Published in final edited form as:

Spine (Phila Pa 1976). 2015 May 1; 40(9): 601–612. doi:10.1097/BRS.0000000000000831.

Proteomic Analysis of Cerebrospinal Fluid in Canine Cervical Spondylomyelopathy

Paula Martin-Vaquero, DVM, PhD, Dipl. ACVIM (Neurology)^{*‡}, Ronaldo C. da Costa, DMV, PhD, Dipl. ACVIM (Neurology)^{*}, Matthew J. Allen, Vet MB, PhD^{*}, Sarah A. Moore, DVM, Dipl. ACVIM (Neurology)^{*}, Jeremy K. Keirse, MS[†], and Kari B. Green, PhD[§]

^{*}Department of Veterinary Clinical Sciences, The Ohio State University, College of Veterinary Medicine, Columbus, OH

[†]Mass Spectrometry and Proteomics Facility, The Ohio State University, College of Medicine, Department of Molecular and Cellular Biochemistry, Columbus, OH

[§]Department of Chemistry, University of Florida

[‡]Dr. Martin-Vaquero's current address is Centro Médico Veterinario Delicias, Calle Delicias 35, C.P. 28045, Madrid, Spain

Abstract

Study Design—Prospective study.

Objective—To identify proteins with differential expression in the cerebrospinal fluid (CSF) from 15 clinically normal (control) dogs and 15 dogs with cervical spondylomyelopathy (CSM).

Summary of Background Data—Canine CSM is a spontaneous, chronic, compressive cervical myelopathy similar to human cervical spondylotic myelopathy. There is a limited knowledge of the molecular mechanisms underlying these conditions. Differentially expressed CSF proteins may contribute with novel information about the disease pathogenesis in both dogs and humans.

Methods—Protein separation was performed with two-dimensional electrophoresis. A Student's *t*-test was used to detect significant differences between groups ($P < 0.05$). Three comparisons were made: 1) control versus CSM-affected dogs, 2) control versus non-corticosteroid treated CSM-affected dogs, and 3) non-corticosteroid treated CSM-affected versus corticosteroid treated CSM-affected dogs. Protein spots exhibiting at least a statistically significant 1.25-fold change between groups were selected for subsequent identification with capillary-liquid chromatography tandem mass spectrometry.

Results—A total of 96 spots had a significant average change of at least 1.25-fold in one of the three comparisons. Compared to the CSF of control dogs, CSM-affected dogs demonstrated increased CSF expression of eight proteins including vitamin D-binding protein, gelsolin, creatine kinase B-type, angiotensinogen, alpha-2-HS-glycoprotein, SPARC, calyculin-1, and

complement C3, and decreased expression of pigment epithelium-derived factor, prostaglandin-H2 D-isomerase, apolipoprotein E, and clusterin.

In the CSF of CSM-affected dogs, corticosteroid treatment increased the expression of haptoglobin, transthyretin isoform 2, cystatin C-like, apolipoprotein E, and clusterin, and decreased the expression of angiotensinogen, alpha-2-HS-glycoprotein, and gelsolin.

Conclusions—Many of the differentially expressed proteins are associated with damaged neural tissue, bone turnover, and/or compromised blood-spinal cord barrier. The knowledge of the protein changes that occur in CSM and upon corticosteroid treatment of CSM-affected patients will aid in further understanding the pathomechanisms underlying this disease.

Keywords

biomarker; cervical spine; DIGE; dog; electrophoresis; Great Dane; mass spectrometry; myelopathy; osseous-associated cervical spondylomyelopathy; proteomics; spinal cord; stenosis; wobbler syndrome

Introduction

Cervical spondylotic myelopathy is a degenerative condition leading to cervical spinal canal narrowing and chronic, progressive spinal cord (SC) compression.¹⁻³ While surgical intervention can attenuate disease progression and improve the neurological status, many patients are left with substantial neurological impairment.^{3,4} Therefore, researchers are actively investigating adjuvant neuroprotective approaches to improve outcome.^{3,4} Gaps in the knowledge of the disease pathobiology have limited therapeutic advances.^{3,5} Animal models of cervical spondylotic myelopathy have assisted in further understanding the disease pathomechanisms but many of these models have limitations, as they often do not accurately reflect the chronic, progressive nature of the human condition, nor the clinical diversity of the disease.^{2,3,5-8} As such, there is an urgent need to establish new animal models that more accurately simulate this disease.

Canine cervical spondylomyelopathy (CSM) is a spontaneous large animal model of cervical spondylotic myelopathy. In CSM, static and dynamic compressions of the cervical SC and nerve roots originate ataxia, weakness, and pain.⁹ There are two forms of canine CSM.⁹ In disc-associated CSM, there is ventral SC compression caused by intervertebral disc protrusion.⁹⁻¹² Osseous-associated CSM is caused by osteoarthritic changes of the cervical vertebrae, originating lateral and/or dorsolateral SC compression.^{9,12-15} Medical and surgical therapies are available for canine CSM but there is no consensus on the best treatment option.^{9,16-23} As with cervical spondylotic myelopathy, treatment of canine CSM yields variable results, and recurrences and clinical deterioration may be seen months to years after therapy.^{9,16,17,23} Thus, it is necessary to optimize current treatments and investigate novel therapies that will improve recovery and outcome in CSM-affected dogs. In both the human and canine conditions, a major roadblock to this is the limited understanding of the molecular mechanisms underlying the disease pathogenesis.^{2,3,9,11}

Proteomics approaches utilizing cerebrospinal fluid (CSF) to investigate the pathogenesis of neurological diseases are rapidly growing.²⁴⁻²⁸ However, limited data exists on the use of

proteomics in human cervical spondylotic myelopathy, or its canine counterpart, CSM.^{29,30} Our objective was to compare the CSF proteome of clinically normal (control) dogs and dogs with osseous-associated CSM to identify CSF proteins that could enhance our understanding of the disease pathogenesis. We hypothesized that the CSF proteome of CSM-affected dogs would differ significantly from that of control dogs. When compared to rodent models of human cervical spondylotic myelopathy, canine CSM more closely approximates the human condition. Canine CSM is a spontaneous chronic, progressive cervical myelopathy, human size is more comparable to canines than to rodents, and CSM-affected dogs receive high quality medical care including diagnostic evaluations and treatments similar to those of people with cervical spondylotic myelopathy. The similarities between canine CSM and human cervical spondylotic myelopathy suggest that results from this study could also be relevant for the human condition.

Materials and Methods

Collection and Storage of CSF

The investigation was conducted in accordance with the guidelines and with approval of the institution's Clinical Research Advisory Committee and the Institutional Animal Care and Use Committee. Two groups of client-owned Great Danes were prospectively enrolled between April 2011 and October 2012. Written owner consent was obtained prior to enrollment. The first group included 15 dogs defined as clinically normal based on a normal neurologic examination and no history of neurologic disease. The second group included 15 dogs with clinical signs and neurologic examination findings consistent with CSM and confirmation via magnetic resonance imaging (MRI). Whether or not patients were being administered corticosteroids at the time of enrollment was recorded. Cerebellomedullary cistern CSF samples (1-2 mL) were collected under general anesthesia into sterile tubes without anticoagulant. The CSF from each dog was immediately centrifuged at 3000 rpm for 8 minutes to remove cellular materials. The supernatants were collected and stored at -80°C until analyzed.

Sample Preparation, Labeling, and Two-Dimensional (2D) Electrophoresis Separation

Proteins were extracted from CSF samples using a 2D cleanup kit (GE, 80-6484-51), resuspended in 100 μL of lysis buffer (30M Tris pH 8.5, 7M Urea, 2M Thiourea, 4% CHAPS) and quantitated by Bradford assay using BSA as a standard. Cy-dye labeling, isoelectric focusing and gel electrophoresis of 2D-cleaned CSF samples were performed according to standard DIGE protocols.³¹ Labeled samples were combined (1 Cy3, 1 Cy5 and 25 μg of Cy2 sample/gel) and diluted 2X with rehydration buffer [7M Urea, 2M thiourea, 2% CHAPS, 1% pH 3-10 IPG buffer (GE Healthcare), 50mM DTT, 1% saturated bromophenol blue solution] to a final volume of 450 μL and then isoelectric focused on an IPGphor II (GE Healthcare) instrument using standard isoelectric focusing protocols for pH 3-10 strips (GE Healthcare). Finally, pH strips were reduced and alkylated, placed in 20x24cm 12% SDS-PAGE gels, and run in a Dalt-12 electrophoresis system (GE Healthcare) at 2 watts per gel for 45 minutes, followed by 15 watts per gel until the dye front reached the bottom (~4 hours).

Image Acquisition and DeCyder Analysis

Gels were scanned in a Typhoon 9400 variable mode scanner (GE Healthcare) using the appropriate settings for CyDye fluorophors at 100-micron resolution. Preparative gels (for spot picking and protein identification) were stained with Lava purple general protein stain (Gel Company) according to standard protocols. Gel images were loaded into DeCyder 2D software (GE Healthcare) and analyzed individually. Log-standardized abundance was the variable subjected to statistical analysis. A Student's *t*-test was used to detect differences between CSM-affected and control GD ($P<0.05$). The following three comparisons were made in Decyder: 1) control versus CSM-affected dogs, 2) control versus non-corticosteroid treated CSM-affected dogs, and 3) non-corticosteroid treated CSM-affected versus corticosteroid treated CSM-affected dogs. For each comparison, spots exhibiting a statistically significant change of at least 1.25-fold were selected for subsequent identification.

Protein Identification

The Ettan Spot Handling Workstation was used to core, digest and extract protein spots of interest (User Manual, Amersham Biosciences). Gel spots were washed, dehydrated, digested using 50 μ L of sequencing grade-modified trypsin (5 μ g/mL in 50 mM ammonium bicarbonate) containing 0.01% ProteaseMax (Promega, Madison WI), and extracted according to standard protocols. Peptide sequences were determined by capillary-liquid chromatography tandem mass spectrometry (Cap-LC/MS/MS) using an UltiMate™ 3000 LC system and an LTQ mass spectrometer (both from Thermo Scientific) equipped with a CaptiveSpray source (Bruker Daltonics Billerica, MA) operated in positive ion mode according to standard methods in the OSU CCIC Mass Spectrometry and Proteomics Facility.³² The MS/MS data acquired was converted into mascot generic files (.mgf) using MS Convert (ProteoWizard) and the resulting files were searched against all NCBI nr other mammalia using Mascot Daemon by Matrix Science version 2.2.1 (Boston, MA) setting the mass tolerance of the precursor ions and fragment ions at 1.8 and 0.8 Da, respectively. Considered modifications were oxidation, deamidation and carbamidomethylation. Protein identifications were checked manually and only proteins with a Mascot score of 100 or higher with a minimum of two unique bold red peptides were accepted.

Results

The characteristics of the dogs enrolled are summarized in Table 1. Analysis with DeCyder revealed 96 total spots with a statistically significant average change of at least 1.25-fold in one of the three comparisons (Tables 2-4). Table 2 summarizes the proteins that demonstrated differential expression when comparing the CSF of control and CSM-affected dogs. Table 3 includes the proteins that were differentially expressed in the CSF of control dogs versus non-corticosteroid treated CSM-affected dogs. Proteins exhibiting differential expression between the CSF of non-corticosteroid treated CSM-affected and corticosteroid treated CSM-affected dogs are shown in Table 4. Positive values for fold change indicate greater expression in the CSF of CSM-affected dogs when compared to control dogs (Table 2), non-corticosteroid treated CSM-affected dogs compared to control dogs (Table 3), and corticosteroid treated CSM-affected dogs compared to non-corticosteroid treated CSM-

affected dogs (Table 4). Compared to the CSF of control dogs, CSM-affected dogs demonstrated increased CSF expression of eight proteins including vitamin D-binding protein (DBP), gelsolin, creatine kinase B-type (CK-BB), angiotensinogen, alpha-2-HS-glycoprotein, SPARC, calstactin-1, and complement C3, and decreased expression of pigment epithelium-derived factor (PEDF), prostaglandin-H2 D-isomerase (PGH2), apolipoprotein E (APOE), and clusterin. Apolipoprotein E and clusterin showed decreased expression in CSM-affected dogs when compared to control dogs (Table 3) but their expression increased in those CSM-affected dogs that were receiving corticosteroids (Tables 2 and 4). Haptoglobin, transthyretin isoform 2, and cystatin C-like were upregulated in CSM-affected dogs but their upregulation appeared to be related to corticosteroid-treatment (Tables 2 and 4), since this upregulation was not observed when the effect of corticosteroids was removed (Table 3). Corticosteroid treatment decreased the CSF expression of angiotensinogen, alpha-2-HS-glycoprotein, and gelsolin in CSM-affected dogs. Table 5 classifies the key proteins identified in this study according to their main functional category.

Discussion

Several proteins showed differential expression in the CSF of clinically normal and CSM-affected dogs. Additionally, corticosteroid administration appeared to alter the expression of some proteins in CSM-affected dogs.

Vitamin D-binding protein participates in vitamin D transport, actin scavenging, and macrophage and osteoclast activation.³³⁻³⁵ This protein was upregulated in CSM-affected dogs consistent with various human neurological diseases.^{27,33,34,36,37} Since DBP has limited passage through the intact blood-brain barrier,^{34,38} elevated CSF DBP in canine CSM suggests blood-SC barrier compromise. Blood-SC barrier disruption was reported in a rodent model of human cervical spondylotic myelopathy³ and it may be present in CSM-affected dogs. Gelsolin was also upregulated in CSM-affected dogs. Both DBP and gelsolin act as actin regulatory proteins and actin scavengers.^{27,34,39,40} Actin is released secondary to axonal degeneration,^{34,39} which is a feature of canine CSM.¹² The upregulation of CSF DBP and gelsolin in CSM-affected dogs may be secondary to axonal damage and actin excess, and result from an attempt to promote actin reorganization and tissue regrowth.⁴¹ As such, these two proteins could be useful as indirect markers of damaged neural tissue.

Creatine kinase B-type is predominantly found in neurons and astrocytes,^{42,43} and was upregulated in CSM-affected dogs. Increased CSF CK-BB has been reported in other human and animal myelopathic disorders.⁴⁴⁻⁴⁶ High CSF CK-BB activity is associated with white matter damage and myelin degeneration,⁴⁶ which are present in the SC of CSM-affected dogs,¹² and may explain the results obtained in this study.

Angiotensinogen is a serine protease inhibitor.⁴⁷ Serine proteases promote cartilage destruction in osteoarthritis.^{48,49} Here, greater expression of CSF angiotensinogen in CSM-affected dogs, which had marked osteoarthritic changes of their cervical vertebrae, might be a compensatory increase in an attempt to inhibit the excess of protease activity that occurs in osteoarthritis. Similarly, alpha-2-HS-glycoprotein (also called fetuin-A) was upregulated in

CSM-affected dogs. Alpha-2-HS-glycoprotein regulates calcium metabolism and osteogenesis and increases during high bone turnover.⁵⁰⁻⁵³ The upregulation of alpha-2-HS-glycoprotein in canine CSM may also be secondary to the presence of osteoarthritic changes of the cervical vertebrae.

The glycoprotein SPARC (osteonectin) participates in bone development and mineralization and tissue remodeling/turnover.⁵⁴⁻⁵⁶ Decreased expression of SPARC is associated with osteopenia, whereas elevated expression is seen in osteoarthritis.^{55,57-59} Increased CSF SPARC in non-corticosteroid treated CSM-affected dogs suggests this protein may be involved in the pathogenesis of the vertebral osteoarthritic changes seen in canine CSM. Dexamethasone can decrease SPARC expression,⁵⁷ which may explain the lack of SPARC upregulation in corticosteroid treated CSM-affected dogs. Calsynenin-1 is a calcium-binding transmembrane protein of the neuronal postsynaptic membrane,^{60,61} and also showed increased expression in CSM-affected dogs. Altered CSF concentrations of calsynenin-1 were present in various human neurodegenerative diseases.^{25,62}

Complement C3 is a central component of the complement cascade.⁶³ Human and dogs with osteoarthritis had increased expression of complement C3.^{49,64,65} In this study, all CSM-affected dogs had osteoarthritic changes of their cervical vertebrae. The upregulation of complement C3 in CSM-affected dogs suggests this protein could play a role in these osteoarthritic changes. Inhibition of complement C3 ameliorated clinical signs of arthritis in mice,⁶⁶ and has been suggested as a targeted therapeutic approach in human osteoarthritis and traumatic SC injury.^{67,68} Complement inhibition could also be an attractive therapeutic strategy in canine CSM.

The PEDF CSF concentration was downregulated in CSM-affected dogs, consistent with a study that showed decreased PEDF CSF concentration in human cervical spondylotic myelopathy.²⁹ PEDF has neuroprotective functions capable of protecting SC motor neurons from glutamate-induced injury.^{69,70} Glutamate excitotoxicity can sustain SC neural degeneration in human cervical spondylotic myelopathy,² and it is possible that the same occurs in canine CSM. Lower CSF PEDF in CSM-affected dogs might make them more vulnerable to SC glutamate excitotoxicity. Given the potent neuroprotective functions of PEDF, this molecule is being considered as a potential therapeutic agent in human neurodegenerative diseases,^{71,72} and might be useful in canine CSM.

The PGH2 CSF concentration was also decreased in CSM-affected dogs. Prostaglandin-H2 D-isomerase (prostaglandin D synthetase or β -trace protein) is a glycoprotein with high abundance in CSF, which is often altered in neurological disorders.⁷³⁻⁷⁹ When there is blood-CSF barrier leakage, PGH2 CSF concentration decreases due to increased diffusion from CSF to serum.^{73,74} The blood-SC barrier may be compromised in canine CSM causing leakage of PGH2 from CSF to serum and lower PGH2 CSF in CSM-affected dogs.

Apolipoprotein E and clusterin were downregulated in CSM-affected dogs but their expression increased secondary to corticosteroid use. In humans, the APOE4 isoform is associated with an elevated risk for several neurological disorders.^{80,81} In human cervical spondylotic myelopathy, the APOE4 isoform increased the risk of developing clinical signs

of myelopathy, and was associated with a worse surgical outcome.^{82,83} Given the involvement of APOE in human neurological disorders and the differential APOE expression between control and CSM-affected dogs, additional studies may be warranted to further investigate a possible role for this protein in the pathogenesis of canine CSM. Corticosteroid use can induce APOE expression,⁸⁴⁻⁸⁶ consistent with the results of this study.

Clusterin is a glycoprotein with various functions including an anti-apoptotic role, involvement in neuronal survival, and complement inhibitory properties.⁸⁷⁻⁹² Neuronal and oligodendrocyte apoptosis occur in human cervical spondylotic myelopathy⁹³ and canine CSM⁹⁴, and are implicated in disease progression.³ The lower CSF clusterin concentrations identified in CSM-affected dogs may compromise neuronal and oligodendrocyte survival by promoting apoptosis. Moreover, low clusterin may originate increased complement activation, which is supported by the results of this study, since CSM-affected dogs had increased complement C3 CSF expression. Clusterin treatment accelerated recovery of nerve function in a rodent model of peripheral neuropathy and it represents a promising targeted therapy.^{87,90,95} The use of corticosteroids increased the expression of clusterin in CSM-affected dogs, consistent with other studies.⁹⁵

Haptoglobin, transthyretin isoform 2, and cystatin C-like were upregulated in CSM-affected dogs in a corticosteroid-dependent manner. Haptoglobin is an acute phase protein⁹⁶⁻⁹⁸ and cystatin C is a proteinase inhibitor abundant in CSF.⁹⁹⁻¹⁰¹ In this study, these proteins were only upregulated in corticosteroid-treated CSM-affected dogs, which is consistent with a corticosteroid-related increase as described in other studies.¹⁰²⁻¹⁰⁴ Transthyretin is a neuroprotective negative acute phase protein and it is often downregulated in human neurological diseases.^{26,97} Conversely, transthyretin was upregulated in CSM-affected dogs. This upregulation is likely secondary to corticosteroid use, since both stress and corticosteroids can increase transthyretin expression in the choroid plexus.¹⁰⁵ Corticosteroid treatment can also decrease the CSF concentrations of multiple proteins.^{106,107}

Limitations of this study include the limited sample size and that several CSM-affected dogs were receiving corticosteroids at the time of CSF collection. This could have hindered the identification of additional differentially expressed proteins but allowed us to identify proteins that were specifically altered by corticosteroid treatment in CSM-affected dogs.

This study compared the CSF proteome of clinically normal and CSM-affected dogs. Many of the differentially expressed proteins are associated with damaged neural tissue, bone turnover, and/or compromised blood-SC barrier, which suggests the potential value of these proteins as biomarkers for canine CSM. The knowledge of the protein changes that occur in CSM and upon corticosteroid treatment of CSM-affected patients will aid in further understanding the pathomechanisms underlying this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The manuscript submitted does not contain information about medical device(s)/drug(s). Funds received in support of this work: Great Dane Club of America, the Gray Lady Foundation, an Intramural Canine grant from The Ohio State University, College of Veterinary Medicine, and the Award Number Grant UL1TR000090 for The Ohio State University Center for Clinical and Translational Science (CCTS) from the National Center For Advancing Translational Sciences.

Relevant financial activities outside the submitted work: grants/grants pending, payment for development of educational presentations, stock, travel/accommodations/meeting expenses, employment, consultancy

REFERENCES

1. Fehlings MG, Tetreault LA, Wilson JR, et al. Cervical spondylotic myelopathy: current state of the art and future directions. *Spine*. 2013; 38(Suppl 1):S1–8. [PubMed: 23962994]
2. Kalsi-Ryan S, Karadimas SK, Fehlings MG. Cervical spondylotic myelopathy: the clinical phenomenon and the current pathobiology of an increasingly prevalent and devastating disorder. *Neuroscientist*. 2013; 19:409–21. [PubMed: 23204243]
3. Karadimas SK, Moon ES, Yu WR, et al. A novel experimental model of cervical spondylotic myelopathy (CSM) to facilitate translational research. *Neurobiol Dis*. 2013; 54:43–58. [PubMed: 23466695]
4. Fehlings MG, Wilson JR, Yoon T, et al. Symptomatic progression of cervical myelopathy and the role of nonsurgical management: a consensus statement. *Spine*. 2013; 38(Suppl 1):S19–20. [PubMed: 23963011]
5. Karadimas SK, Erwin WM, Ely CG, et al. Pathophysiology and natural history of cervical spondylotic myelopathy. *Spine*. 2013; 38(Suppl 1):S21–36. [PubMed: 23963004]
6. Karadimas SK, Gialeli CH, Klironomos G, et al. The role of oligodendrocytes in the molecular pathobiology and potential molecular treatment of cervical spondylotic myelopathy. *Curr Med Chem*. 2010; 17:1048–58. [PubMed: 20156160]
7. Klironomos G, Karadimas S, Mavrakis A, et al. New experimental rabbit animal model for cervical spondylotic myelopathy. *Spinal Cord*. 2011; 49:1097–102. [PubMed: 21788956]
8. Lee J, Satkunendrarajah K, Fehlings MG. Development and characterization of a novel rat model of cervical spondylotic myelopathy: the impact of chronic cord compression on clinical, neuroanatomical, and neurophysiological outcomes. *J Neurotrauma*. 2012; 29:1012–27. [PubMed: 21561323]
9. da Costa RC. Cervical spondylomyelopathy (Wobbler syndrome) in dogs. *Vet Clin North Am Small Anim Pract*. 2010; 40:881–913. [PubMed: 20732597]
10. Seim HB, Withrow SJ. Pathophysiology and diagnosis of caudal cervical spondylomyelopathy with emphasis on the Doberman Pinscher. *J Am Anim Hosp Assoc*. 1982; 18:241–51.
11. De Decker S, da Costa RC, Volk HA, et al. Current insights and controversies in the pathogenesis and diagnosis of disc-associated cervical spondylomyelopathy in dogs. *Vet Rec*. 2012; 171:531–7. [PubMed: 23180710]
12. Trotter EJ, de Lahunta A, Geary JC, et al. Caudal cervical vertebral malformation-malarticulation in Great Danes and Doberman Pinschers. *J Am Vet Med Assoc*. 1976; 68:917–30. [PubMed: 1270334]
13. Gutierrez-Quintana R, Penderis J. MRI features of cervical articular process degenerative joint disease in Great Dane dogs with cervical spondylomyelopathy. *Vet Radiol Ultrasound*. 2012; 53:304–11. [PubMed: 22236021]
14. Martin-Vaquero P, da Costa RC, Drost WT. Comparison of noncontrast computed tomography and high-field magnetic resonance imaging in the evaluation of Great Danes with cervical spondylomyelopathy. *Vet Radiol Ultrasound*. 2014 doi:10.1111/vru.12148 [Epub ahead of print].
15. Martin-Vaquero P, da Costa RC. Evaluation of traditional and novel radiographic vertebral ratios in Great Danes with versus without cervical spondylomyelopathy. *Vet Radiol Ultrasound*. 2014 doi:10.1111/vru.12159 [Epub ahead of print].

16. De Risio L, Muñana K, Murray M, et al. Dorsal laminectomy for caudal cervical spondylomyelopathy: postoperative recovery and long-term follow-up in 20 dogs. *Vet Surg.* 2002; 31:418–27. [PubMed: 12209412]
17. Lewis M, Olby NJ, Sharp NJH, et al. Long-term effect of cervical distraction and stabilization on neurological status and imaging findings in giant breed dogs with cervical stenotic myelopathy. *Vet Surg.* 2013; 42:701–709. [PubMed: 23889830]
18. McKee WM, Butterworth SJ, Scott HW. Management of cervical spondylopathy-associated intervertebral disc protrusions using metal washers in 78 dogs. *J Small Anim Pract.* 1999; 40:465–72. [PubMed: 10587923]
19. Dixon BC, Tomlinson JL, Kraus KH. Modified distraction-stabilization technique using and interbody polymethylmethacrylate plug in dogs with caudal cervical spondylomyelopathy. *J Am Vet Med Assoc.* 1996; 208:61–8. [PubMed: 8682707]
20. De Decker S, Bhatti SFM, Duchateau L, et al. Clinical evaluation of 51 dogs treated conservatively with disc-associated wobbler syndrome. *J Small Anim Pract.* 2009; 50:136–42. [PubMed: 19261084]
21. da Costa RC, Parent JM, Holmberg DL, et al. Outcome of medical and surgical treatment in dogs with cervical spondylomyelopathy: 104 cases. *J Am Vet Med Assoc.* 2008; 233:1284–90. [PubMed: 18922055]
22. Jeffery ND, McKee WM. Surgery for disc-associated wobbler syndrome in the dog - an examination of the controversy. *J Small Anim Pract.* 2001; 42:574–81. [PubMed: 11791771]
23. da Costa RC, Parent JM. One-year clinical and magnetic resonance follow-up of Doberman Pinschers with cervical spondylomyelopathy treated medically or surgically. *J Am Vet Med Assoc.* 2007; 231:243–50. [PubMed: 17630890]
24. Romeo MJ, Espina V, Lowenthal M, et al. CSF proteome: a protein repository for potential biomarker identification. *Expert Rev Proteomics.* 2005; 2:57–70. [PubMed: 15966853]
25. Hammack BN, Fung KY, Hunsucker SW, et al. Proteomic analysis of multiple sclerosis cerebrospinal fluid. *Mult Scler.* 2004; 10:245–60. [PubMed: 15222687]
26. Kolarcik C, Bowser R. Plasma and cerebrospinal fluid-based protein biomarkers for motor neuron disease. *Mol Diagn Ther.* 2006; 10:281–92. [PubMed: 17022691]
27. Liu XD, Zeng BF, Zhu HB, et al. Proteomics analysis of the cerebrospinal fluid of patients with lumbar disk herniation. *Proteomics.* 2006; 6:1019–28. [PubMed: 16372267]
28. Nakamura K, Miyasho T, Nomura S, et al. Proteome analysis of cerebrospinal fluid in healthy beagles and canine encephalitis. *J Vet Med Sci.* 2012; 74:751–6. [PubMed: 22251802]
29. Jenis, LG.; Banco, RJ. [Accessed May 28, 2014] Proteomic analysis of CSF biomarkers in cervical myelopathy. Feb. 2012 Available at: <http://www.aaos.org/education/anmeet/anmeet.aspabstract>
30. Eun JP, Ma TZ, Lee WJ, et al. Comparative analysis of serum proteomes to discover biomarkers for ossification of the posterior longitudinal ligament. *Spine.* 2007; 32:728–34. [PubMed: 17414905]
31. Whitehill JG, Popova-Butler A, Green-Church KB, et al. Interspecific proteomic comparisons reveal ash phloem genes potentially involved in constitutive resistance to the emerald ash borer. *PLoS One.* 2011; 6:e24863. [PubMed: 21949771]
32. Reddish JM, St-Pierre N, Nichols A, et al. Proteomic analysis of proteins associated with body mass and length in yellow perch, *Perca flavescens*. *Proteomics.* 2008; 8:2333–43. [PubMed: 18452223]
33. Disanto G, Ramagopalan SV, Para AE, et al. The emerging role of vitamin D binding protein in multiple sclerosis. *J Neurol.* 2011; 258:353–8. [PubMed: 21042807]
34. Yang M, Qin Z, Zhu Y, et al. Vitamin D-binding protein in cerebrospinal fluid is associated with multiple sclerosis progression. *Mol Neurobiol.* 2013; 47:946–56. [PubMed: 23339019]
35. Gomme PT, Bertolini J. Therapeutic potential of vitamin D-binding protein. *Trends Biotechnol.* 2004; 22:340–5. [PubMed: 15245906]
36. Xiao F, Chen D, Lu Y, et al. Proteomic analysis of cerebrospinal fluid from patients with idiopathic temporal lobe epilepsy. *Brain Res.* 2009; 1255:180–9. [PubMed: 19109932]

37. Zhang J, Sokal I, Peskind ER, et al. CSF multianalyte profile distinguishes Alzheimer and Parkinson diseases. *Am J Clin Pathol.* 2008; 129:526–9. [PubMed: 18343778]
38. Pardridge WM, Sakiyama R, Coty WA. Restricted transport of vitamin D and A derivatives through the rat blood-brain barrier. *J Neurochem.* 1985; 44:1138–41. [PubMed: 3838342]
39. Lee WM, Galbraith RM. The extracellular actin-scavenger system and actin toxicity. *N Engl J Med.* 1992; 326:1335–41. [PubMed: 1314333]
40. Peng X, Zhang X, Wang L, et al. Gelsolin in cerebrospinal fluid as a potential biomarker of epilepsy. *Neurochem Res.* 2011; 36:2250–8. [PubMed: 21786170]
41. Ghermann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immune effector cell of the brain. *Brain Res Brain Res Rev.* 1995; 20:269–87. [PubMed: 7550361]
42. Dawson DM, Eppenberger HM, Kaplan NO. Creatine kinase: evidence for a dimeric structure. *Biochem Biophys Res Commun.* 1965; 21:346–353. [PubMed: 5865501]
43. Dawson DM, Fine IH. Creatine kinase in human tissue. *Arch Neurol.* 1967; 16:175–180. [PubMed: 6018047]
44. Pa ao lu A, Pa ao lu H. Enzymatic changes in the cerebrospinal fluid as indices of pathological change. *Acta Neurochir (Wien).* 1989; 97:71–6. [PubMed: 2718797]
45. Witsberger TH, Levine JM, Fosgate GT, et al. Associations between cerebrospinal fluid biomarkers and long-term neurologic outcome in dogs with acute intervertebral disk herniation. *J Am Vet Med Assoc.* 2012; 240:555–62. [PubMed: 22332624]
46. Jackson C, de Lahunta A, Divers T, et al. The diagnostic utility of cerebrospinal fluid creatine kinase activity in the horse. *J Vet Intern Med.* 1996; 10:246–51. [PubMed: 8819050]
47. Wong MK, Takei Y. Characterization of a native angiotensin from an anciently diverged serine protease inhibitor in lamprey. *J Endocrinol.* 2011; 209:127–37. [PubMed: 21220408]
48. Milner JM, Patel A, Rowan AD. Emerging roles of serine proteinases in tissue turnover in arthritis. *Arthritis Rheum.* 2008; 58:3644–56. [PubMed: 19035508]
49. Garner BC, Kuroki K, Stoker AM, et al. Expression of proteins in serum, synovial fluid, synovial membrane, and articular cartilage samples obtained from dogs with stifle joint osteoarthritis secondary to cranial cruciate ligament disease and dogs without stifle joint arthritis. *Am J Vet Res.* 2013; 74:386–94. [PubMed: 23438113]
50. Heiss A, Eckert T, Aretz A, et al. Hierarchical role of fetuin-A and acidic serum proteins in the formation and stabilization of calcium phosphate particles. *J Biol Chem.* 2008; 283:14815–25. [PubMed: 18364352]
51. Szweras M, Liu D, Partridge A, et al. Alpha 2-HS glycoprotein/fetuin, a transforming growth factors-beta/bone morphogenetic protein antagonist, regulates postnatal bone growth and remodeling. *J Biol Chem.* 2002; 277:19991–7. [PubMed: 11901155]
52. Sritara C, Thakkestian A, Ongphiphadhanakul B, et al. Causal relationship between the AHSG gene and BMD through fetuin-A and BMI: multiple mediation analysis. *Osteoporos Int.* 2014; 25:1555–62. [PubMed: 24570294]
53. Dickson IR, Gwilliam R, Arora M, et al. Lumbar vertebral and femoral neck bone mineral density are higher in postmenopausal women with the alpha 2HS-glycoprotein 2 phenotype. *Bone Miner.* 1994; 24:181–8. [PubMed: 8019205]
54. Yan Q, Sage EH. SPARC, a matricellular glycoprotein with important biological functions. *J Histochem Cytochem.* 1999; 47:1495–506. [PubMed: 10567433]
55. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. *Matrix Biol.* 2001; 19:816–27. [PubMed: 11223341]
56. Ribeiro N, Sousa SR, Brekken RA, et al. Role of SPARC in bone remodeling and cancer-related bone metastasis. *J Cell Biochem.* 2014; 115:17–26. [PubMed: 24038053]
57. Nakamura S, Kamihagi K, Satakeda H, et al. Enhancement of SPARC (osteonectin) synthesis in arthritic cartilage. Increased levels in synovial fluids from patients with rheumatoid arthritis and regulation by growth factors and cytokines in chondrocyte cultures. *Arthritis Rheum.* 1996; 39:539–51. [PubMed: 8630101]
58. Nanba Y, Nishida K, Yoshikawa T, et al. Expression of osteonectin in articular cartilage of osteoarthritic knees. *Acta Med Okayama.* 1997; 51:239–43. [PubMed: 9359920]

59. Delany AM, Amling M, Priemel H, et al. Osteopenia and decreased bone formation in osteonectin-deficient mice. *J Clin Invest.* 2000; 105:915–23. [PubMed: 10749571]
60. Vogt L, Schrimpf SP, Meskenaite V, et al. Calsynenin-1, a proteolytically processed postsynaptic membrane protein with a cytoplasmic calcium-binding domain. *Mol Cell Neurosci.* 2001; 17:151–66. [PubMed: 11161476]
61. Hintsch G, Zurlinden A, Meskenaite V, et al. The calsynenins – a family of postsynaptic membrane proteins with distinct neuronal expression patterns. *Mol Cell Neurosci.* 2002; 21:393–409. [PubMed: 12498782]
62. Yin GN, Lee HW, Cho JY, et al. Neuronal pentraxin receptor in cerebrospinal fluid as a potential biomarker for neurodegenerative diseases. *Brain Res.* 2009; 1265:158–70. [PubMed: 19368810]
63. Delanghe JR, Speeckaert R, Speeckaert MM. Complement C3 and its polymorphism: biological and clinical consequences. *Pathology.* 2014; 46:1–10. [PubMed: 24300728]
64. Gobezie R, Kho A, Krastins B, et al. High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. *Arthritis Res Ther.* 2007; 9:R36. [PubMed: 17407561]
65. Gharbi M, Sanchez C, Mazzucchelli G, et al. Identification of differential pattern of protein expression in canine osteoarthritis serum after anterior cruciate ligament transection: a proteomic analysis. *Vet J.* 2014; 197:848–53. [PubMed: 23831215]
66. Song H, Qiao F, Atkinson C, et al. A complement C3 inhibitor specifically targeted to sites of complement activation effectively ameliorates collagen-induced arthritis in DBA/1J mice. *J Immunol.* 2007; 179:7860–7. [PubMed: 18025232]
67. Holers VM. The spectrum of complement alternative pathway-mediated diseases. *Immunol Rev.* 2008; 223:300–16. [PubMed: 18613844]
68. Qiao F, Atkinson C, Song H, et al. Complement plays an important role in spinal cord injury and represents a therapeutic target for improving recovery following trauma. *Am J Pathol.* 2006; 169:1039–47. [PubMed: 16936276]
69. Bilak MM, Corse AM, Bilak SR, et al. Pigment epithelium-derived factor (PEDF) protects motor neurons from chronic glutamate-mediated neurodegeneration. *J Neuropathol Exp Neurol.* 1999; 58:719–28. [PubMed: 10411342]
70. Yamagishi S, Inagaki Y, Takeuchi M, et al. Is pigment epithelium-derived factor level in cerebrospinal fluid a promising biomarker for early diagnosis of Alzheimer's disease? *Med Hypotheses.* 2004; 63:115–7. [PubMed: 15193361]
71. Yabe T, Sanagi T, Yamada H. The neuroprotective role of PEDF: implication for the therapy of neurological disorders. *Curr Mol Med.* 2010; 10:259–66. [PubMed: 20236058]
72. Tombran-Tink J, Barnstable CJ. PEDF: a multifaceted neurotrophic factor. *Nat Rev Neurosci.* 2003; 4:628–36. [PubMed: 12894238]
73. Tuman H, Nau R, Felgenhauer K. Beta-trace protein in cerebrospinal fluid: a blood-CSF barrier-related evaluation in neurological diseases. *Ann Neurol.* 1998; 44:882–9. [PubMed: 9851432]
74. Tuman H, Reiber H, Nau R, et al. Beta-trace protein concentration in cerebrospinal fluid is decreased in patients with bacterial meningitis. *Neurosci Lett.* 1998; 242:5–8. [PubMed: 9509992]
75. Orenes-Piñero E, Manzano-Fernández S, López-Cuenca Á, et al. β -Trace protein: from GFR marker to cardiovascular risk predictor. *Clin J Am Soc Nephrol.* 2013; 8:873–81. [PubMed: 23335042]
76. Saso L, Leone MG, Sorrentino C, et al. Quantification of prostaglandin D synthetase in cerebrospinal fluid: a potential marker for brain tumor. *Biochem Mol Biol Int.* 1998; 46:643–56. [PubMed: 9844724]
77. Rajagopal MU, Hathout Y, MacDonald TJ, et al. Proteomic profiling of cerebrospinal fluid identifies prostaglandin D2 synthase as a putative biomarker for pediatric medulloblastoma: A pediatric brain tumor consortium study. *Proteomics.* 2011; 11:935–43. [PubMed: 21271676]
78. Mase M, Yamada K, Shimazu N, et al. Lipocalin-type prostaglandin D synthase (beta-trace) in cerebrospinal fluid: a useful marker for the diagnosis of normal pressure hydrocephalus. *Neurosci Res.* 2003; 47:455–9. [PubMed: 14630351]
79. Huang YC, Lyu RK, Tseng MY, et al. Decreased intrathecal synthesis of prostaglandin D2 synthase in the cerebrospinal fluid of patients with acute inflammatory demyelinating polyneuropathy. *J Neuroimmunol.* 2009; 206:100–5. [PubMed: 19049845]

80. Lopez MF, Krastins B, Ning M. The role of apolipoprotein E in neurodegeneration and cardiovascular disease. *Expert Rev Proteomics*. 2004; 11:371–81. [PubMed: 24754513]
81. Beffert U, Danik M, Krzykowski P, et al. The neurobiology of apolipoproteins and their receptors in the CNS and Alzheimer's disease. *Bran Res Brain Rev*. 1998; 27:119–42.
82. Setzer M, Hermann E, Seifert V, et al. Apolipoprotein E gene polymorphism and the risk of cervical myelopathy in patients with chronic spinal cord compression. *Spine*. 2008; 33:497–502. [PubMed: 18317192]
83. Setzer M, Vrionis FD, Hermann EJ, et al. Effect of apolipoprotein E genotype on the outcome after anterior cervical decompression and fusion in patients with cervical spondylotic myelopathy. *J Neurosurg Spine*. 2009; 11:659–66. [PubMed: 19951017]
84. Ruzdijic S, Perovic M, Mladenovic A, et al. The impact of aging, dietary restriction, and glucocorticoids on ApoE gene expression in rat brain. *Ann N Y Acad Sci*. 2005; 1053:231–2. [PubMed: 16179527]
85. Zuckerman SH, Evans GF, O'Neal L. Exogenous glucocorticoids increase macrophage secretion of apo E by cholesterol-independent pathways. *Atherosclerosis*. 1993; 103:43–54. [PubMed: 8280184]
86. Martin-Sanz P, Vance JE, Brindley DN. Stimulation of apolipoprotein secretion in very-low-density and high-density lipoproteins from cultured rat hepatocytes by dexamethasone. *Biochem J*. 1990; 271:575–83. [PubMed: 2244866]
87. Charnay Y, Imhof A, Vallet PG, et al. Clusterin in neurological disorders: molecular perspectives and clinical relevance. *Brain Res Bull*. 2012; 88:434–43. [PubMed: 22617144]
88. Liu L, Persson JK, Svensson M, et al. Glial cell responses, complement, and clusterin in the central nervous system following dorsal root transection. *Glia*. 1998; 23:221–38. [PubMed: 9633807]
89. Zinkie S, Gentil BJ, Minotti S, et al. Expression of the protein chaperone, clusterin, in spinal cord cells constitutively and following cellular stress, and upregulation by treatment with Hsp90 inhibitor. *Cell Stress Chaperones*. 2013; 18:745–58. [PubMed: 23595219]
90. Dati G, Quattrini A, Bernasconi L, et al. Beneficial effects of r-h-CLU on disease severity in different animal models of peripheral neuropathies. *J Neuroimmunol*. 2007; 190:8–17. [PubMed: 17714795]
91. Xiao F, Chen D, Lu Y, et al. Proteomic analysis of cerebrospinal fluid from patients with idiopathic temporal lobe epilepsy. *Brain Res*. 2009; 1255:180–9. [PubMed: 19109932]
92. Yu W, Chen D, Wang Z, et al. Time-dependent decrease of clusterin as a potential cerebrospinal fluid biomarker for drug-resistant epilepsy. *J Mol Neurosci*. Feb 2.2014 Epub ahead of print. DOI 10.1007/s12031-014-0237-3.
93. Yu WR, Liu T, Kiehl TR, et al. Human neuropathological and animal model evidence supporting a role for Fas-mediated apoptosis and inflammation in cervical spondylotic myelopathy. *Brain*. 2011; 134:1277–92. [PubMed: 21490053]
94. da Costa, RC.; Armstrong, J.; Russell, D., et al. Is apoptosis present in the spinal cord of dogs with cervical spondylomyelopathy?; Proceedings of the American College of Veterinary Internal Medicine Forum; Seattle, WA, USA. 2013, June 12-15; www.vin.com(abstract)
95. Redondo M, Téllez T, Roldan MJ, et al. Anticlusterin treatment of breast cancer cells increases the sensitivities of chemotherapy and tamoxifen and counteracts the inhibitory action of dexamethasone on chemotherapy-induced cytotoxicity. *Breast Cancer Res*. 2007; 9:R86. [PubMed: 18078515]
96. Chang KH, Tseng MY, Ro LS, et al. Analyses of haptoglobin level in the cerebrospinal fluid and serum of patients with neuromyelitis optica and multiple sclerosis. *Clin Chim Acta*. 2013; 417:26–30. [PubMed: 23262373]
97. Bai S, Liu S, Qin Z, et al. Proteome analysis of haptoglobin in cerebrospinal fluid of neuromyelitis optica. *Mol Biol Rep*. 2010; 37:1619–25. [PubMed: 19444643]
98. Tumani H, Pfeifle M, Lehmsiek V, et al. Candidate biomarkers of chronic inflammatory demyelinating polyneuropathy (CIDP): proteome analysis of cerebrospinal fluid. *J Neuroimmunol*. 2009; 214:109–12. [PubMed: 19589605]

99. Guo SL, Han CT, Jung JL, et al. Cystatin C in cerebrospinal fluid is upregulated in elderly patients with chronic osteoarthritis pain and modulated through matrix metalloproteinase 9-specific pathway. *Clin J Pain*. 2014; 30:331–9. [PubMed: 23887333]
100. Ghys L, Paepe D, Smets P, et al. Cystatin C: a new renal marker and its potential use in small animal medicine. *J Vet Intern Med*. 2014 doi: 10.1111/jvim.12366 [Epub ahead of print].
101. Wilson ME, Boumaza I, Bowser R. Measurement of cystatin C functional activity in the cerebrospinal fluid of amyotrophic lateral sclerosis and control subjects. *Fluids Barriers CNS*. 2013; 10:15. [PubMed: 23497730]
102. Harvey JW, West CL. Prednisone-induced increases in serum alpha-2-globulin and haptoglobin concentrations in dogs. *Vet Pathol*. 1987; 24:90–2. [PubMed: 2435050]
103. Bjarnadóttir M, Grubb A, Olafson I. Promoter-mediated, dexamethasone-induced increase in cystatin C production by HeLa cells. *Scand J Clin Lab Invest*. 1995; 55:617–23. [PubMed: 8633186]
104. Risch L, Herklotz R, Blumberg A, et al. Effects of glucocorticoid immunosuppression on serum cystatin C concentrations in renal transplant patients. *Clin Chem*. 2001; 47:2055. [PubMed: 11673383]
105. Martinho A, Gonçalves I, Costa M, et al. Stress and glucocorticoids increase transthyretin expression in rat choroid plexus via mineralocorticoid and glucocorticoid receptors. *J Mol Neurosci*. 2012; 48:1–13. [PubMed: 22371232]
106. Lu J, Katano T, Nishimura W, et al. Proteomic analysis of cerebrospinal fluid before and after intrathecal injection of steroid into patients with postherpetic pain. *Proteomics*. 2012; 12:3105–12. [PubMed: 22936653]
107. Mitchell GB, Clark ME, Caswell JL. Alterations in the bovine bronchoalveolar lavage proteome induced by dexamethasone. *Vet Immunol Immunopathol*. 2007; 118:283–93. [PubMed: 17604846]

Figure 1.

Three dimensional representations of significant protein expression changes between control and CSM-affected dogs. Proteins that were increased (A) or decreased (B) in CSM-affected dogs are shown in the left 2 columns of Figs. 1A and 1B. For some proteins, corticosteroids and CSM had opposing effects on protein expression (right 2 columns, Figs. 1A and 1B). Corticosteroid treatment increased the expression of several proteins in CSM-affected dogs (C).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript