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BASIC SCIENCE

Proteomic Analysis of Cerebrospinal Fluid in Canine Cervical Spondylomyelopathy

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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 2-dimensional electrophoresis. A Student *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 1 of the 3 comparisons. Compared with the CSF of control dogs, CSM-affected dogs demonstrated increased CSF expression of 8 proteins including vitamin D-binding protein, gelsolin, creatine kinase B-type, angiotensinogen, α-2-HS-glycoprotein, SPARC (secreted protein, acidic, rich in cysteine), calsyntenin-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, α-2-HS-glycoprotein, and gelsolin.

Conclusion. 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.

Key words: biomarker, cervical spine, DIGE, dog, electrophoresis, Great Dane, mass spectrometry, myelopathy, osseous-associated cervical spondylomyelopathy, proteomics, spinal cord, stenosis, Wobbler syndrome.

Level of Evidence: N/A

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Cervical spondylotic myelopathy is a degenerative condition leading to cervical spinal canal narrowing and chronic, progressive spinal cord (SC) compression.[1–3] Although 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 because 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.

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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 2 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 exist 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 with 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 with 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 with canines than with 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's consent was obtained prior to enrollment. The first group included 15 dogs defined as clinically normal based on a normal neurological examination and no history of neurological disease. The second group included 15 dogs with clinical signs and neurological examination findings consistent with CSM and confirmation *via* magnetic resonance imaging. 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 revolutions per minute for 8 minutes to remove cellular materials. The supernatants were collected and stored at −80°C until analyzed.

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

Proteins were extracted from CSF samples using a 2-D cleanup kit (GE, 80-6484-51), resuspended in 100 μL of lysis buffer (30 M Tris pH 8.5, 7 M Urea, 2 M Thiourea, 4% CHAPS [3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate]) and quantitated by Bradford assay using BSA as a standard. Cy-dye labeling, isoelectric focusing, and gel electrophoresis of 2-D-cleaned CSF samples were performed according to standard DIGE (difference gel electrophoresis) protocols.[31] Labeled samples were combined (1 Cy3, 1 Cy5, and 25 μg of Cy2 sample/gel) and diluted 2× with rehydration buffer (7 M Urea, 2 M thiourea, 2% CHAPS, 1% pH 3–10 immobilized pH gradient [IPG] buffer [GE Healthcare, Pataskala, OH], 50 mM dithiotreitol, and 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 20 × 24 cm 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 hr).

Image Acquisition and DeCyder Analysis

Gels were scanned in a Typhoon 9400 variable mode scanner (GE Healthcare) using the appropriate settings for CyDye fluorophores at 100-micron resolution. Preparative gels (for spot picking and protein identification) were stained with Lava purple general protein stain (Gel Company, San Francisco, CA) according to standard protocols. Gel images were loaded into DeCyder 2-D software (GE Healthcare) and analyzed individually. Log-standardized abundance was the variable subjected to statistical analysis. A Student *t* test was used to detect differences between CSM-affected and control Great Danes ($P < 0.05$). The following 3 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, Piscataway, NJ). Gel spots were washed, dehydrated, digested using 50 μL of sequencing grade-modified trypsin (5 μg/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 (tandem mass spectrometry) data acquired were converted into mascot generic files (.mgf) using MS Convert (ProteoWizard), and the resulting files were searched against all NCBInr 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 proteins with only a Mascot score of 100 or higher with a minimum of 2 unique bold red peptides were accepted.

TABLE 1. Characteristics of Clinically Normal (Control) Great Dane Dogs and Great Danes With CSM

	Control	CSM-Affected
Number of dogs enrolled	15	15
Male/female ratio	8/7	13/2
Weight (kg)	52 (40.5–73)*	56.8 (42–79.3)*
Age at the time of enrollment (yr)	2.3 (1–6.4)*	4 (1–7.2)*
Age at the onset of clinical signs (yr)	N/A	1.7 (0.4–4.2)*
Duration of clinical signs (yr)†	N/A	1.9 (0–5)*
Dogs receiving corticosteroids	0/15	8/15

**Data presented as median (range).*
†Prior to diagnostic confirmation with magnetic resonance imaging.
CSM indicates cervical spondylomyelopathy; N/A = not applicable.

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 1 of the 3 comparisons (Tables 2–4 and Figure 1). 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

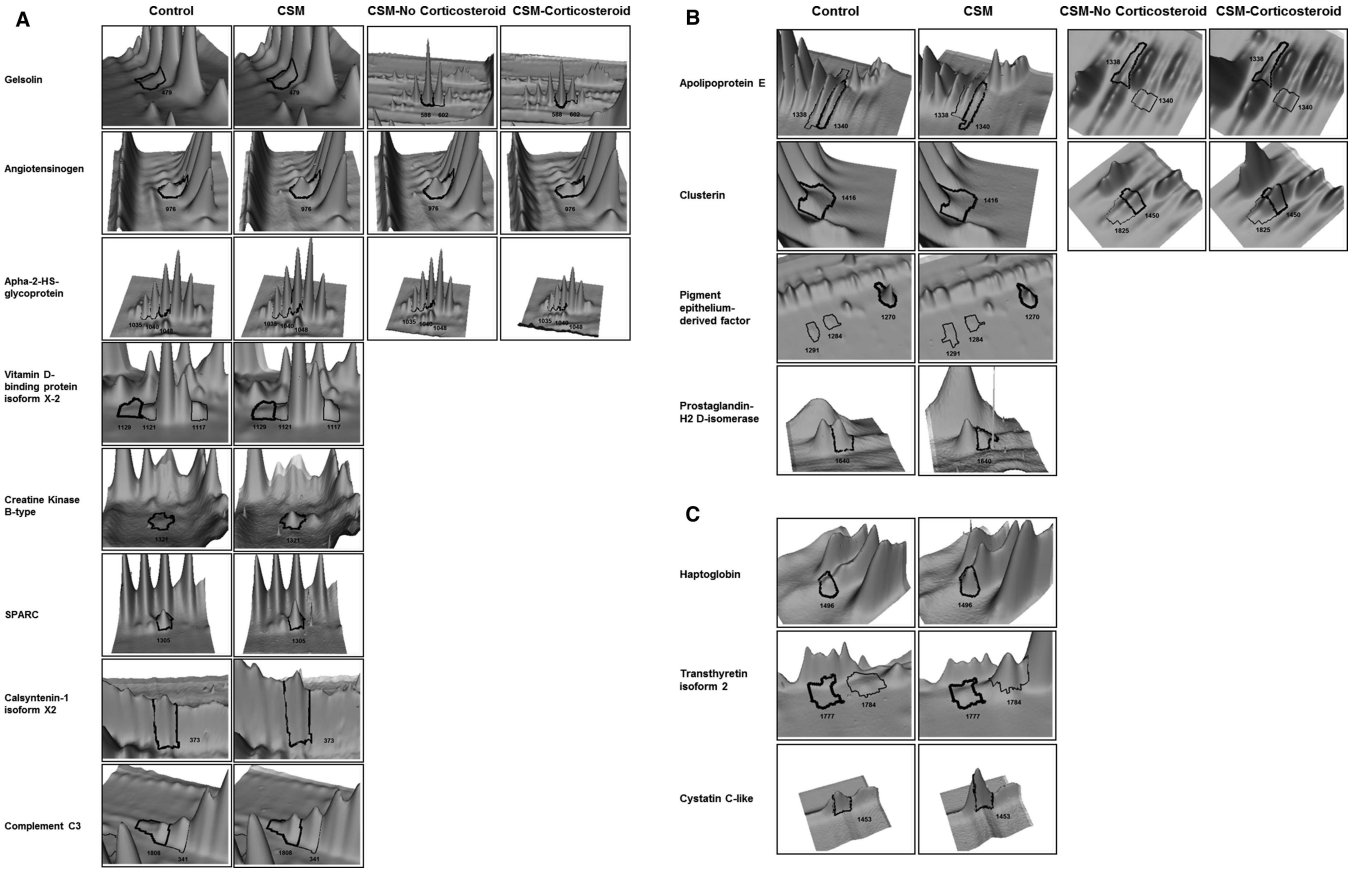

Figure 1. Three-dimensional representations of significant protein expression changes between control and CSM-affected dogs. Proteins who were increased **(A)** or decreased **(B)** in CSM-affected dogs are shown in the left 2 columns **(A** and **B)**. For some proteins, corticosteroids and CSM had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

TABLE 2. Proteins With Significant ($P < 0.05$) Differential Expression When Comparing the Cerebrospinal Fluid of Clinically Normal Great Danes and Great Danes With Cervical Spondylomyelopathy

Spot No.	Protein Name	Accession No.	pI/Molecular Weight*	Fold Change†	*P*
101	Serotransferrin isoform 1	gi|545539001	8.08/87339	−1.42	0.011
341	Complement C3	gi|545535669	6.19/181723	1.57	<0.001
413	Albumin (fragment)	gi|2147092	5.51/30901	1.33	0.012
479	Gelsolin	gi|545518174	8.49/94331	1.32	0.0076
	Serum albumin isoform X1	gi|545520919	5.51/68562		
	Coagulation factor V isoform X1	gi|345803274	5.53/250267		
673	Albumin	gi|3319897	5.36/67857	1.37	0.013
867	Serum albumin isoform X1	gi|545520919	5.51/70558	1.34	0.023
1027	Dipeptyl peptidase 1	gi|22653678	6.64/49380	1.29	0.01
	Hemopexin	gi|73988725	6.88/51305		
1129	Vitamin D–binding protein isoform X2	gi|73975215	5.2/54536	1.29	0.04
	Angiotensinogen	gi|545494757	5.65/51945		
	Fetuin-B	gi|74003556	5.64/43162		
1212	Apolipoprotein A1	gi|3915607	5.2/30178	−1.25	0.033
1234	No ID‡—likely complement C3	…	…	−1.8	<0.001
1235	No ID—likely complement C3	…	…	−2.96	0.01
1240	Complement C3	gi|545535669	6.19/181723	−3.13	0.0036
1242	Complement C3	gi|545535669	6.19/181723	−1.43	0.0065
	Albumin	gi|3319897	5.36/67857		
1259	Procollagen C-endopeptidase enhancer 1 isoform 3	gi|73957867	6.82/49353	−1.27	0.041
	Transthyretin isoform 2	gi|57089193	6.42/15972		
1270	Pigment epithelium-derived factor	gi|119637837	8.69/44923	−1.29	0.0067
1284	Pigment epithelium-derived factor	gi|119637837	6.36/46343	−1.32	0.0056
1291	Pigment epithelium-derived factor	gi|119637837	6.36/46343	−1.37	0.011
1321	Creatine kinase B-type	gi|359320104	5.47/42674	1.53	0.0037
1348	Apolipoprotein E	gi|3915605	5.2/35332	1.25	0.04
1450	Clusterin precursor	gi|50979240	5.65/52327	3.02	0.0059
	Haptoglobin	gi|123511	5.72/36890		
1453	Cystatin-C-like	gi|359322597	9.46/16607	2.5	0.02
1459	No ID	…	…	1.89	<0.001
1487	No ID—likely haptoglobin isoform 2	…	…	2.48	0.0021
1496	Haptoglobin isoform 2	gi|73957095	5.83/38814	2.11	0.0014
1519	No ID	…	…	−1.62	0.0036
1532	Kallikrein-6 precursor	gi|308210822	7.57/27062	−1.28	0.0022
1611	No ID	…	…	−1.46	0.011

(*Continued*)

TABLE 2. (*Continued*)

Spot No.	Protein Name	Accession No.	pI/Molecular Weight*	Fold Change†	*P*
1632	Prostaglandin-H2 D-isomerase precursor	gi|50978842	7.62/21414	−1.47	0.012
1640	Prostaglandin-H2 D-isomerase precursor	gi|50978842	7.62/21414	−1.62	0.011
1650	Apolipoprotein A1	gi|73955106	5.28/30163	−1.31	<0.001
1655	Prostaglandin-H2 D-isomerase precursor	gi|50978842	7.62/21414	−1.67	0.018
1743	No ID	…	…	−1.51	0.003
1774	Prostaglandin-H2 D-isomerase	gi|3914338	7.68/21402	−1.26	0.025
	Transthyretin isoform 2	gi|57089193	6.42/15858		
1777	Transthyretin isoform 2	gi|57089193	6.42/15858	5.07	<0.001
1778	Transthyretin isoform 2	gi|57089193	6.42/15858	4.22	0.0019
1783	Apolipoprotein E isoform X1	gi|57036446	5.27/37201	8.72	0.0077
1784	Transthyretin isoform 2	gi|57089193	6.42/15858	7.21	0.0039
1808	Complement C3	gi|545535669	6.19/180183	1.55	<0.001
1809	Complement C1q subcomponent subunit A	gi|57043319	9.25/26150	3.02	0.017
1812	Apolipoprotein E	gi|3915605	5.2/35332	−1.42	0.039
1820	No ID	…	…	−1.35	0.0025
1825	Clusterin precursor	gi|50979240	5.65/52327	1.1	0.045
1828	Haptoglobin	gi|123511	5.72/36890	4.85	0.0031
	Clusterin precursor	gi|50979240	5.65/52327		

**pI: Isoelectric point. Molecular weight is expressed in Daltons.*

†Negative fold change values indicate greater expression in control Great Danes (GDs) than in cervical spondylomyelopathy–affected GDs. Positive fold change values indicate greater expression in cervical spondylomyelopathy–affected GDs than in control GDs.

‡No ID: Unknown protein.

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 than that of control dogs (Table 2), non–corticosteroid-treated CSM-affected dogs than that of control dogs (Table 3), and corticosteroid-treated CSM-affected dogs than that of non–corticosteroid-treated CSM-affected dogs (Table 4). Compared with the CSF of control dogs, CSM-affected dogs demonstrated increased CSF expression of 8 proteins including vitamin D–binding protein (DBP), gelsolin, creatine kinase B-type (CK-BB), angiotensinogen, α-2-HS-glycoprotein, SPARC (secreted protein, acidic, rich in cysteine), calsyntenin-1, and complement C3, and decreased expression of pigment epithelium–derived factor (PEDF), prostaglandin-H2 D-isomerase (PGH2), apolipoprotein E (APOE), and clusterin. APOE and clusterin showed decreased expression in CSM-affected dogs when compared with 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 seemed to be related to corticosteroid treatment (Tables 2 and 4) because this upregulation was not observed when the effect of corticosteroids was removed (Table 3). Corticosteroid treatment decreased the CSF expression of angiotensinogen, α-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. In addition, corticosteroid administration seemed to alter the expression of some proteins in CSM-affected dogs.

DBP 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] Because 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 myelopathy,[3] and it may

TABLE 3. Proteins With Significant ($P < 0.05$) Differential Expression When Comparing the Cerebrospinal Fluid of Clinically Normal Great Danes (GDs) and Non–Corticosteroid-Treated Cervical Spondylomyelopathy–Affected GDs

Spot No.	Protein Name	Accession No.	pI/Molecular Weight*	Fold Change†	*P*
189	No ID‡—likely immunoglobulin gamma heavy chain D	…	…	−1.27	0.0091
192	Immunoglobulin gamma heavy chain D	gi|17066530	6.23/52169	−1.29	0.025
264	No ID—likely serum albumin precursor	…	…	1.49	0.034
274	Serum albumin isoform X1	gi|545520919	5.51/70558	1.28	0.0028
293	No ID—likely serum albumin precursor	…	…	1.34	0.043
294	No ID	…	…	1.4	0.0023
341	Complement C3	gi|545535669	6.19/181723	1.88	0.023
373	Calsyntenin-1 isoform X2	gi|545499365	4.73/108218	1.36	0.025
386	Serum albumin isoform X1	gi|545520919	5.51/70558	1.43	0.042
508	Serum albumin isoform X1	gi|545520919	5.51/70558	1.32	0.029
	Complement C4-A	gi|545520262	6.56/194554		
	Serum albumin	gi|472346475	5.62/70729		
976	Angiotensinogen	gi|545494757	5.65/51945	1.39	0.04
	Alpha-2-HS-glycoprotein	gi|545553759	5.12/40021		
1023	EGF containing fibulin-like extracellular matrix protein 2 isoform X2	gi|345783107	4.86/51819	−1.52	0.04
1030	No ID—likely α-2-HS-glycoprotein	…	…	1.41	0.024
1035	Alpha-2-HS-glycoprotein	gi|545553759	5.12/40021	1.33	0.0047
1040	Alpha-2-HS-glycoprotein	gi|545553759	5.12/40021	1.3	0.006
1048	Alpha-2-HS-glycoprotein	gi|545553759	5.12/40021	1.26	0.019
1117	Vitamin D–binding protein isoform X2	gi|73975215	5.2/54536	1.31	0.026
1121	Vitamin D–binding protein isoform X2	gi|73975215	5.2/54536	1.27	0.048
1212	Apolipoprotein A1	gi|3915607	5.2/30178	−1.42	0.02
1235	No ID—likely complement C3	…	…	−7.99	0.032
1240	Complement C3	gi|545535669	6.19/181723	−7.46	0.022
1242	Complement C3	gi|545535669	6.19/181723	−1.8	0.028
	Albumin	gi|3319897	5.36/67857		
1267	CD5 molecule-like	gi|545505255	5.45/39212	−1.34	0.012
1305	SPARC (osteonectin)	gi|545496016	4.71/35606	1.26	0.035
1321	Creatine kinase B-type	gi|359320104	5.47/42674	2.29	0.047
1338	Apolipoprotein E	gi|3915605	5.2/35332	−1.37	0.0034
1340	Apolipoprotein E	gi|3915605	5.2/35332	−1.29	0.013
1416	Clusterin precursor	gi|50979240	5.65/52327	−1.51	0.035
1767	No ID	…	…	1.3	0.021
1808	Complement C3	gi|545535669	6.19/180183	1.85	0.014

**pI: Isoelectric point. Molecular weight is expressed in Daltons.*

†Negative fold change values indicate greater expression in control Great Danes (GDs) than in non–corticosteroid-treated cervical spondylomyelopathy (CSM)-affected GDs. Positive fold change values indicate greater expression in non–steroid-treated CSM-affected GDs than in control GDs.

‡No ID: unknown protein.

EGF indicates epidermal growth factor; SPARC, secreted protein, acidic, rich in cysteine.

TABLE 4. Proteins With Significant ($P < 0.05$) Differential Expression When Comparing the Cerebrospinal Fluid of Non–Corticosteroid-Treated Cervical Spondylomyelopathy (CSM)-Affected Great Danes (GDs) and Corticosteroid-Treated CSM-Affected GDs

Spot No.	Protein Name	Accession No.	pI/Molecular Weight*	Fold Change†	*P*
114	Albumin (fragment)	gi|2147092	5.51/30901	−1.56	0.014
241	Serum albumin precursor	gi|55742764	5.52/70556	−1.45	0.047
243	No ID‡—likely serum albumin precursor	…	…	−1.4	0.038
251	No ID—likely serum albumin precursor	…	…	−1.3	0.048
252	No ID—likely serum albumin precursor	…	…	−1.34	0.048
264	No ID—likely serum albumin precursor	…	…	−1.39	0.021
355	Gelsolin	gi|545518174	8.49/95129	−1.49	0.0017
507	Gelsolin	gi|545518174	8.49/95129	−1.49	0.032
588	Gelsolin	gi|545518174	8.49/95129	−1.43	0.019
602	Gelsolin	gi|545518174	8.49/95129	−1.42	0.016
660	Serotransferrin isoform 1	gi|545539001	11/87339	1.88	0.021
688	Serotransferrin isoform 1	gi|545539001	8.08/87339	−1.3	0.018
	Clusterin precursor	gi|50979240	5.65/52327		
715	No ID	…	…	1.26	0.038
756	Clusterin precursor	gi|50979240	5.65/52327	1.37	0.043
	WAP, Kazal, immunoglobulin, Kunitz and NTR domain–containing protein 2 isoform 1	gi|73966420	6.01/65777		
802	Serotransferrin isoform 1	gi|545539001	8.08/87339	1.35	0.044
	Complement C3	gi|545535669	6.19/181723		
924	Fibrinogen α chain	gi|73978329	5.76/97324	1.53	0.044
976	Angiotensinogen	gi|545494757	5.65/51945	−1.43	0.024
	Alpha-2-HS-glycoprotein	gi|545553759	5.12/40021		
1030	No ID—likely alpha-2-HS-glycoprotein	…	…	−1.75	0.0028
1035	Alpha-2-HS-glycoprotein	gi|545553759	5.12/40021	−1.47	0.0081
1040	Alpha-2-HS-glycoprotein	gi|545553759	5.12/40021	−1.36	0.021
1061	Immunoglobulin gamma heavy chain B	gi|17066526	8.52/52553	1.99	0.028
1153	No ID—likely apolipoprotein A1	…	…	−1.35	0.01
1229	No ID	…	…	1.46	0.048
1237	No ID	…	…	−1.7	0.011
1248	No ID	…	…	−1.33	0.0037
1259	Procollagen C-endopeptidase enhancer 1 isoform 3	gi|73957867	6.82/49353	−1.58	0.046
	Transthyretin isoform 2	gi|57089193	6.42/15972		
1430	No ID	…	…	−1.68	0.023
1433	Apolipoprotein E	gi|3915605	5.2/35332	1.43	<0.001
1445	No ID	…	…	1.28	0.012

(*Continued*)

TABLE 4. (*Continued*)

Spot No.	Protein Name	Accession No.	pI/Molecular Weight*	Fold Change†	*P*
1450	Clusterin	gi|50979240	5.65/52327	5.11	0.0011
	Haptoglobin	gi|123511	5.72/36890		
1453	Cystatin-C-like	gi|359322597	9.46/16607	3.16	0.0061
1454	No ID	…	…	1.47	0.0074
1457	No ID—likely apolipoprotein E	…	…	1.63	0.001
1469	Collagen α-1(I) chain precursor	gi|50978774	5.67/32134	2.24	0.0068
1487	No ID	…	…	3.69	<0.001
1496	Haptoglobin isoform 2	gi|73957095	5.83/38814	3.03	0.001
1745	No ID	…	…	−1.78	0.0033
1783	Apolipoprotein 3 isoform X1	gi|57036446	5.27/37201	6.63	0.018
1784	Transthyretin isoform 2	gi|57089193	6.42/15858	6.28	0.0097
1824	Apolipoprotein E	gi|3915605	5.2/35332	1.54	<0.001
1825	Clusterin precursor	gi|50979240	5.65/52327	1.7	0.011
1828	Haptoglobin	gi|123511	5.72/36890	6.6	0.0056
	Clusterin precursor	gi|50979240	5.65/52327		
1829	No ID	…	…	1.31	0.044

**pI: Isoelectric point. Molecular weight is expressed in Daltons.*

†Negative fold change values indicate greater expression in non–corticosteroid-treated cervical spondylomyelopathy (CSM)-affected Great Danes (GDs) than in corticosteroid-treated CSM-affected GDs. Positive fold change values indicate greater expression in corticosteroid-treated CSM-affected GDs than in non–corticosteroid-treated CSM-affected GDs.

‡No ID: Unknown protein.

WAP indicates whey acidic protein; NTR, netrin-like domain.

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 2 proteins could be useful as indirect markers of damaged neural tissue.

CK-BB 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, α-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 α-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 that 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. Calsyntenin-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 calsyntenin-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

TABLE 5. Functional Categories of Proteins Differentially Expressed Between (Control) Great Danes and Great Danes With Cervical Spondylomyelopathy

Functional Category	Protein Name
Signal protein	Angiotensinogen
	Complement C3
	Pigment epithelium–derived factor
	Prostaglandin-H2 D-isomerase
	Cystatin C-like
Transport and binding protein	Calsyntenin-1
	Transthyretin isoform 2
Signal/transport and binding protein	Vitamin D–binding protein
	Gelsolin
	Creatine kinase B-type
	Alpha-2-HS-glycoprotein
	SPARC
	Apolipoprotein E
	Clusterin
	Haptoglobin
Antioxidant protein	Haptoglobin

SPARC indicates secreted protein, acidic, rich in cysteine.

cervical vertebrae. The upregulation of complement C3 in CSM-affected dogs suggests that 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. PGH2 (prostaglandin D synthetase or ß-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 because of 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.

APOE 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 antiapoptotic role, involvement in neuronal survival, and complement inhibitory properties.[36,87–91] Neuronal and oligodendrocyte apoptosis occur in human cervical spondylotic myelopathy[92] and canine CSM,[93] 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 because 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.[87,90,94] The use of corticosteroids increased the expression of clusterin in CSM-affected dogs, consistent with other studies.[94]

Haptoglobin, transthyretin isoform 2, and cystatin C-like were upregulated in CSM-affected dogs in a corticosteroid-dependent manner. Haptoglobin is an acute-phase protein,[95–97] and cystatin C is a proteinase inhibitor abundant in CSF.[98–100] 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.[101–103] Transthyretin is a neuroprotective negative acute-phase protein and it is often downregulated in human neurological diseases.[26,96] Conversely, transthyretin was upregulated in CSM-affected dogs. This upregulation is likely secondary to corticosteroid use because both stress and corticosteroids can increase transthyretin expression in the choroid plexus.[104] Corticosteroid treatment can also decrease the CSF concentrations of multiple proteins.[105,106]

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.

➢ Key Points

- ❑ Eight proteins were significantly upregulated in the CSF of CSM-affected dogs when compared with control dogs, including vitamin D–binding protein, gelsolin, creatine kinase B-type, angiotensinogen, α-2-HS-glycoprotein, SPARC, calsyntenin-1, and complement C3.
- ❑ The CSF of CSM-affected dogs demonstrated 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, α-2-HS-glycoprotein, and gelsolin.
- ❑ This study provides an overview of differentially expressed proteins in the CSF of clinically normal and CSM-affected dogs and suggests the potential value of these proteins as biomarkers for canine CSM and their involvement in the disease pathogenesis.

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