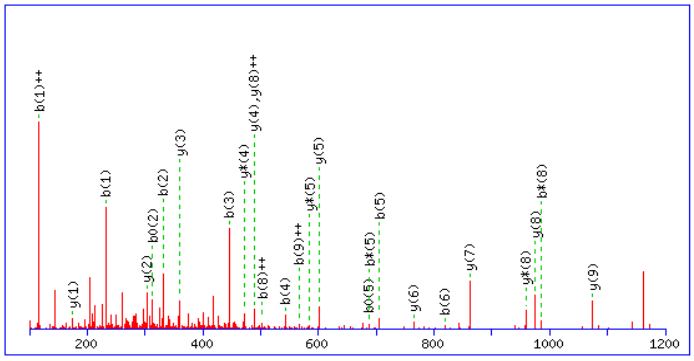


Peptide: RPKPQQFFGLM
Name: Substance P (AA #1-11)
Mascot ion score: 30
Souce : 4_120308SpikePeptidesDigiTRAQ

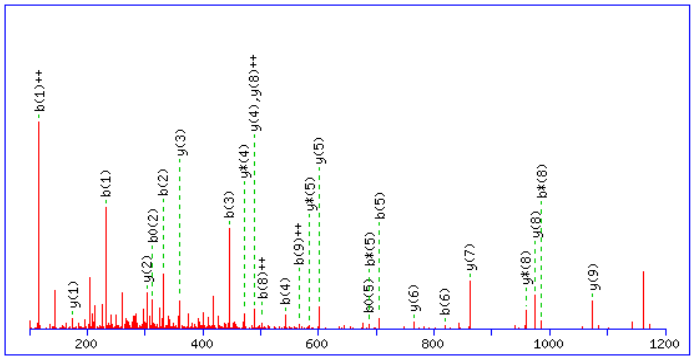
Theoretical ratio: 1 / 1.67 / 2.33 / 0.33
Experimental ratio: 1 / 2.20 / 2.93 / 0.74

Example spectra from the dataset X X_120308SpikePeptidesDigiTRAQ are displayed. These peptides were identified when the mass spectra from standard peptides, searched on the UniProt database.

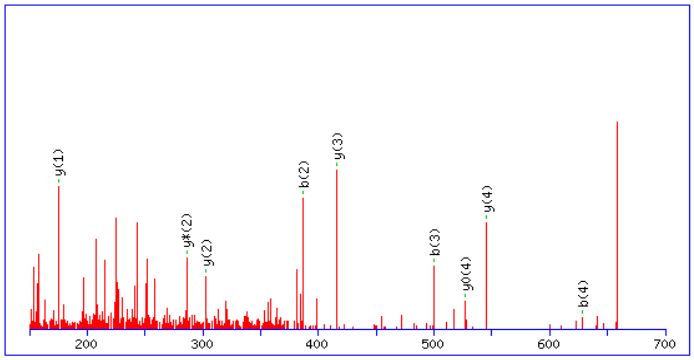
8.13 FIGURE 7. EXAMPLE SPECTRA OF < 10 kD CSF PEPTIDES



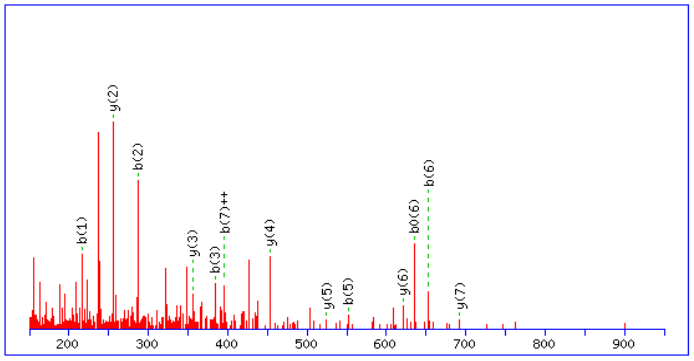
WAEGGGHSR
Secreteogranin-1
Mascot ion score: 41
1_061207CSF10kDfilterWITH digestiTRAQ
Retention time: 20.96



SVNPYLQGQR
Secreteogranin-5
Mascot ion score: 67
1_061207CSF10kDfilterWI THdigestiTRAQ
Retention time: 35.02



LELQR
Somatostatin
Mascot ion score: 23
1_061207CSF10kDfilterWI THdigestiTRAQ
Retention time: 33.41

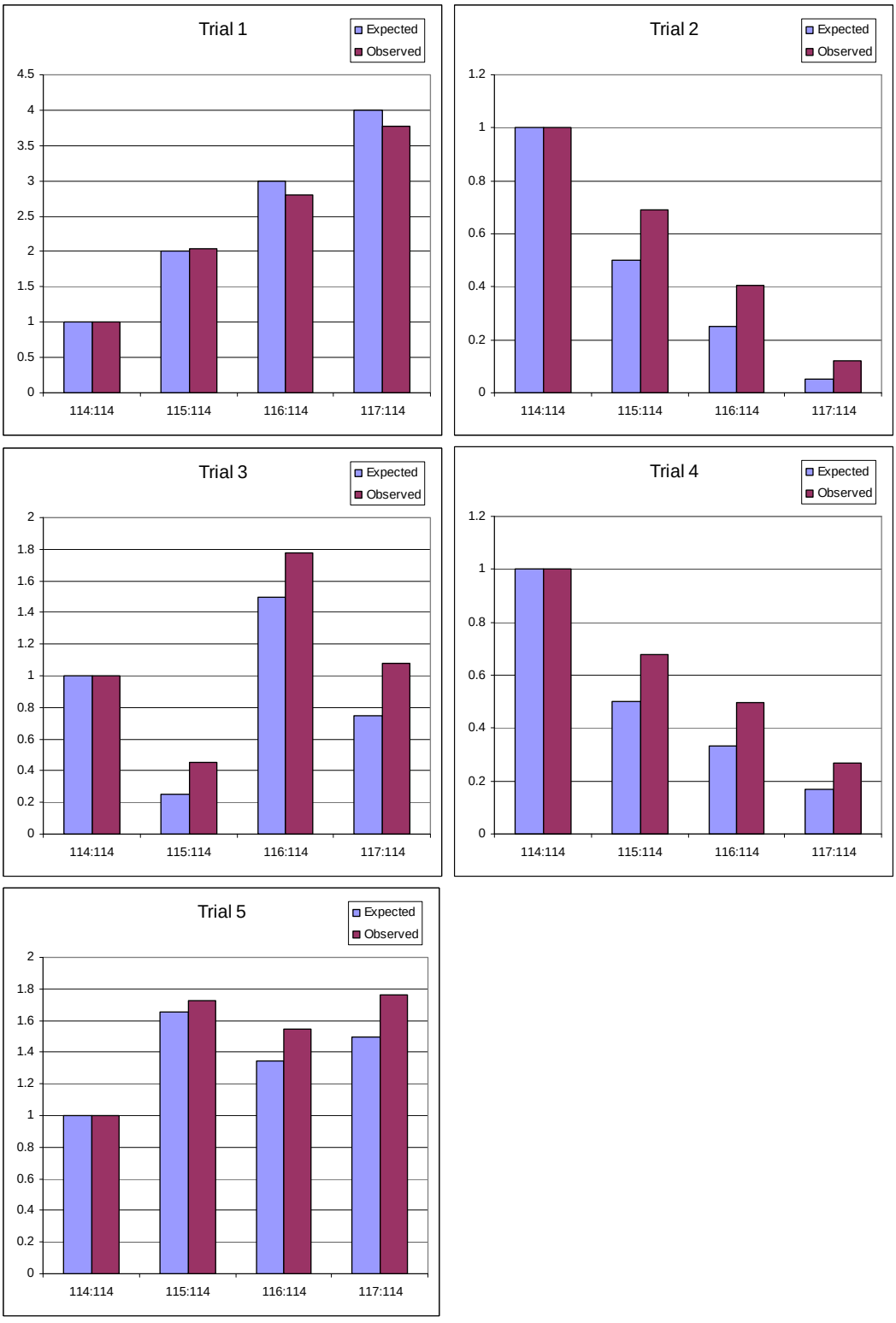


AAPAPTHV
VGF nerve growth factor inducible
Mascot ion score: 28
1_061207CSF10kDfilterWI THdigestiTRAQ
Retention time: 24.14

Example spectra from the dataset 1_061207CSF10kDfilterWITHdigestiTRAQ are displayed. These peptides were identified when the mass spectra from 10 kD CSF filtrate were searched on the SwePep database.

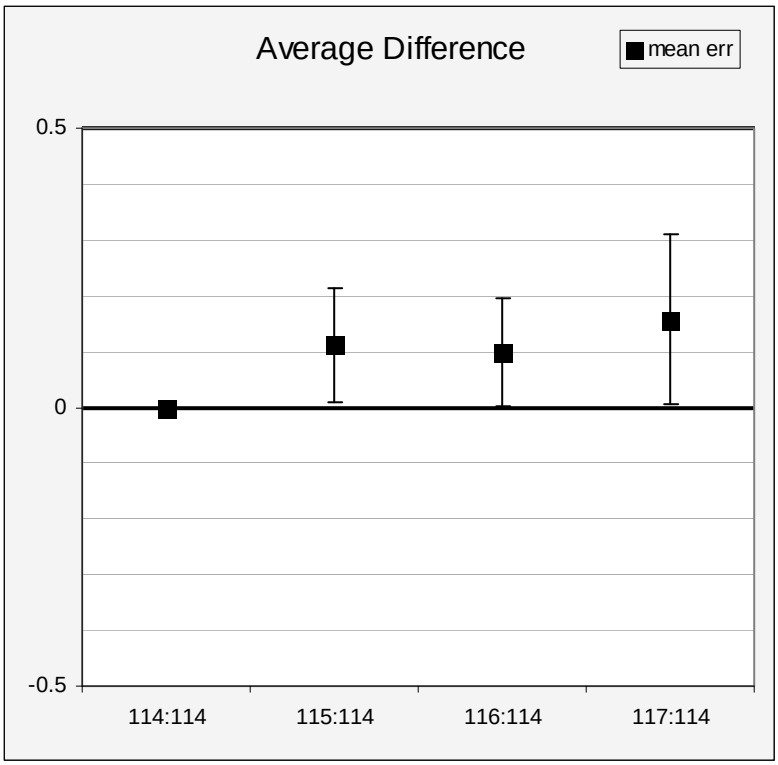
8.14 FIGURE 8. iTRAQ RATIOS FOR STANDARD PROTEINS

A



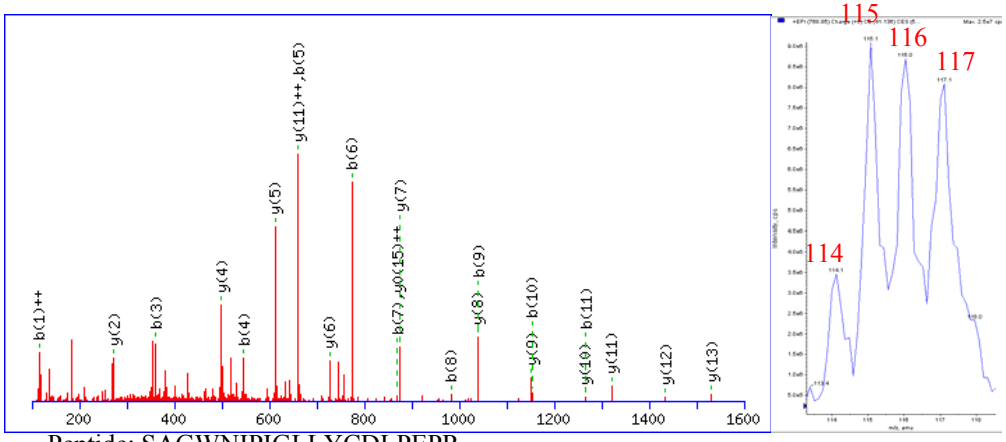
The average experimentally obtained ratios were plotted with theoretical ratios. All ratios were normalized to label 114, which was designated as a ratio of 1.

B



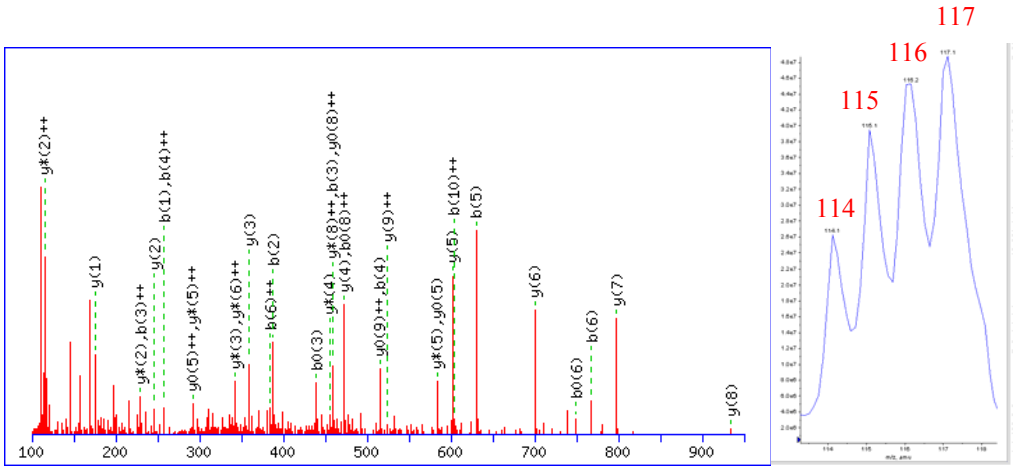
The average deviation from expected values (± SD) of the peptide ratios when normalized to label 114 was plotted in logarithmic$_{10}$ space.

8.15 FIGURE 9. EXAMPLE SPECTRA OF iTRAQ LABELED STANDARD PROTEINS



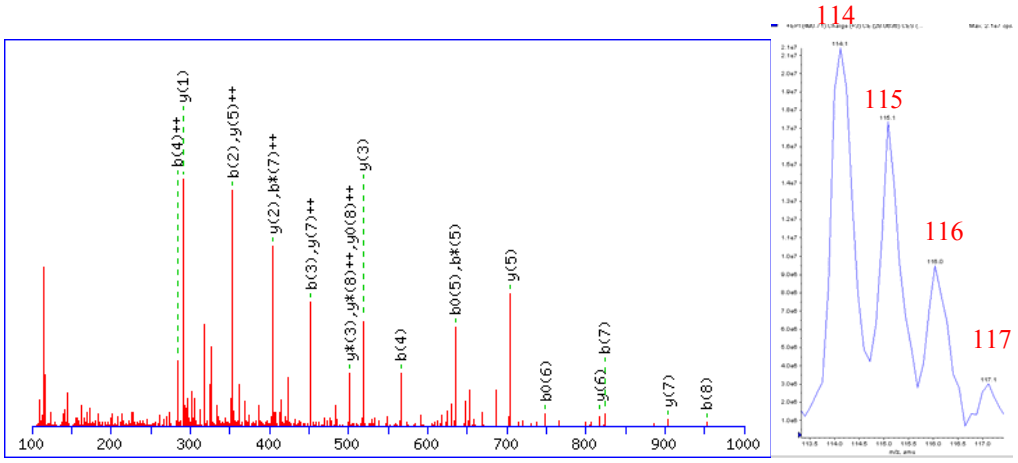
Peptide: SAGWNIPIGLLYCDLPEPR
Name: Transferrin
Mascot ion score: 90
Source: 1_110408protmixiTRAQ

Theoretical ratio: 1 / 3 / 5 / 7
Experimental ratio: 1 / 1.8 / 2.4 / 3.1



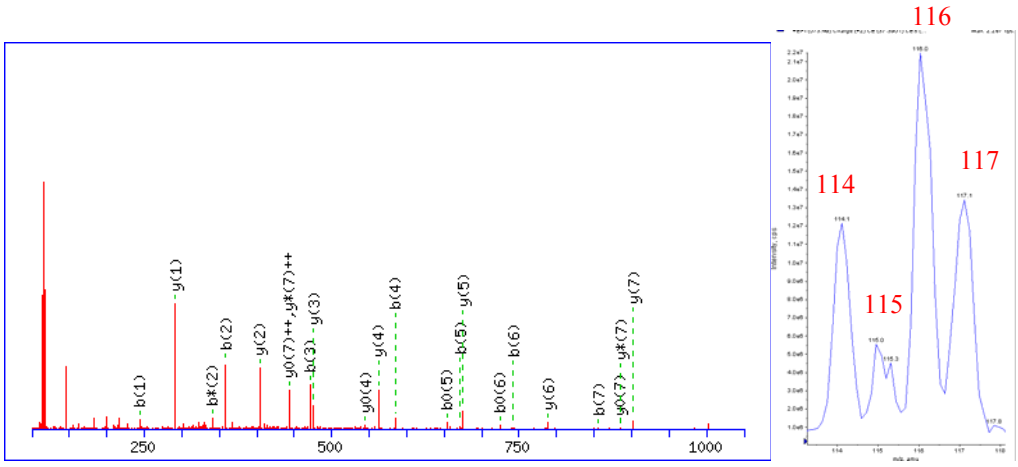
Peptide: LEAGDHPVELLAR
Name: SPARC
Mascot ion score: 59
Source: 1_110408protmixiTRAQ

Theoretical ratio: 1 / 3 / 5 / 7
Experimental ratio: 1 / 2.2 / 3.2 / 4.1



Peptide: AHVNSLGENLK
Name: Interleukin 10
Mascot ion score: 46
Source: 2_110408protmixiTRAQ

Theoretical ratio: 1 / 0.33 / 0.20 / 0.14
Experimental ratio: 1 / 0.67 / 0.38 / 0.10

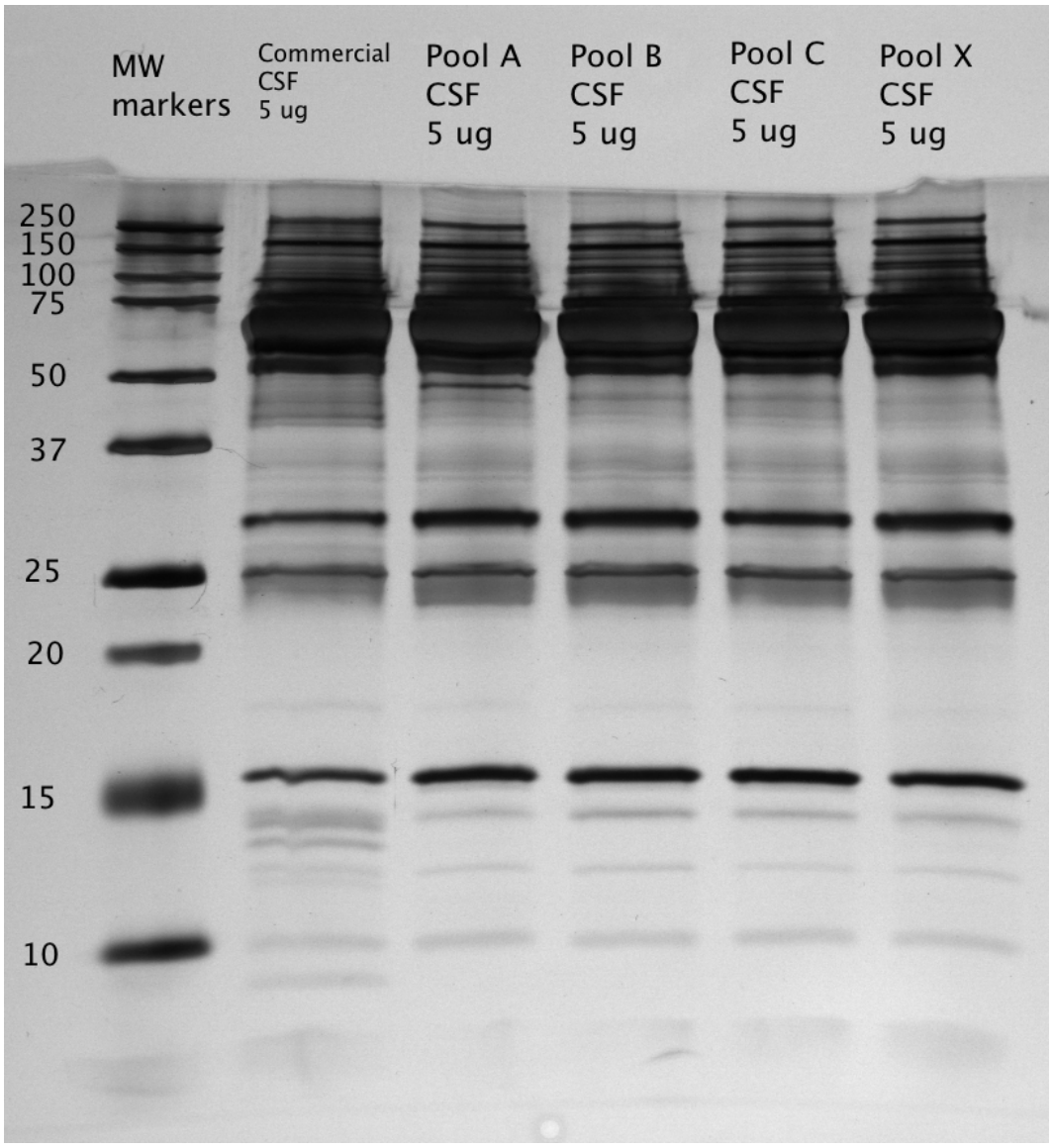


Peptide: VNLLSAIK
Name: Tumor necrosis factor
Mascot ion score: 56
Source: 3_110408protmixiTRAQ

Theoretical ratio: 1 / 0.20 / 1.40 / 0.60
Experimental ratio: 1 / 0.36 / 1.60 / 1.06

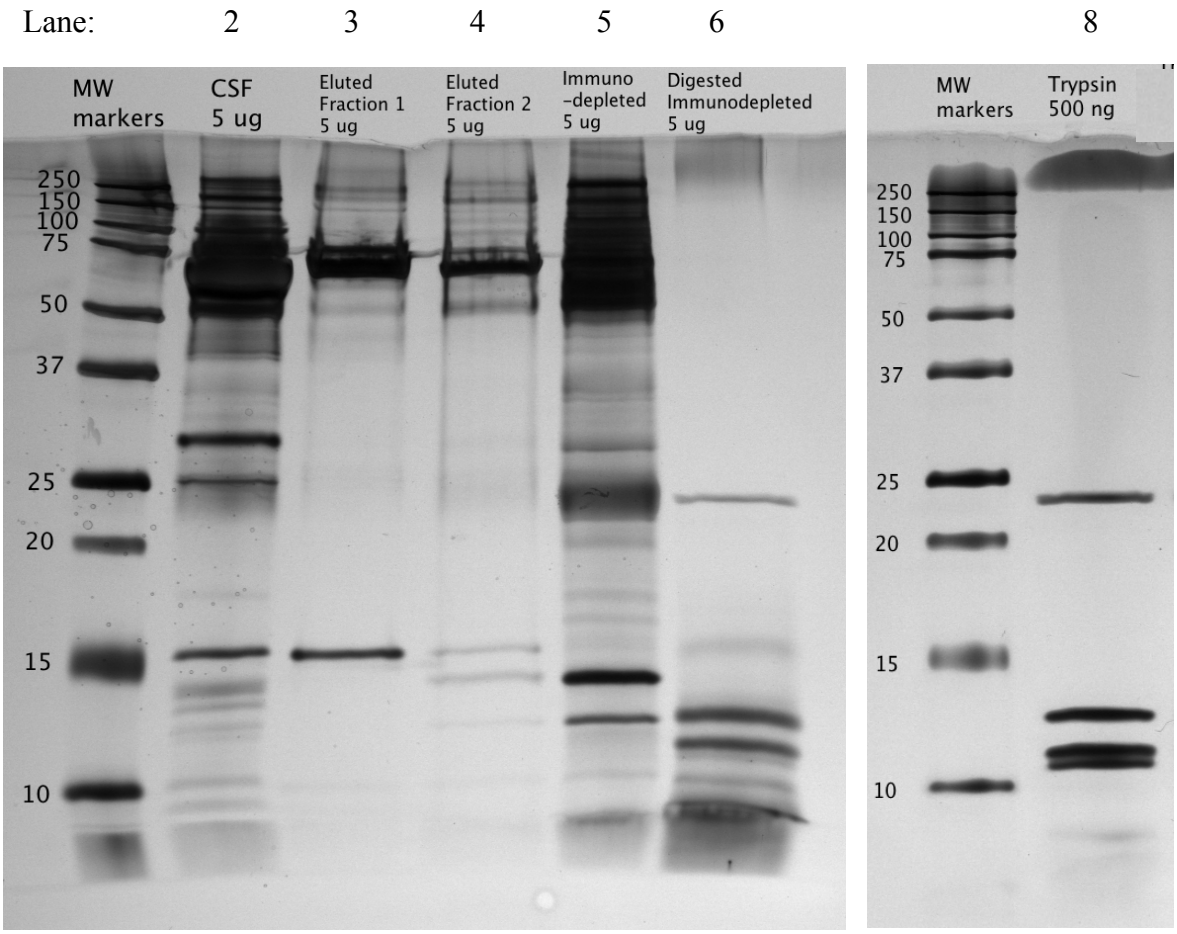
Example spectra from the dataset X_110408protmixiTRAQ are displayed. These peptides were identified when the mass spectra from standard proteins, searched on the UniProt database.

8.16 FIGURE 10. 1D GEL ELECTROPHORESIS PROFILE OF CSF



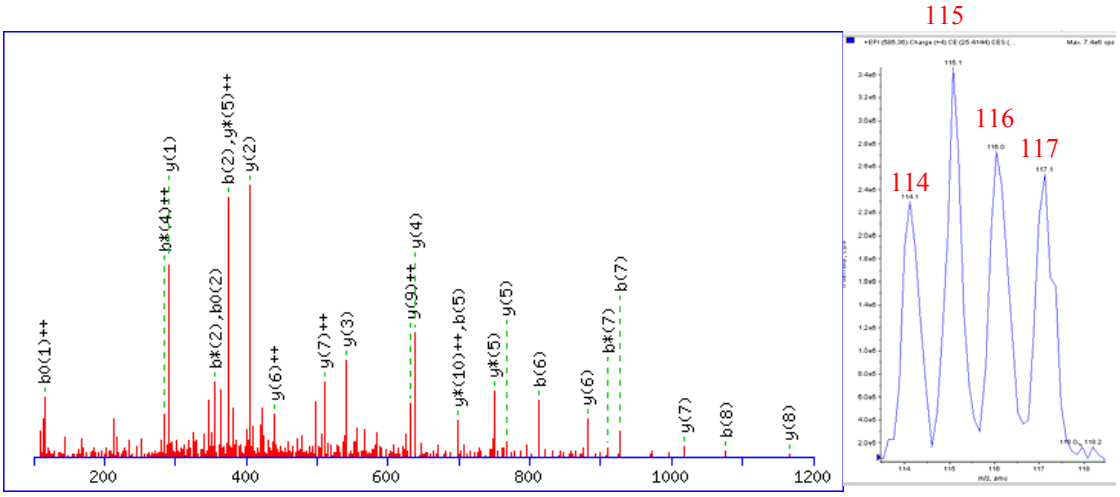
1D gel electrophoresis of the CSF used in the studies was performed. Visualization by silver staining demonstrates that the CSF from age matched controls (pool A), from subjects with asymptomatic degenerative disc disease (pool B), from subjects with symptomatic degenerative disc disease (pool C), and CSF from young healthy controls (pool X), is very similar macroscopically. The commercial pool of CSF however, differs markedly.

8.17 FIGURE 11. VISUALIZATION OF IMMUNODEPLETION

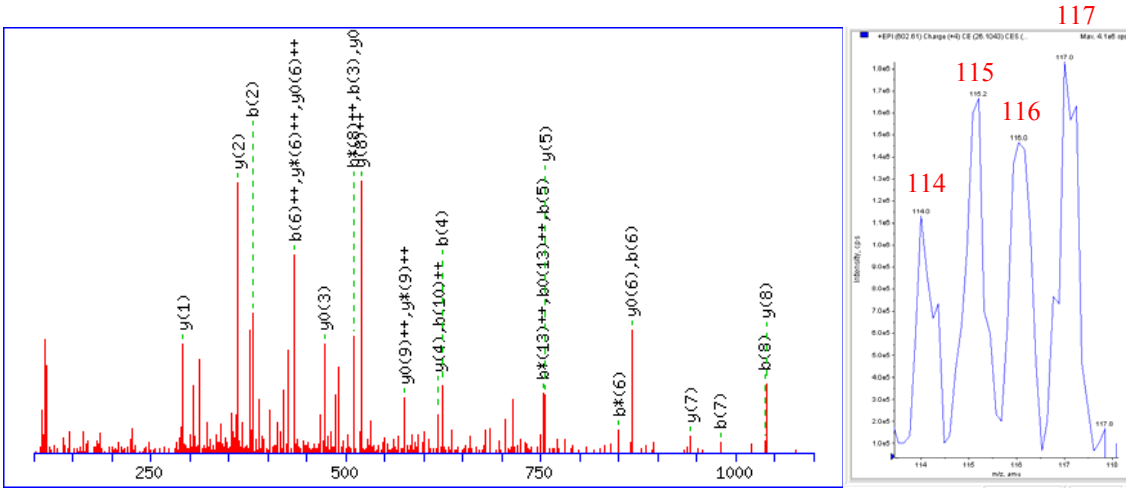


Lane 2 shows CSF before depletion with the Proteoprep 20 immunodepletion column. Lanes 3 and 4 show CSF proteins that were removed by serial passes through the immunodepletion column. The 5th lane shows the immunodepleted CSF. Lane 6 shows the immunodepleted CSF after trypsin digestion. Lane 8 shows that the presence of trypsin accounts for the bands above 10 kD seen in lane 6.

8.18 FIGURE 12. EXAMPLE SPECTRA OF IMMUNODEPLETED CSF PROTEINS



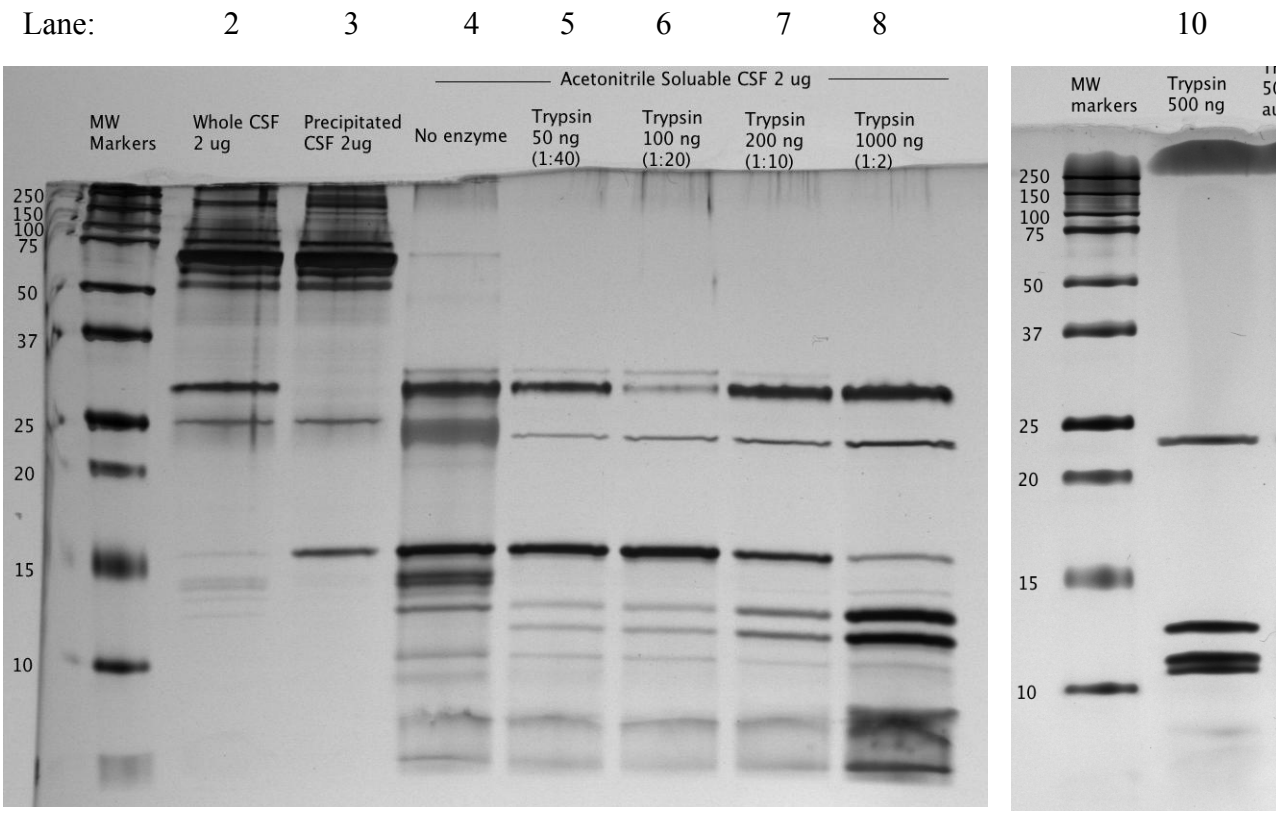
TQPNLDNCPFHDQPHLK
Cystatin C
MASCOT ion score: 60
250_mMNaCl180408depletedCSF2DLCiTRAQ
Retention time: 81.08



VHENENIGTTEPGEHQEAK
SPARC protein
MASCOT ion score: 66
500_mMNaCl180408depletedCSF2DLCiTRAQ
Retention time: 38.23

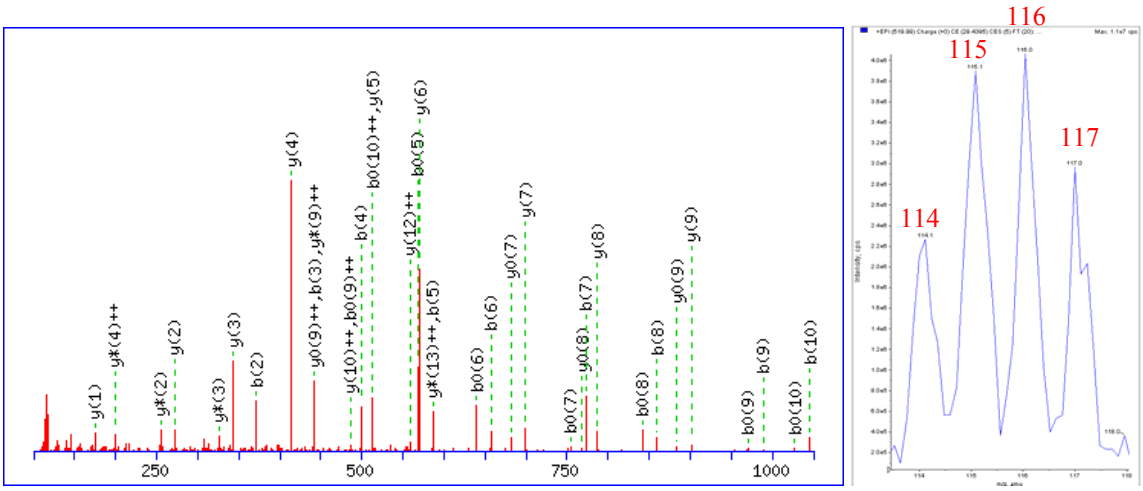
Two example MS/MS mass spectra from the immunodepleted CSF are shown. The iTRAQ reporter region from 113-119 is shown in detail on the right.

8.19 FIGURE 13. VISUALIZATION OF ACETONITRILE PRECIPITATION

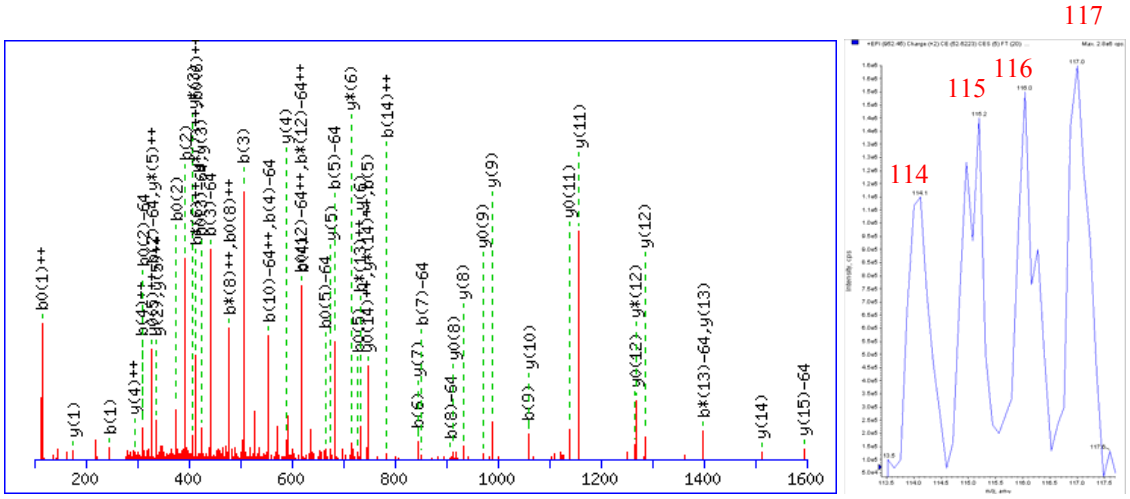


Lane 2 shows CSF before acetonitrile precipitation. Lanes 3 shows CSF proteins that were precipitated with acetonitrile. The 4th lane shows the CSF proteins that where soluble in acetonitrile. Lanes 5 – 8 show acetonitrile soluble CSF with increasing amounts of trypsin. Increasing amount of trypsin did not alter the presence of two protein bands at approximately 30 and 15 kD, suggesting these proteins were resistant to trypsin cleavage. Lane 10 shows that the presence of trypsin accounts for the bands between 25 and 10 kD in lanes 5 – 8.

8.20 FIGURE 14. EXAMPLE SPECTRA OF ACETONITRILE PRECIPITATION CSF PROTEINS



ILAGSADSEGVAAPR
ProSAAS
MASCOT ion score: 83
100_mMNaCl170408acnsolubleCSF2DLCiTRAQ
Retention time: 56.46



TMLLQPAGSLGSYSYR
Prostaglandin D2 synthase
MASCOT ion score: 101
250_mMNaCl170408acnsolubleCSF2DLCiTRAQ
Retention time: 66.91

Two example MS/MS mass spectra from the immunodepleted CSF are shown. The iTRAQ reporter region from 113-119 is shown in detail on the right.

9 DISCUSSION

9.1 PEPTIDE AND PROTEIN STANDARDS

The goal of the peptide and protein standards was to establish the accuracy of the iTRAQ method for determining abundance, and to explore the possibility of using such standards as a control group for absolute quantification of proteins of a particular interest. According to literature, isotopic labeling techniques have successfully determined changes in relative abundance changes down to ~1.5 fold changes, over a dynamic range of proteins from 10 – 100.[125] In the current study, the fold changes ranged from 3 to 7, and the dynamic range of the peptides in the standard was 1, as all peptide species were at equal concentrations to one another. Agreeing with this, changes in peptide abundance were observable. The majority of peptide ratio changes were significant in all trials, except in two cases, trial 2 114:117 and trial 3 114:117. In both of these cases, the observed ratio was less than 0.2 fold away from 1. Similar results were obtained with regard to protein ratios.

Six out of the eight peptides were observed by mass spectrometry. The only peptides which were not observed were leucine-enkephalin and methionine-enkephalin. The likely reason for why these peptides were not observed is because of their small size and their low lipophilicity. During the second LC separation, peptides are separated from the salts used to elute the peptides from the SCX column, by use of a C18 lipophilic column. The salts do not bind the C18 column because they are not lipophilic, and are removed to prevent damage to the mass spectrometer. As both leucine-enkephalin and methionine-enkephalin are only 5 amino acids in length, and only have either one or two hydrophobic amino acids according to the hydropathy scale,[158] these peptides likely failed to bind the C18 column and were lost with the salt.

In the case of the protein standards, differences from expected values were also observed like with the peptide standards. However, when comparing figures 1B and 4B, the average standard deviation in the error was about two times smaller than with the peptide standards. Even so, the difference between expected and observed ratios for protein standards was not significantly different from zero.

With the protein standards, all the proteins in the sample were successfully identified with Mascot. However, when the Interrogator algorithm was used with the ProQuant software to analyze iTRAQ data by Applied Biosystems, nerve growth factor was not identified, and thus only five out of the six proteins were observed. This result is not surprising, because even Mascot could only find one peptide to identify NGF, and it is likely the Interrogator algorithms was unable to detect this one peptide.

There are a number of possible explanations to explain why the experimental ratios of peptide standards did not exactly match theoretical data. As mix A, B, C and D were all made with volumes of individually labeled peptides less than 10 μl, small errors in pipetting could result in large variability. For example, a small amount of liquid sticking to the outside of the pipette upon transferring to the new vial, at these small volumes could result in significant error. In addition, when the mixes were created, greater than 80% of the solution was ethanol. As ethanol is volatile at room temperature, leaks can occur because the vapor pressure of the sample acts to push against the air cushion of the pipette, which results in pushing the sample out of the tip. In addition, ethanol has lower surface tension than water due to lack of hydrogen bonding, and thus it is not held in the pipette as well as aqueous solutions.

Another source of error comes from the iTRAQ labels. Intrinsically they have a degree of error associated with them. The isotopic chemical labels are not 100% pure, and instead of purities of 92.9%, 92.3%, 92.4% and 92.3% for reagents 114, 115, 116, and 117 respectively. The ProQuant software, however, can correct for these inaccuracies in labeling to a degree. Additionally, the efficiency of the labeling of the various iTRAQ labels may not be equivalent in all cases. It is possible that some of the labels are more or less reactive than the others.

The accuracy of iTRAQ labels also depends on the number of spectra found, and their associated error factor. Statistically, the greater the number of spectra, the higher the confidence of the ratio can be. This poses a theoretical limitation to the accuracy of neuropeptide quantitation, because they are so small, they are unlikely to have many unique peptides for quantification. At the same time, this theoretically poses less of a problem for protein quantification where it is expected that each protein can produce many different unique peptide spectra. This helps to explain why the variation for the

protein standards is less than that of the peptide standards. The accuracy of iTRAQ ratios in the peptide standards permitted significance to be detected at observed ratios of 1:2. In the protein standards, ratios were found to be significantly different even at ratios of 25:27. Thus, iTRAQ experiments with proteins are much more accurate than with peptides.

While protein determination inaccuracy, pipetting error, and labeling efficiency can contribute to error in the iTRAQ ratios, the ProQuant software can apply a bias to correct for these errors. The software assumes that the average protein abundance across all the samples is identical. In the case of these standards, we are certain that the protein abundances were not equal across samples, and thus the software biases were not applied. Normally the software bias would partially control for differences due to protein determination inaccuracy, pipetting error, and labeling efficiency.

The protein and peptide standards were explored for two reasons: First, to give an idea of the accuracy of iTRAQ to theoretical values, second, to explore the possibility of using such standards as a control group for the absolute quantification of proteins of particular interest. While the idea of a standard group which permits absolute quantification of proteins is practical, the assumptions of the software bias puts difficulties in utilizing such a standard. Such an assumption is only valid when each individual sample labeled by iTRAQ is similar in protein amounts and type. Thus, a standard protein or peptide control cannot be used in conjunction with the software bias correction. If there is no bias correction, small variations during pipetting, labeling or protein quantification steps could result in significant false protein expression changes. Thus the only way to ensure bias correction is applied and to include standard proteins or peptides for absolute quantitation, a modified concoction of CSF which had the standard proteins removed, and then added back into the CSF at known amounts must be created. In addition to the difficulty of creating such a concoction, the standard proteins or peptides need to be added to the modified CSF at amounts similar to physiological levels, as isotopic labeling methods are accurate for ratios of up to 1:100.[125] Yet if this was done, it is still possible that these proteins or peptides of interest would still be below the dynamic range of proteins observable by mass spectrometry. Even if the dynamic range was not problematic in the creation of a standard CSF concoction, the use of recombinant

proteins may be a source of error. Due to post-translational differences in processing, recombinant proteins can differ from native proteins by function,[159] structure,[160] and glycosylation.[161] These post-translational differences may also alter observable peptide sequences, resulting in peptides which are not observed in native proteins. Alternatively, peptides may be produced that are not observed in recombinant proteins. This has the potential to result in iTRAQ ratios which are not reflective of the true protein abundances.

To explore specific peptides or proteins, methods such as multiple reaction monitoring (MRM) are more suitable than a peptide or protein standard.[162] With MRM, synthetic tryptic peptides of proteins or peptides of interest are synthesized with a ^{13}C labeled amino acid, as well as the ^{12}C counterpart. Alternatively, these ^{13}C and ^{12}C tryptic peptides can be created using isotopic labeling of tryptic peptides. Standard solutions including both of these peptides are created, with the ^{13}C peptide is used as a reference standard, and the amount of the ^{12}C amino acid being varied to create a standard curve. These standard solutions are separated by liquid chromatography as the biological sample will be, and the retention time of the peptide along with its mass spectra is recorded. Next, the ^{13}C peptide is spiked into individual biological samples. When the biological sample is analyzed by mass spectrometry, the representative peptide is selectively monitored for using the mass spectra and retention time determined previously. The peptide is identified and its absolute abundance is determined according to the previously established standard curve. In this manner, selected proteins or peptides can be quantitated absolutely in a complex mixture, even at very low abundance.[163] Because individual samples are compared to the reference sample, and samples are not pooled, one can separate error due to the experimental method, from actual differences in the experimental subjects.

9.2 CSF ULTRAFILTRATION

Differential neuropeptide expression proteomics is promising because developing such a method would be useful in identifying situations where there exist metabolic defects in neuropeptide processing which contribute to a CNS disorder. The evaluate the possibility of detecting neuropeptides in CSF, commercially obtained CSF was ultrafiltrated and labeled with iTRAQ. In these experiments, four peptides were observed by mass spectrometry which corresponded to secretograinin-1, secretogranin-5,

somatostatin and VGF nerve growth factor inducible. Previously, in a study where CSF peptides were isolated with 5 kD ultrafiltration and hydrophobic liquid chromatography, in addition to 8 other proteins, secretogranin-1 and VGF nerve growth factor inducible were found.[164] Note that in that study, 4 ml of total CSF were used, while in our study only 1/8th of this amount was utilized. Another major difference was that in our study, iTRAQ reagents were used to label the filtered peptides.

Each of the neuropeptides found in this study could be potential biomarkers for disease. For example, some polymorphisms of the secretogranin-1 gene have been found to be associated with schizophrenia.[165] Additionally, decreased brain secregranin-1 immunoreactivity has been found in schizophrenic[166] and Alzheimer's disease patients.[167] In a rat epilepsy model, secretogranin-1 mRNA has been found to be upregulated in granule cells of the hippocampus.[168] With regards to secretogranin-5, secretogranin-5 expression was inversely correlated with body weight, demonstrating a clear action of secretogranin-5 on body weight homeostasis.[169] Furthermore, in patients with ALS, increased secretogranin-5 levels have been observed in CSF.[137] Somatostatin levels have also been suggested to be involved in some diseases, for example somatostatin levels are decreased in patients with affective illnesses,[136] Alzheimer's disease,[170] and increased in Parkinson's disease.[171] Moreover, VGF nerve factor inducible has been thought to be a putative biomarker of ALS muscle weakness, according to recent studies.[172]

One of the downfalls of the CSF ultrafiltration technique for sample preparation is that few peptides are found. The difficulty in isolating peptides from CSF is that there is a high salt-content of CSF, similar to plasma levels.[93] Most separatory techniques rely on size or hydrophobicity differences to separate components from one another.[173] As neuropeptides are small, they may be lost when using techniques to desalt samples by molecular size, such as dialysis or ultrafiltration. Methods which remove salts according to hydrophobicity are the best choice for isolating peptides from ultrafiltrate. However, not all peptides are hydrophobic, and thus may not be successfully retained on the hydrophobic resin.

Another compounding problem is the extremely low peptide concentrations in CSF. For example, in CSF, neuropeptide Y have been reported to be approximately around 125 pg/ml,[174] corticotropin releasing hormone levels have been reported to be

approximately 50 pg/ml,[175] and substance P levels have been reported around 65 pg/ml.[176] Comparatively, in CSF, secretogranin-1 has been found at concentrations around 20 ng/ml,[177] and secretogranin-5 at 7 ng/ml.[178] Surprisingly, CSF somatostatin levels have been reported at approximately 40 pg/ml.[170,179,180] The absolute concentration of VGF nerve growth factor inducible has not been determined in the literature.

That somatostatin was detected in ultrafiltrate CSF while its concentration in whole CSF is so low suggests that it may be a false-positive identification. Supporting this possibility are the low Mascot ion scores of the peptide, and the many unexplained peaks in its spectrum. This raises another possible confound – the false positive rate. A good estimate of the false positive rate is to search the spectra using a randomized database. Interestingly, this turned up zero peptide hits. While this result increases the confidence that somatostatin was observed, it does not rule out the possibility that somatostatin was a false positive identification. Also another possibility, is that the somatostatin concentration in the commercial CSF was abnormally high.

Another potential problem of the CSF ultrafiltration method is that neuropeptides may be bound by carrier proteins and may be unable to flow through the ultrafiltration filter. Thus, the amount of peptides in the low molecular weight fraction may be reduced. In studies with human serum, this effect has been observed.[181-183] This problem can be circumvented with denaturing conditions where the serum ultrafiltration is performed in the presence of an agent that disrupts protein-protein interactions. For example, when denatured with acetonitrile, the peptide amounts found in the low molecular weight fraction is greatly increased.[181-183]

Neuropeptidomics of CSF is a very promising sample preparation technique; however, there are some theoretical considerations which must be addressed before it can be utilized successfully. First, is the software bias of ProQuant. Without the software bias, the ProQuant software may identify changes which occurred due to differences in labeling efficiency, or other types of random experimental error. To utilize the software bias, an assumption is made that the overall expression change is equal in across groups. This assumption is not likely to be valid when the sample analyzed has few peptides, which is the case with ultrafiltrated CSF. For the assumption to be valid, there should be a large amount of ultrafiltrate peptides in each group, but to make things troublesome,

there is no simple method to determine peptide amounts from ultrafiltrated CSF to accurately equalize peptide amounts. This is of particular importance when dealing with pooled samples, as experimental error and group differences cannot be separated statistically. Another challenge that needs to be overcome is improving the relative quantitation accuracy of the ultrafiltrated peptides. To calculate a p-value, the ProQuant software requires multiple mass spectra of the neuropeptide of interest. For proteins, because they are large and are likely to have several acquired mass spectra, the accuracy of the calculated ratios will be higher than in the case of peptides, were the number of mass spectra obtained is limited by the number of unique peptide fragments. With the development of MRM technology these downfalls can be averted. Individual samples can be labeled and compared to a standard, so that experimental error and group differences can be teased apart statistically.

9.3 CSF IMMUNODEPLETION

According to Figure 11, the immunodepletion was successful in removing abundant proteins from CSF. Unmodified CSF had a large band of protein between 75 and 50 kD, which is likely albumin. Albumin has a molecular mass of 69 kD, and a band at this molecular weight was observed to be removed by immunodepletion. A number of other proteins were removed from CSF also, resulting in an increase in the number of protein bands seen in the immunodepleted CSF. Furthermore, the digestion of the immunodepleted CSF was successful, as observed by the majority of the immunodepleted CSF being removed by a 1:40 enzyme to substrate ratio, with mostly 3 bands remaining. The remaining bands are trypsin.

The goal of this experiment was to identify and quantitate proteins in immunodepleted CSF from experimental subjects with and without pain and disc degeneration. As a whole, many interesting proteins were found, a number of which were identified as changed in degenerative disc disease or pain. In the group with disc degeneration and pain, apolipoproteins A-IV, D, and E were increased in the painful disc degeneration group compared to the control group. Apolipoproteins are important in remylination, which occurs after axonal injury. In fact, it is known that apolipoproteins A-IV[184], D,[185] and E[186] accumulate in nerves after peripheral nerve injury, as Schwann

cells attempt to remyelinate, while apolipoproteins D and E expression is induced.[187] In addition, past reports have also demonstrated that an increase in apolipoprotein A-IV occurs in the CSF of painful herniated discs.[188] These findings suggest that painful disc degeneration may involve altered lipid metabolism as a result of nerve injury, but that the same processes do not occur in control or asymptomatic disc degeneration.

Greater hemopexin levels were observed in the CSF of subjects with painful disc degeneration compared to the control group. Hemopexin is a scavenger for free heme. Free heme is known to causes oxidative damage.[189] Interestingly, hemopexin is increased in experimental models of peripheral, but not central, nerve injury.[190-192] Thus, in addition to the increase in apolipoproteins, the increase of hemopexin is additional evidence that suggests that painful disc degeneration may be a result of nerve damage at the degenerating discs.

Prostaglandin-H2 D-isomerase was decreased in the CSF of subjects with painful and asymptomatic degenerative disc disease, compared to controls. This finding is somewhat paradoxical because according to past literature, the levels of prostaglandin-H2 D-isomerase increase in CSF with peripheral inflammation.[193,194] However, in these studies only studied acute changes in prostablandin-H2 D-isomerase. It is possible with chronic inflammation, prostaglandin-H2 D-isomerase levels decrease lower than in the control state. In fact, in CSF of patients with lumbar disc herniation, prostaglandin-H2 D-isomerase levels also were observed to be lower.[188]

Another interesting protein that was significantly higher in the groups with degenerative disc disease when compared to controls, is alpha-1 antichymotrypsin. Alpha-1 antichymotrypsin is a serine protease inhibitor, which is known to be increased in inflammatory conditions such as rheumatoid arthritis.[195] While the role of alpha-1 antichymotrypsin in degenerative disc disease has yet to be elucidated, its increased levels add to the growing body of evidence that there is ongoing inflammation in degenerating discs.[196]

Cystatin C is a small secreted cysteine protease inhibitor, with concentration in CSF approximately 7 μg/ml.[197] CSF concentrations of cystatin C have been thought to be indicative of pain,[96] although this has been disputed.[198] In this study, cystatin C levels were higher in the degenerative disc disease groups compared to the group without disc

degeneration, suggesting that cystatin C levels may not be a marker for pain. Nevertheless, when comparing painful to asymptomatic disc degeneration, cystatin C was still greater in the group with pain, but the difference was not significant. These results raise the possibility that cystatin C levels may be indicative of disc degeneration, and not pain per se. In support of this, cystatin C levels have previously been found to be elevated in the CSF of patients with lumbar disc herniation.[188] Cystatin C binds cysteine proteases such as cathepsins B, H and L, and is thought to be involved with tissue remodeling.[197] Interestingly, cathepsins D, K and L are observed at disc degeneration sites by immunohistochemistry.[199] It is possible that increased local cysteine protease levels result in a compensatory increase in cystatin C levels.

ProSAAS is an endogenous inhibitor of prohormone convertase 2, an enzyme primarily found in neuroendocrine tissues.[200] Prohormone convertases are thought to be involved with the proteolytic cleavage of proteins to mature peptides, and thus likely plays a role in neuropeptide maturation.[201] In a past experiment, proSAAS was decreased in the CSF of patients with lumbar disk herniation.[188] In this experiment, proSAAS was not changed in the patients with degenerative disc disease without pain; however, proSAAS was increased in patients with painful degenerative disc disease. It is possible that the chronic pain state results in an alteration in neuropeptide processing.

Superoxide dismutase, a free radical scavenger, levels were decreased in the asymptomatic disc degeneration group compared to the control group. Levels in the painful disc degeneration group were almost significantly decreased when compared to control, at $p = 0.07$. These results are in agreement with a past study that found superoxide dismutase levels decreased in the CSF of those with lumbar disc herniation.[188] Additionally, in synovial fluid of persons with osteoarthritis superoxide dismutase levels are also decreased.[202] Also, overexpression of SOD in transgenic mice increases the development of neuropathic pain,[203] consistent with the higher SOD expression in the pain group. While the expression levels of SOD in the pain group were similar to that of the control group, it is possible that the SOD in the pain groups may be deactivated, which would be consistent with animal models.[204]

Gelsolin is a calcium regulated actin binding protein with a number of functions,[205] playing roles in motility,[206] ion channel organization,[207] lipid signaling,[208]

and apoptosis.[209] Gelsolin is present in the CSF,[210] and is secreted by both neurons[211] and chondrocytes.[212] Interestingly, it was found increased in both degenerative disc disease groups regardless of pain, suggesting elevated CSF gelsolin may be involved in disc degeneration. In fact, gelsolin is known to have anti-inflammatory actions,[213] and could be upregulated in response to counteract local inflammation at the degenerating discs.

Chromogranin A levels were increased in both painful and asymptomatic degenerative disc disease groups. Chromogranins are expressed ubiquitously in secretory cells of the immune system, nervous system, and the endocrine system.[214] While chromogranin A has not yet been implicated in degenerative disc disease, there is a possibility for chromogranin A to be involved. Chromogranin A levels are increased in rheumatoid arthritis.[215] Additionally, chromogranin A levels correlate with soluble tumor necrosis factor receptors, which are markers of systemic inflammation.[216]

β-2-microglobulin levels have been previously found to be elevated in CSF of dogs with disc degeneration.[217] In our study, β-2-microglobulin levels were elevated in the group with painful disc degeneration. β-2-microglobulin is a component of the class I major histocompatability antigens, and is normally expressed on immune cells.[218] The protein is shed at low concentrations during cell membrane turnover,[219] which occurs in elevated frequency when the immune system is activated,[220] making it a marker of inflammation. This finding is suggestive of a chronic inflammatory state in the intervertebrals discs, which can be detected in the CSF. In fact, it is well known that inflammation plays a role in disc degeneration.[196]

In CSF from patients with asymptomatic degenerative disc disease, levels of neural cell adhesion molecule L1-like protein (CHL1) were increased over control. In the CSF of groups with painful degenerative disc disease, CHL1 was also increased, but not low enough to reach significance ($P = 0.0566$). CHL1 protein is an important molecule for axonal growth,[221,222] and neuronal survival in vitro.[223] It is expressed by some neurons as well as glia.[221] In fact, it is upregulated in states of nerve injury, and the upregulation is prolonged such that axonal reconnection is likely required to return CHL1 levels to control levels.[224] Our results suggest that nerve damage occurs in degenerative disc disease, hence increasing CHL1 levels. In painful degenerative disc disease, it is possible that axons have made reconnections that normalize the CHL1 levels, but these

reconnections are improper and result in pain. In asymptomatic degenerative disc disease, perhaps these reconnections do not occur and thus there is a persistent increase in CHL1 levels.

Fibronectin is an extracellular matrix molecule that plays important roles in development,[225] as well as during response to injury. For example, fibronectin is increased locally in osteoarthritis joints,[226,227] and is also increased in human degenerating discs.[228] Fibronectin has also been implicated in neuropathic pain. For example, in a rat model of peripheral nerve injury, fibronectin levels increase in the CNS.[229] Additionally, intrathecal administration of fibronectin results in mechanical allodynia and increased expression of $P2X_4$ receptors in microglia.[229] Interestingly, we found increased levels of fibronectin in CSF of persons with painful degenerative disc disease, but not asymptomatic degenerative disc disease. Thus, there is a possibility that the pain experienced in persons with disc degeneration is a result of fibronectin from degenerating discs entering the spinal cord.

Calsyntenin-1 was downregulated in the CSF of the asymptomatic degenerative disc disease group when compared to the control group. Calsyntenin-1 is actually a transmembrane protein found in post-synaptic membranes of both inhibitory and excitatory cells.[230] It is expressed ubiquitously by almost all CNS neurons.[231] Calsyntenin-1 has an intracellular calcium binding domain, and thus may play a role in the modulation of Ca^{2+} signaling.[230] Additionally, the extracellular domain of calsyntenin-1 can be proteolytically cleaved which internalizes the calcium binding domain.[230] Our data suggest that in persons with asymptomatic disc degeneration there may be less proteolytic cleavage of calsyntenin-1. However, the functional consequences of this observation are unknown as the functions of calsyntenin-1 have for the most part yet to be characterized.

Amyloid-like protein 1 was increased in the CSF of both asymptomatic and painful disc degeneration groups. Amyloid-like protein 1 is a glycoprotein secreted into the CSF.[232] While the study of amyloid-like protein 1 has mostly been limited to its relation to neurodegeneration, we can still make some inferences on possible reasons for why it was increased in patients with disc degeneration. Because amyloid-like protein 1 was highly upregulated in the frontal cortex of manganese exposed non-human primates,

amyloid-like protein 1 upregulation likely can occur as a response to oxidative stress.[233] This would be consistent with past work demonstrating that oxidative stress accumulates in degenerating discs.[234]

Serine/cysteine proteinase inhibitor clade G member 1 (SERPING1), was decreased in the CSF of the group with asymptomatic degenerative disc disease. SERPING1 has a number of anti-inflammatory actions, including inhibition of complement activation, inhibition of the kallikrein-kinin system, and suppression of leukocyte infiltration.[235] Because SERPING1 levels are only decreased in degenerative disc disease, our results suggest the inflammatory state of the lumbar intervertebral discs may not be the same in painful and in asymptomatic degenerative disc disease.

In CSF of subjects with painful degenerative disc disease, uncharacterized protein C2orf55 levels were increased, suggesting that it may be involved with the development or maintenance of pain. However, as the function of this protein has yet to be elucidated, speculation of this protein's role in degenerative disc disease is difficult.

In this study, 83 proteins were observed after immunodepletion. In a previous study by Ogata *et al*, CSF was depleted with the Agilent multiple affinity removal column, which removed six abundant proteins, identifying 219 proteins.[236] There are a number of explanations which explain this difference in number of proteins identified; among them are differences in fractionation procedure, the quality of the mass spectra obtained in our sample, and limitations of the immunodepletion technique.

In terms of technical differences, the Ogata *et al* study used a more thorough SCX fractionation technique with a greater amount of starting CSF total protein. Their study used 2 ml of pooled CSF per iTRAQ channel. In comparison, our study used 4 times less CSF total protein. The Ogata *et al* study also used an offline SCX fractionation procedure which resulted in 20 fractions, possibly resulting in greater resolution of peptides, and our study utilized an online SCX fractionation procedure with 14 fractions. Online SCX fractionations utilize step gradients, which do not produce the superior resolution SCX fractionation that offline linear gradients can.[237] On the other hand, however, the downfall of offline SCX fractionation techniques is that they are not automated, and greater amount of protein is required for the chromatography.

Some carrier proteins present in abundance in CSF, such as albumin, bind proteins and smaller peptides.[238] In fact, proteins which are eliminated by immunodepletion, such as albumin, immunoglobulins and apolipoproteins, have been observed to be associated with other proteins.[239] Thus, removing abundant proteins may also result in the loss of low abundant proteins and peptides which have biomarker potential. In addition, the proteoprep 20 utilizes polyclonal IgGs to remove abundant proteins. As IgGs may associate with peptides and small proteins low abundance proteins,[239] these is much opportunity for low abundant proteins and peptides to be lost by non-specific binding.

In this experiment, expression levels of a number of proteins in CSF were changed in both asymptomatic and painful disc degeneration, when compared to the control group. A total of 83 proteins were identified and quantitated in Proteoprep 20 immunodepleted CSF. A number of proteins involved in inflammation, or thought to play a role in inflammation had expression levels that were altered in subjects with disc degeneration. For example, gelsolin, alpha-1 antichymotrypsin, cystatin C, chromogranin A, prostaglandin D2 synthase, superoxide dismutase, amyloid-like protein 1, and SERPING1 levels were altered in disc degeneration CSF. Our results are in agreement with past experiments investigating CSF from patients with herniated discs,[188] as well as the vast amount of literature implicating inflammatory mediators in disc degeneration.[71,196,240]

9.4 ACETONITRILE PRECIPITATION

From the 1D electrophoresis in Figure 13, we can see that incubation of CSF with acetonitrile successfully precipitates larger proteins, leaving behind smaller proteins in the soluble fraction. With our protocol, the separation of large from small proteins occurred at approximately 40 kD, although it was not a clear cut separation. Also observed in the 1D electrophoresis, there were a number of protein bands which did not disappear upon addition of trypsin, suggesting that trypsin was unable to digest these proteins. There was one such protein at approximately 30 kD, and another at 15 kD. The protein at 30 kD was likely apolipoprotein E, which is 34 kD in size, and highly abundant, constituting 3% of CSF proteins.[119] The protein at approximately 15 kD is likely

apolipoprotein A-II, a protein which is 17 kD in size and making up 2% of CSF proteins.[119] The total amount of lipids in CSF is on the order of 10 μg/ml.[241] Reducing the volume of the CSF to 20 μl for trypsin digestion and iTRAQ labeling, would result in a 40-fold increase in lipid and apolipoprotein concentrations, favoring the formation of chylomicrons. The formation of chylomicrons can explain why the apolipoproteins were trypsin resistant, as the surrounding lipids could impede access to the apolipoproteins, preventing their digestion.

The goal of this experiment was to identify and quantitate proteins in acetonitrile precipitated CSF from experimental subjects with and without pain and disc degeneration. Overall, the results of the acetonitrile precipitation experiment agree with the ProteoPrep 20 experiment and other past proteomics experiments.[188] For example, cystatin C levels were found to be increased compared to controls in the acetonitrile precipitation experiment, in both the asymptomatic disc degeneration group, as well as in the symptomatic disc degeneration group, and this was also what was observed in the Proteoprep 20 experiment. Additionally, prostaglandin D2 synthase was lower in the group with asymptomatic disc degeneration, also agreeing with the Proteoprep 20 study. A past proteomics study comparing control subjects to those with disc herniation found that the group with disc herniation also had increased cystatin C levels and decreased prostaglandin D2 synthase levels.[188] ProSAAS protein levels also agreed in both the ProteoPrep 20 immunodepletion and the acetonitrile precipitation experiments. ProSAAS levels remained the same in patients with asymptomatic disc degeneration, but were elevated in patients with painful disc degeneration.

Apolipoprotein A-I was found to be decreased in the asymptomatic degenerative disc group, when compared to the control CSF. Yet, apolipoproteins A-II, C-III, D, and E, were not significantly changed. This result does not agree with the Proteoprep 20 experiment, where all the apolipoproteins detected were increased in the group with painful degenerative disc disease when compared to controls. However, because lipids are very soluble in organic solvents such as acetonitrile, it is likely that the acetonitrile precipitation extracted all the lipoproteins and lipids into the low molecular weight fraction. This concentration of lipids and lipoproteins could have allowed for the formation of chylomicrons, which might prevent trypsin digestion of the apolipoproteins.

Without complete digestion of the apolipoproteins in the acetonitrile precipitation experiment, their ratios may not accurately reflect their true relative amounts.

Insulin-like growth factor-II levels were increased in the CSF of persons with painful degenerative disc disease, when compared to both the control and asymptomatic groups. Insulin-like growth factor-II is expressed by glial cells of the CNS and spinal cord, with a particular abundance in the choroid plexus.[242] As a result it is also abundant in CSF.[243] In experimental nerve injury in rats, peripheral nerve injury is known to result in sprouting of noradrenergic axons into dorsal root ganglia,[244] which is dependent on neurotrophic factors nerve growth factor, brain derived nerve factor, and neurotrophin-3.[245] Insulin-like growth factor-II has neurogenic effects similar to nerve growth factor, increasing neurite outgrowth in both sympathetic and sensory neurons.[246] Thus, our results indicate that insulin-like growth factor-II may be released locally in response to peripheral nerve damage at the intervertebral discs.

Orosomucoid-1 was decreased in the CSF of asymptomatic degenerative disc disease, compared to the control group and painful disc degeneration group. Orosomucoid is an acute phase protein, meaning it is an anti-inflammatory protein synthesized in response to acute inflammation.[247] Currently, it is thought that acute phase proteins such as orosomucoid are released to counteract the inflammation and reduce its side-effects, and also to prevent the establishment of chronic inflammation.[248] Because orosomucoid levels are decreased in asymptomatic degenerative disc disease, this suggests that there is less of an inflammatory response in the asymptomatic group, and this may explain why these individuals are asymptomatic. In the case of painful degenerative disc disease, individuals may have higher inflammatory activity, resulting in pain.

α-2-HS-glycoprotein, also known as fetuin-A, is a reverse acute phase reactant, meaning its levels fall in acute inflammation.[249] Interestingly, the levels of α-2-HS-glycoprotein were decreased in asymptomatic degenerative disc disease CSF, suggesting ongoing inflammation may be present in persons with degenerative disc disease. Additionally, α-2-HS-glycoprotein is an antagonist of transforming growth factor-β, as well as bone morphogenetic protein,[250] both of which are known to affect intervertebral disc chondrocytes. Bone morphogenetic protein-2 increases cell proliferation and

proteoglycan synthesis of intervertebral disc cells in cell culture,[251,252] and is used in humans for lumbar interbody fusion.[253,254] Similarly, transforming growth factor-β1 also increases disc cell proliferation in culture,[255] as well as improved interbody bone matrix formation in a sheep model of intervertebral interbody fusion.[256] Because α-2-HS-glycoprotein levels are lower in degenerative disc disease, this could suggest that this may be an incomplete compensatory response to enhance proliferation and proteoglycan synthesis in degenerating discs. This failed compensatory response could result in changes within the disc that facilitate the development of pain.

Prosaposin is a myelinotrophic protein that is secreted after nerve injury, providing trophic support. Prosaposin is secreted by sciatic nerve after transaction in a rat model.[257] In a rat diabetic model, prosaposin mRNA was elevated in peripheral nerve.[258] In our study, the elevated CSF levels of prosaposin in the group with painful degenerative disc disease suggest that nerve injury may be present at the intervertebral discs, and this may contribute to pain. Importantly, there was no increase in prosaposin levels in the CSF of the asymptomatic degenerative disc disease group.

Amyloid-like protein 2 was increased in the CSF of the painful degenerative disc disease group. Cell culture experiments have shown that neuronal PC12 cells, upon trophic factor withdrawal, an apoptotic stimulus, synthesize amyloid-like protein 2.[259] Amyloid-like protein 2 also has neurotrophic activity, and can stimulate neurite outgrowth,[260] and likely plays a role in axonal pathfinding and synaptogenesis.[261] Concordantly, amyloid-like protein 2 may play a role in sprouting of noradrenergic axons into dorsal root ganglia,[244] which is dependent on neurotrophic factors nerve growth factor, brain derived nerve factor, and neurotrophin-3.[245] Amyloid-like protein 2 may be released locally in response to peripheral nerve damage at the intervertebral discs.

N_2,N_2-dimethylguanosine tRNA methyltransferase was increased in the CSF of the asymptomatic degenerative disc disease group. In a mutant mouse generated to be deficient in N_2,N_2-dimethylguanosine tRNA methyltransferase, it was found that these mice had reduced grip strength and performed poorly on the rotorod.[262] However, the effect of increased N_2,N_2-dimethylguanosine tRNA methyltransferase is unknown.

A total of 52 proteins were observed utilizing the acetonitrile precipitation method for protein fractionation. There are a number of reasons for why the number of proteins

found was lower than the 1539 proteins found in a previous study that utilized acetonitrile precipitation.[153] Firstly, in the Abdi *et al* study, CSF proteins were split into three fractions with two precipitation steps, whereas in our study there was only two fractions. Additionally, in the Abdi *et al* study, all three fractions were analyzed by 2D LC MS/MS, whereas in our study, only one fraction was analyzed. It is also important to note that our 2D LC was performed online, with a step-gradient, whereas the 2D LC in Abdi *et al* was performed offline, with a linear gradient. Linear gradients offer superior peptide separation compared to step-gradients.[237] Furthermore, Abdi *et al* precipitated a large amount amount of CSF. 100 μg of each CSF fraction and group was labeled. In our study, only 10 μg of the acetonitrile soluble proteins were labeled. Differences in mass spec technique can also help explain the fewer proteins observed. Abdi *et al* used MALDI to analyze the CSF proteins, whereas in our study we used ESI. MALDI is less sensitive to salts and contaminants, and in addition, is not limited by elution time. With ESI, if many peptides elute from the chromatography column concurrently, peptides with higher intensity ions will be favored over those with lower intensity, and some peptides may be skipped. With MALDI, the mass spectra can sample the peptides many times to obtain information from all the peptides as the peptides are spotted on a plate before analysis. In addition, Abdi *et al* used very low tolerances for protein confirmation. For instance, only one peptide was required to confirm the identity of a protein, and this was the criteria for 793 of the 1539 proteins identified. Instead, in our study, we used a cutoff of 90% confidence as evidence of the protein. To summarize, due to differences in sample processing, mass spectrometry techniques, and data analysis, we observed much fewer proteins than in the Abdi *et al* study.

While only 52 proteins were observed in total, many proteins with differential expression where found. Of these proteins that were differentially expressed, the majority of these have biological activity which could be related to disc degeneration or chronic pain. Cystatin C, prostaglandin D2 synthase, and proSAAS levels were consistent with past reports and the proteoprep 20 experiment. Acute phase proteins such as orosomucoid and α-2-HS-glycoprotein could be involved in disc degeneration as part of the inflammatory response. Growth factors insulin-like growth factor II, prosaposin, and

amyloid-like protein 2 could be factors released from damaged neurons or degenerating discs, suggesting a possible mechanistic link to the production of chronic pain.

9.5 IMPLICATIONS

The main purpose of these experiments was to identify factors that could explain why two individuals could undergo lumbar intervertebral disc degeneration, and yet one individual would develop chronic pain, while the other does not. By using the proteoprep 20 immunodepletion column and by using acetonitrile precipitation, abundant proteins were removed to reveal lower abundance proteins. We successfully observed a number of possible biochemical mediators that may play a role in low back pain due to disc degeneration.

A substantial number of proteins altered in painful disc degeneration where found to be altered in painful degenerative disc disease that can be related to nerve injury and inflammation. Proteins such as apolipoprotein A-IV, apolipoprotein D, apolipoprotein E, hemopexin, neural cell adhesion molecule L1-like protein, insulin-like growth factor II, amyloid-like protein 2, and prosaposin were increased in painful disc degeneration. These proteins have been implicated in nerve damage and growth. Consistent with our results, is the large literature demonstrating that the innervation of painful lumbar discs is deeper and more extensive than those of normal discs. In healthy disc, only the outer part of the disc, the annulus fibrosus is innervated by sensory nerve fibers.[263] In patients with chronic discogenic low back pain, histochemical studies have shown that sensory nerve fibers penetrate into the inner layer of the annulus fibrosus, and even into the nucleus pulposus.[56,264,265] Thus, our results substantiate the idea that nerve growth is involved with discogenic low back pain. This raises the possibility that biochemical markers of nerve growth and regeneration may be useful to screen patients who are likely to develop low back pain.

Furthermore, it is well known that neuropeptides play an important role in the processing and propagation of nociceptive information in the CNS. It is interesting to note that we observed alterations in proSAAS levels in the group with painful degenerative disc disease. Altered levels of proSAAS, a protein involved with neuropeptide processing, could suggest that neuropeptide processing may be changed in

low back pain. In fact, altered neuropeptide processing has been described previously in CSF of patients with painful herniated lumbar discs.[266] It is also possible that particular neuropeptide levels are altered in the CSF of persons with low back pain. For example, preoperative β-lipotropin levels in CSF are thought to be predictive of postoperative pain.[267] Perhaps a neuropeptide marker could also be found for disc degeneration and low back pain.

In our experiments, we found that inflammatory related proteins were altered in CSF of persons with disc degeneration, whether or not they had low back pain. For example, levels of prostaglandin H2-D isomerase, cystatin C, α_1-antichymotrypsin, superoxide dismutase, gelsolin, chromogranin A, β_2-microglobulin, orosomucoid, and alpha-2-HS-glycoprotein, are altered in CSF of individuals with disc degeneration regardless of pain state. While it is well known disc degeneration involves inflammatory processes,[70,196] because the presence of pain did not alter the levels of inflammatory proteins, our results suggest that inflammation itself is not the trigger for pain. Instead, perhaps it is abnormal nerve innervation or altered neuropeptide processing plays the important role in the generation of low back pain, and inflammation occurs merely as a byproduct of the disc degeneration. Nevertheless, it is important to keep in mind that inflammatory mediators are known to influence the release of neurotrophic factors,[268-270] so it may be premature to draw any conclusions yet.

10 FUTURE DIRECTIONS

The results from both the ProteoPrep 20 experiment and the acetonitrile precipitation experiments summarized in Table 6 are preliminary, and will require validation in a larger sample set. To accomplish such a task, MRM technology is perhaps the best suited. This technology allows researchers to create lists of proteins which spectra are then targeted by the mass spectrometer for identification.

This technology has very recently arisen in various forms. Sigma-Aldrich has developed a strategy, called protein AQUA, where synthetic tryptic peptides from selected proteins of interest are created with isotope labeled amino acids. These AQUA peptides are then added to the biological protein sample, and are used as an internal reference standard. Thermo Scientific has developed a similar strategy called

HeavyPeptide. In additional, Applied Biosystems has created new labeling reagents which are MRM compatable, called mTRAQ. Using MRM to target putative biomarkers in CSF would allow high throughput quantitation of protein levels in non-pooled CSF samples, allowing validation of multiple proteins across many samples. In addition, MRM could also be beneficial in illuminating differences at the neuropeptide level, as the mass spectrometer can be set to select proteins or peptides that normally are not abundant enough to be selected in unbiased tandem mass spectrometry.

11 CONCLUSION

The results of this work demonstrate that it is feasible to use proteomics to examine what is occurring at the protein level in degenerative disc disease. This is the first proteomics experiment of CSF from persons with disc degeneration and low back pain. While there has previously been a related study of CSF from persons with painful intervertebral disc herniation, our study is an improvement over their experimental design because of the inclusion of an asymptomatic group with disc degeneration. Thus, we were able to observe differences at the protein level in asymoptomatic degenerative disc disease, and in painful degenerative disc disease. In general, in the CSF of persons with degenerative disc disease, mostly proteins involved with inflammation were observed to be differentially expressed. In those with painful disc degeneration, proteins associated with neuronal damage and mediators of neuropeptide processing were altered. Our results suggest nerve damage and neuropeptide processing may be playing a role in generation and maintenance of chronic back pain due to disc degeneration.

While there were numerous proteins that exhibited differential expression in painful degenerative disc disease, whether or not any of these proteins can meet the criteria of being a biomarker will require validation. For a biomarker to be useful, it must identify at risk individuals with a high degree of accuracy. A false positive result in this context could result in a patient receiving unnecessary surgical intervention, which may even be detrimental or a cause of pain. Likewise, a false negative can result in a patient not receiving important care. To avoid these two possibilities, it is important that the biomarker correlates well with the pathology. As a result, levels of the biomarker should distinctly place individuals into separate groups. In our experimental design, we pooled

the CSF in the different groups so that the intragroup variance was not determinable. Thus, to confirm if any of the differentially expressed proteins can serve as biomarkers, experiments need to be replicated using a method that does not utilize pooling. Additionally, replication should involve larger sample sizes, and relaxed subject selection criteria that are more similar to the cases observed in the clinic, to ensure that the results of the experiments are relevant to the average patient.

Further questions that require answering remain. For example, if the pain in degenerative disc disease is indeed a result of nerve injury, which nerves are the ones being injured, and how is this occurring? Can surgical fusion of the painful discs stop the generation of pain if the source of the pain is neurogenic? Are there biochemical markers that could differentiate individuals who would benefit from surgery, and individuals who would not? These questions and many more will need to be answered in order to help bring new and improved therapies for low back pain to the clinical setting.

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13 APPENDIX

Human ethics certification:

McGill

Faculty of Medicine
3655 Promenade Sir William Osler
Montreal, QC H3G 1Y6

Faculté de médecine
3655, Promenade Sir William Osler
Montréal, QC, H3G 1Y6

Fax/Télécopieur: (514) 398-3595

CERTIFICATION OF ETHICAL ACCEPTABILITY FOR RESEARCH INVOLVING HUMAN SUBJECTS

The Faculty of Medicine Institutional Review Board (IRB) is a registered University IRB working under the published guidelines of the Tri-Council Policy Statement, in compliance with the Plan d'action ministériel en éthique de la recherche et en intégrité scientifique, (MSSS, 1998) and the Food and Drugs Act (17 June 2001); and acts in accordance with the U.S. Code of Federal Regulations that govern research on human subjects. The IRB working procedures are consistent with internationally accepted principles of good clinical practice.

At a full Board meeting on February 26, 2007, the Faculty of Medicine Institutional Review Board, consisting of:

SERGE GAUTHIER, MD	PAUL BRASSARD, MD
MARTIN CHASEN, MD	PIERRE DESCHAMPS, BCL, LSCR
VINCENT GRACCO, PHD	KATHERINE GRAY-DONALD, PHD
MARYLNNE GURSKY, BN, M.ED	MARIGOLD HYDE, B.SC.
HARVEY SIGMAN, MD	SALLY TINGLEY, BCOMM

Examined the research project **A02-M29-07B** entitled ***Analysis of Spinal Fluid and Intervertebral Discs Collected from Low Back Pain Patients (and Controls) at the University of Minnesota***

As proposed by: Dr. Laura S. Stone (Applicant) to _____ (Granting Agency, if any)

And consider the experimental procedures to be acceptable on ethical grounds for research involving human subjects.

February 26, 2007
Date

Chair, IRB

Dean of Faculty

Institutional Review Board Assurance Number: FWA 00004545