


**STANDARD ARTICLE**

# MicroRNA expression in the cerebrospinal fluid of dogs with and without cervical spondylomyelopathy

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**Abstract**

**Background:** Osseous-associated cervical spondylomyelopathy (OA-CSM) is a common condition of the cervical vertebral column that affects giant dog breeds. MicroRNAs (miRNAs) are small RNAs that regulate gene expression, and recent data suggest that circulating miRNAs present in biological fluids may serve as potential biomarkers for disease. The miRNA profiles of cerebrospinal fluid (CSF) from healthy dogs and dogs clinically affected by OA-CSM have not been described.

**Objective:** To characterize the expression levels of miRNAs present in the CSF of normal Great Danes and identify differentially expressed miRNAs in the CSF of Great Danes clinically affected with OA-CSM.

**Animals:** Client-owned dogs: 12 control, 12 OA-CSM affected.

**Methods:** Cerebrospinal fluid samples were collected prospectively. MicroRNA expression was evaluated using the NanoString nCounter platform and quantitative real-time PCR.

**Results:** We identified 8 miRNAs with significant differential expression. MiR-299-5p and miR-765 had increased expression levels in the CSF of OA-CSM-affected dogs, whereas miR-494, miR-612, miR-302-d, miR-4531, miR-4455, and miR-6721-5p had decreased expression levels in OA-CSM affected dogs compared to clinically normal dogs. Quantitative real-time PCR was performed to validate the expression levels of 2 miRNAs (miR-494 and miR-612), and we found a 1.5-fold increase in miR-494 expression and a 1.2-fold decrease in miR-612 in the CSF of the OA-CSM affected group ( $P = .41$  and  $.89$ , respectively).

**Conclusions and Clinical Importance:** Data generated from our study represent an initial characterization of the miRNA profile of normal canine CSF and suggest that a distinct CSF miRNA expression profile is associated with OA-CSM.

**KEYWORDS**

cerebrospinal fluid, cervical spondylomyelopathy, Great Danes, microRNA, miR-494, miR-612, Wobbler syndrome

**Abbreviations:** CSF, cerebrospinal fluid; CSM, cervical spondylomyelopathy; DA-CSM, disc-associated cervical spondylomyelopathy; GD, Great Dane; miRNA, micro ribonucleic acid; MRI, magnetic resonance imaging; OA-CSM, osseous-associated cervical spondylomyelopathy; qRT-PCR, quantitative real time polymerase chain reaction; RBC, red blood cell; TNCC, total nucleated cell count.

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## 1 | INTRODUCTION

MicroRNAs (miRNAs) are highly conserved, small (18-25 nucleotides) RNAs that play important roles in regulating gene expression in animals. MicroRNAs repress gene expression by binding to complementary sequences present in the 3' untranslated region of target mRNAs, which promotes translational repression, transcript degradation, or both.<sup>1,2</sup> Distinct tissue-specific miRNA expression profiles and circulating miRNAs in biological fluids such as plasma, serum, urine, and cerebrospinal fluid (CSF) have been identified in a number of species including dogs.<sup>1,3</sup> Circulating miRNAs found in these fluids are thought to contribute to disease pathogenesis. For example, the expression of miR-7-5p, miR-331-5p, and miR-145-5p in the CSF is associated with Parkinson's disease in humans, suggesting that circulating (or secreted) miRNAs may play a role in cellular cross talk, disease pathogenesis, or both.<sup>4</sup> Consequently, circulating (or secreted) miRNAs present in bodily fluids may serve as biomarkers for the diagnosis and monitoring of disease.<sup>5,6</sup> To date, few studies have characterized miRNA expression in canine CSF. A study conducted in dogs with meningoencephalomyelitis found that several miRNAs implicated in the pathogenesis of the disease counterpart in humans, miR-21 and miR-181c, are detectable in the CSF and serum of affected dogs.<sup>7</sup> A recent study evaluated the expression of 14 miRNAs with known roles in central nervous system (CNS) diseases of humans, in the CSF of 20 dogs with various neurological conditions. Seven of these miRNAs were amplified from canine CSF samples, and increased miR-10b-5p expression was identified in CSF samples from dogs with neoplastic disease.<sup>8</sup>

Canine cervical spondylomyelopathy (CSM) is a common condition in large and giant breed dogs causing narrowing of the vertebral canal leading to progressive cervical spinal cord compression. Two forms of the disease exist: osseous- and disc-associated CSM (OA-CSM and DA-CSM). The OA-CSM form is found predominantly in young adult Great Danes and other giant breeds, whereas the disc-associated form is seen mainly in middle-aged Dobermans.<sup>9</sup> Few treatment advances have been made in recent years. A number of surgical and medical management techniques have been described for DA-CSM,<sup>10-13</sup> but surgical treatment has comparable long-term survival to medical management and no important advances in treatment for dogs with OA-CSM have been made in the last decade.<sup>9,14,15</sup> Studies have investigated molecular factors thought to play a role in the pathogenesis of OA-CSM in dogs, identifying dysregulation of inflammatory cytokines interleukin-6, monocyte chemoattractant protein-1, and chemokine ligand 2 and alterations in vitamin-D binding protein, angiotensinogen, and complement C3 proteins.<sup>16,17</sup> A better understanding of the pathophysiology and mechanisms underlying OA-CSM will be critical to identify potential targets for treatment and develop biomarkers to monitor response to treatment and to identify treatment responders.

No prior studies have investigated the potential role of miRNAs in the pathogenesis of OA-CSM. We sought to characterize the expression levels of miRNAs present in the CSF of clinically normal Great Danes and identify differentially expressed miRNAs in the CSF of Great Danes clinically affected with OA-CSM.

## 2 | MATERIALS AND METHODS

### 2.1 | Cerebrospinal fluid collection and storage

Cerebrospinal fluid samples were prospectively collected as part of a different study,<sup>16,17</sup> and remaining CSF was banked for prospective evaluation of the miRNA expression profile of Great Danes. The study 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. Samples were collected from 12 clinically normal and 12 CSM-affected Great Danes. Dogs were defined as clinically normal based on findings of a normal neurological examination, no history of neurological disease, and normal cervical spine magnetic resonance imaging (MRI). The OA-CSM affected dogs were identified and included in the study if they exhibited clinical signs and neurological examination findings consistent with a diagnosis of cervical myelopathy. All dogs included in the study underwent standardized gait grading,<sup>16</sup> cerebellomedullary cistern CSF collection, and MRI of the cervical vertebral column. The OA-CSM was confirmed on MRI by 2 investigators (P.M.V. and D.V.). Spinal cord compression was graded as mild, moderate, or severe as previously described.<sup>16</sup> The sites of spinal cord compression and the presence or absence of spinal cord signal changes were determined based on sagittal and transverse T2-weighted images of all intervertebral disc spaces of the cervical vertebral column. The site with the most severe spinal cord compression was used for statistical analysis. After MRI, cerebellomedullary cistern CSF samples (1-2 mL) were collected using sterile technique under general anesthesia into fluid collection tubes without additives. The CSF from each dog was immediately centrifuged at 3000 rpm for 8 minutes to remove cellular materials. An aliquot of each CSF sample was evaluated by a board-certified veterinary clinical pathologist to determine total nucleated cell count, red blood cell (RBC) count, protein concentration, and cytology findings. This technique was described previously as part of a different study.<sup>16</sup> The remaining CSF aliquots were stored at  $-80^{\circ}\text{C}$  for subsequent miRNA analysis.

### 2.2 | RNA isolation

Cerebrospinal fluid samples were thawed over ice and centrifuged before RNA isolation. The samples were centrifuged for 30 seconds at 8000 rpm to remove any cellular debris. Total RNA was isolated from 250  $\mu\text{L}$  of canine CSF using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, California). To allow for normalization of CSF miRNAs, a spike-in solution containing non-mammalian synthetic miRNAs, ath-miR-159a, cel-miR-248, cel-miR-254, osa-miR-414, and osa-miR-442 (Integrated DNA Technologies, Skokie, Illinois) was added to each sample. The final concentration of ath-miR159a was 0.2  $\text{pg}/\mu\text{L}$ , cel-miR-248 was 0.4  $\text{pg}/\mu\text{L}$ , cel-miR-254 was 0.8  $\text{pg}/\mu\text{L}$ , osa-miR414 was 1.6  $\text{pg}/\mu\text{L}$ , and osa-miR442 was 3.2  $\text{pg}/\mu\text{L}$ . Total RNA then was cleaned up using the Norgen BioTek RNA Clean-up and Concentration Kit according to the manufacturer's protocol (Norgen BioTek, Thorold, Ontario, Canada). The samples were assessed for any

contaminants that could affect the assay using Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts).

### 2.3 | NanoString nCounter assay

Prior studies have characterized miRNA expression in normal canine tissues using the NanoString nCounter Human v3 miRNA Expression Assay (NanoString, Seattle, Waltham), validating this assay as a platform to profile the expression of miRNAs in samples from dogs.<sup>3,18</sup> This assay allows detecting and measuring expression levels of up to 800 human miRNAs; the mature sequences of 168 which are 100% conserved between human and dog (Sanger miRbase V16). For miRNA profiling of canine CSF samples, 3 µL of RNA were annealed with multiplexed DNA tags (miR-tag) and target-specific bridge probes. Mature miRNAs were bound to specific miR-tags using a ligase enzyme, and all tags in excess then were removed using the enzyme clean-up step. The tagged miRNA product then was diluted (ratio 1:5), and 5 µL were combined with 20 µL of reported probes in hybridization buffer and 5 µL of capture probes. Samples were incubated overnight (16-20 hours) at 65°C to allow the sequence-specific probes to complex with tagged miRNA targets. Excess probes were removed using a 2-step magnetic bead-based purification protocol performed on an automated fluidic handling system (nCounter Prep Station). Fluorescent target/probe complexes were immobilized, and each sample cartridge was imaged with a charge-coupled device camera and analyzed using the nCounter Digital Analyzer. For each cartridge, a high-density scan encompassing 325 fields of view was performed. Array values were scale normalized using the sum of the control probe values to obtain a normalization factor for each profile.

### 2.4 | NanoString data analysis

Abundances of miRNA were quantified using the NanoString nCounter gene expression software.<sup>19</sup> Boxplot analysis did not detect obvious batch effect or poor sample integrity. Therefore, all data were used for analysis. Raw data were normalized using internal positive control probes included in each assay and then a filtering step was applied. Internal negative control probes were used to determine a background threshold (2 standard deviations above the mean negative control probe count value), and if >90% of the samples had miRNA expression lower than the background threshold cutoff value, those miRNAs were filtered out.

### 2.5 | Quantitative real-time PCR

Cerebrospinal fluid miRNAs detected by the NanoString nCounter Human v3 miRNA Expression Assay that were differentially expressed in clinically normal dogs compared to OA-CSM dogs were independently validated using TaqMan miRNA quantitative real-time PCR (qRT-PCR) assays (TaqMan Advanced miRNA assays; ThermoFisher, Waltham, Massachusetts). Single sequence comparisons of human and canine miRNA genes annotated and registered in the web database, miRbase (Sanger Institute, release 16.0), were confirmed to be present in the canine genome using BLAT genome browser (<https://genome.ucsc.edu/cgi-bin/hgBlat>). The expression levels of hsa-miR-299-5p,

hsa-miR-494-3p, hsa-miR-612, and hsa-miR-302d-3p were determined using miRNA sequence-specific Taqman probes according to manufacturer's instructions (TaqMan Advanced miRNA assays: MI0000744, MI0003134, MI0003625, MI0000774; see Table 1 for mature miRNA sequences). Sequences for the remaining miRNAs (hsa-miR-765, hsa-miR-4531, hsa-miR-4455, and hsa-miR-6721-5p) were not identified in the canine genome and therefore we were unable to use commercially available Taqman primer-probe sets to evaluate expression levels. Briefly, miRNA-specific cDNA was generated from 5 µL of total RNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher), followed by real-time PCR with Taqman probes according to the manufacturer's protocol. All reactions were run in triplicate wells and included non-template controls. All samples were normalized to synthetic non-mammalian oligo spike-in cel-miR-254 (TaqMan Advanced miRNA assay: MIMAT0000310, mature sequence: UGCAAUUCUUUCGCGACUGUAGG).

### 2.6 | Statistical analysis

Differentially expressed miRNAs between comparison groups were determined by 2-sided *t*-test and fold changes using log-transformed values. A 2-tailed Student's *t* test was performed comparing lesion localization, severity of clinical signs, and the presence or absence of increased

**TABLE 1** Demographics of Great Dane dogs with and without OA-CSM

	OA-CSM-affected dogs	Clinically normal dogs
Number of dogs enrolled	12	12
Male/female ratio	10/2	7/5
Weight (kg)	56 (45-79.3)	50.75 (40.5-73)
Age at the time of enrolment (years)	3 (1.3-7)	1.75 (1-6)
Age at the onset of clinical signs (years)	1.5 (0.9-4.1)	NA
Gait grade		
<2 (mild)	6	NA
2-4 (moderate)	3	NA
4-6 (severe)	3	NA
Intramedullary spinal cord signal change (present)	4	NA
Main site of compression		
C3-C4	1	NA
C4-C5	2	NA
C5-C6	2	NA
C6-C7	7	NA
CSF analysis		
RBC	28.5 (0-25 200)	4.5 (0-122)
WBC	1 (0-9)	0 (0-3)
Protein (mg/dL)	12.2 (10.1-19.7)	12.3 (8.8-17)

Abbreviations: C, cervical vertebrae; CSF, cerebrospinal fluid; NA, not applicable; OA-CSM, osseous-associated cervical spondylomyelopathy; RBC, red blood cell; WBC, white blood cell.

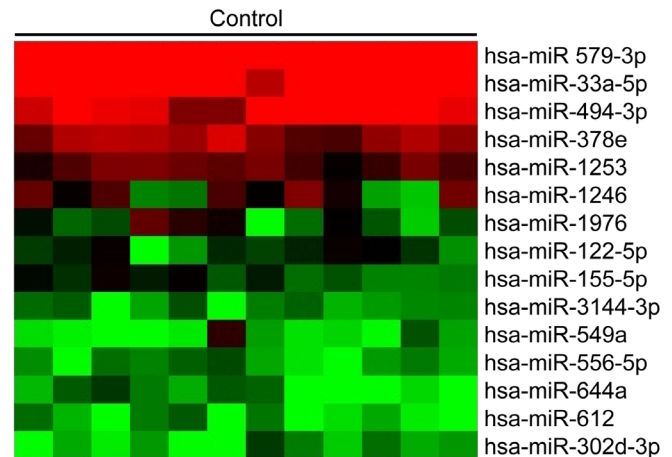
intramedullary signal intensity in the OA-CSM affected dogs. The severity of clinical signs was graded based on the gait analysis as previously described.<sup>16</sup> Dogs with moderate and severe OA-CSM were grouped and compared to the mildly affected dogs. For the comparison of lesion localization, the number of dogs with the most compressive lesion located at C6-C7 was compared to the other localizations. Real-time PCR miRNA data were first normalized to internal control (miR-254) and the delta-delta Ct method<sup>20</sup> was used to compare miRNA expression by using the Student's *t* test. *P* ≤ .05 were considered significant.

### 3 | RESULTS

The detailed characteristics of the OA-CSM affected and clinically normal Great Danes are summarized in Table 1. Gait scores were as follows: 6 were categorized as mild (gait score 1 or 2), 3 were categorized as moderate (gait score 3 or 4), and 3 were categorized as severe (gait score of 5 or 6). Four of the 12 dogs had evidence of increased intramedullary signal intensity on MRI. The most severe spinal cord compression was present at C6-C7 in 7 dogs, at C4-C5 and C5-C6 in 2 dogs, respectively, and at C3-C4 in 1 dog. In this group of 12 OA-CSM affected dogs, 2 had evidence of disc degeneration at the main site of compression. Six dogs had evidence of disc degeneration with no compression of the spinal cord and 4 dogs had no evidence of disc degeneration. The gait score and MRI of all clinically normal dogs were within normal limits except for 1 dog that had evidence of partial disc degeneration. All CSF analyses of all dogs were within reference range (<5 white blood cells/μL, and protein concentration <25 mg/dL), except 1 control sample that had 9 white blood cells/μL with 25 200 RBCs/μL. Adjusting the white blood cell count according to the blood contamination resulted in a normal white blood cell count. See Table 1 for median and ranges of red and white blood cell counts and protein concentrations.

#### 3.1 | Global miRNA expression in the CSF of clinically normal dogs

To begin to characterize the expression levels of miRNA present in the CSF of clinically normal Great Danes, global miRNA expression



**FIGURE 1** Highly expressed micro-RNAs (miRNAs) detectable in cerebrospinal fluid (CSF) of clinically normal Great Danes. Top 15 miRNAs expressed in the CSF of 12 clinically normal Great Danes. Color areas indicate relative expression of each gene after log 2 transformation with respect to the gene median expression (red above, green below, and black equal to the mean). The miRNAs in red are highly expressed, whereas those in green have a relatively lower expression

profiling was performed on CSF samples from 12 clinically normal dogs using the NanoString nCounter platform. Using this method, we detected 153 miRNAs in control dog CSF and the 15 miRNAs that showed the highest levels of expression in CSF are represented in Figure 1.

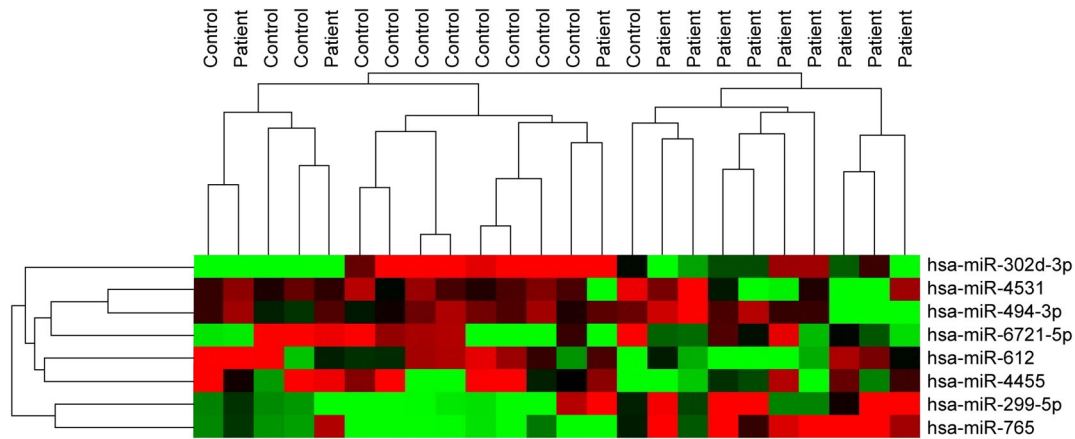
#### 3.2 | Differential miRNA expression in the CSF of Great Danes affected by OA-CSM

We identified 8 miRNAs that showed significant differential expression in the CSF of dogs affected by OA-CSM compared to that of clinically normal dogs (Table 2). Unsupervised hierarchical cluster analysis readily separated patient CSF samples based on whether they were from clinically normal or OA-CSM-affected dogs, providing support for the notion that a unique miRNAs expression signature is associated with dogs affected by OA-CSM (Figure 2).

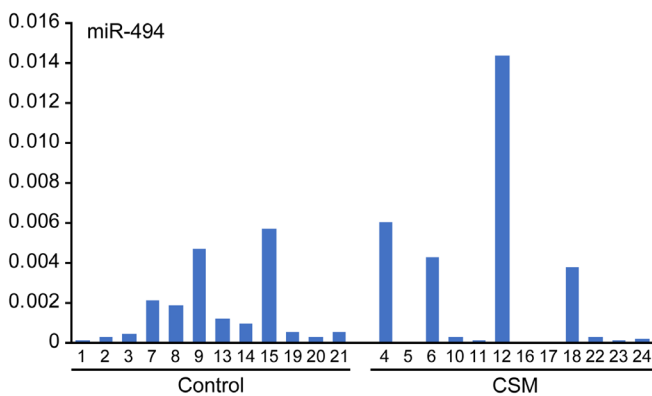
**TABLE 2** miRNA signature associated with OA-CSM

miRNA	Mature miRNA sequence	Fold-change—Gene expression affected/clinically normal	<i>P</i>	Chromosomal location
hsa-miR-299-5p	UGGUUUACCGUCCCAUACAU	2.37	.05	8
hsa-miR-765	UGGAGGAGAAGGAAGGUGAUG	2.31	.03	N/A
hsa-miR-494-3p	UGAAACAUACACGGGAAACCUC	-1.71	.04	8
hsa-miR-612	GCUGGGCAGGGCUUCUGAGCUCCUU	-1.77	.04	5
hsa-miR-4531	AUGGAGAAGGCUUCUGA	-1.78	.008	N/A
hsa-miR-4455	AGGGUGUGUGUUUUUU	-2.21	.04	N/A
hsa-miR-6721-5p	UGGGCAGGGGCUUUAUUGUAGGAG	-3.30	.03	N/A
hsa-miR-302d-3p	UAAGUGCUUCCAUGUUUGAGUGU	-3.91	.007	32

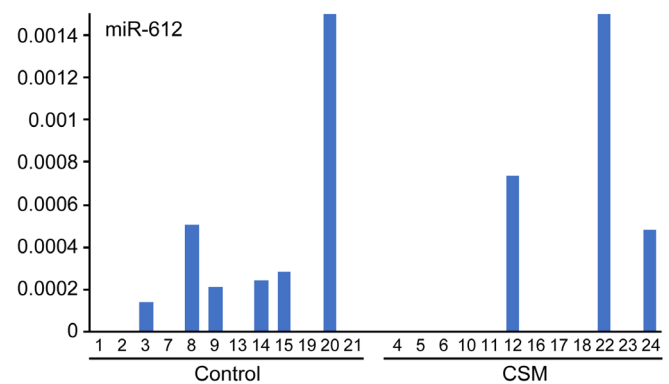
Abbreviations: miRNA, microRNA; N/A, sequences not found in canine genome; OA-CSM, osseous-associated cervical spondylomyelopathy.



**FIGURE 2** Differential cerebrospinal fluid (CSF) micro-RNA (miRNA) expression of clinically normal versus osseous-associated cervical spondylomyelopathy (OA-CSM)-affected Great Danes. The miRNA signature is presented here from 12 clinically normal Great Danes compared to 12 OA-CSM-affected Great Danes. These results are based from the NanoString assay, and 8 miRNA were found to be significantly dysregulated ( $P < .05$ ). Unsupervised hierarchical cluster analysis separated the samples into 2 groups and demonstrated a unique miRNA expression profile associated with the presence or absence of OA-CSM. Two miRNAs (miR-299-5p and miR-765) were upregulated, and 6 miRNAs (miR-302d-3p, miR-4531, miR-494-3p, miR-6721-5p, miR-612, and miR-4455) were downregulated



**FIGURE 3** Real-time PCR evaluating mature miR-494-3p expression in clinically normal and osseous-associated cervical spondylomyelopathy (OA-CSM)-affected canine cerebrospinal fluid. Real-time PCR was performed in 12 clinically normal and 12 OA-CSM-affected Great Danes to confirm the expression of miR-494-3p. The mean expression of miR-494-3p was 1.5 fold higher in the OA-CSM-affected group compared to the clinically normal Great Danes using a Student's *t* test ( $P = .41$ )



**FIGURE 4** Real-time PCR evaluating mature miR-612 expression in clinically normal and osseous-associated cervical spondylomyelopathy (OA-CSM) affected canine CSF. Real-time PCR was performed in 12 clinically normal and 12 OA-CSM-affected Great Danes to confirm the expression of miR-612. The mean expression of miR-612 was 1.2-fold lower in the OA-CSM-affected group compared to the clinically normal Great Danes using a Student's *t*-test ( $P = .89$ )

### 3.3 | Independent validation of the NanoString nCounter assay results by qRT-PCR

Four of the miRNAs that were found to be differentially expressed in the CSF of clinically normal versus OA-CSM-affected dogs were evaluated by qRT-PCR. MiR-494-3p and miR-612 were confirmed to be expressed, but their expression levels were discordant when compared to qRT-PCR, which demonstrated the miR-494-3p expression was 1.5-fold higher in the OA-CSM group compared to the normal dogs ( $P = .41$ ), and miR-612 was expressed 1.2-fold lower in the OA-CSM-affected group compared to the normal dogs ( $P = .89$ ; Figures 3 and 4). MiR-302d-3p and miR-299-5p were expressed at very low levels (cycle threshold  $>35$ ).

### 3.4 | Association of miRNAs with imaging characteristics and severity of clinical findings

We evaluated differences in miRNA expression in canine OA-CSM patients with differential lesion localization or increased intramedullary signal intensity on MRI, but no significant differences in miRNA expression were observed, suggesting that the CSF miRNA signature in dogs with OA-CSM does not reflect differences in lesion location or imaging findings. We further investigated whether dogs with OA-CSM exhibiting more severe clinical signs had differences in CSF miRNA expression levels. Interestingly, we found that hsa-miR-1246 ( $P = .004$ ) and hsa-miR-1322 ( $P = .02$ ) were significantly overexpressed in OA-CSM dogs with more severe gait grade compared to dogs with a mild or moderate



gait grade. No significantly different miRNA profiles were found between different lesion localizations or the presence or absence of increased intramedullary signal intensity. Hsa-miR-1246 and hsa-miR-1322 were significantly differentially expressed in dogs with more severe gait grade ( $P = .004$  and  $.02$ , respectively).

## 4 | DISCUSSION

We performed miRNA expression profiling on CSF from clinically normal dogs and dogs clinically affected by OA-CSM to begin to characterize miRNA expression in canine CSF. Using the NanoString nCounter system, we identified 15 miRNAs with high levels of expression in normal canine CSF and found that a unique miRNA expression signature composed of 8 miRNAs was present in the CSF of dogs affected by OA-CSM. Quantitative RT-PCR further confirmed the expression of miR-494 and miR-612 in canine CSF. Taken together, these data demonstrate that a distinct miRNA expression signature is present in dogs with OA-CSM and is the first description of a global miRNA profile in normal canine CSF and a potential miRNA expression signature in the CSF of OA-CSM affected Great Danes.

We evaluated the miRNA expression pattern in CSF samples from normal and affected Great Danes. Data generated from studies in humans suggest that interrogating the expression of miRNAs in CSF samples may be more accurate in identifying miRNAs that play a causal role in diseases of the CNS as compared to serum or plasma.<sup>5,6,21</sup> It is known that brain-derived miRNA are present in a higher proportion in CSF samples compared to serum samples, and the miRNA profile in human CSF has been found to be almost identical to that of human brain tissue.<sup>6,21</sup> Cerebrospinal fluid is in direct contact with the brain and spinal cord, and provides a relatively cell-free environment compared to blood.<sup>6</sup>

All clinically normal dogs had normal CSF analysis apart from 1 sample with marked blood contamination, which made these samples good candidates to establish the normal miRNA profile of canine CSF. Further studies are required to establish the normal profile of miRNAs across different breeds, but our findings lend insight into the miRNA expression profile of clinically normal Great Danes and serve as an initial characterization of CSF-associated miRNAs in Great Danes clinically affected by OA-CSM. Eight of the 15 miRNAs expressed at high levels in the CSF of clinically normal dogs have been previously reported in human CSF, with miR-302d and miR-556-5p being among the 20 most highly expressed miRNAs detectable in human CSF.<sup>1</sup>

Prior studies evaluating the expression of miRNAs in the CSF of dogs with meningoencephalomyelitis and dogs with various neurological conditions identified increased expression of miR-21, miR-181c, and miR-10b in the CSF of affected dogs.<sup>7,8</sup> In our study, these miRNAs were not found to be differentially expressed in OA-CSM affected dogs or clinically normal dogs. This finding reflects differences in the pathogenesis and inflammatory nature of meningoencephalomyelitis and neoplastic disease processes compared to OA-CSM.

When comparing the miRNA expression of CSF in clinically normal and OA-CSM affected Great Danes, unsupervised hierarchical clustering

recapitulated the 2 groups based on the clinical status of the Great Danes, providing support for the notion that dysregulation of these miRNAs may play a role in OA-CSM. Interestingly, we observed that 1 clinically normal Great Dane was found to cluster with the OA-CSM affected dogs. This dog was a 2.5-year-old female spayed Great Dane with the following CSF results: 219 RBC/ $\mu$ L, 1 WBC/ $\mu$ L, and protein concentration of 12.1 mg/dL. All dogs included in our study were classified as clinically normal based on complete neurologic examination and MRI of the cervical vertebral column (considered the gold standard for diagnosis of OA-CSM<sup>22,23</sup>), but it is possible that this dog may have been asymptomatic or had early onset or dynamic OA-CSM in the absence of detectable imaging changes.<sup>24</sup> Asymptomatic OA-CSM is recognized in people,<sup>25</sup> horses,<sup>26</sup> and dogs,<sup>22,27</sup> and has been described in Doberman Pinschers with DA-CSM<sup>27</sup> and reported in Great Danes with OA-CSM.<sup>22</sup> We subsequently evaluated the expression of 2 OA-CSM-associated miRNAs, miR-494, and miR-612, using qRT-PCR assays. Although the expression levels of these miRNA were not found to be statistically significant when evaluated by qRT-PCR, this finding may be due, in part, to differences in assay sensitivity, false positives with qRT-PCR associated with a 2-step amplification process, and miRNA primer probe efficiency. These results warrant further investigation in a larger cohort of patients.

The role of these dysregulated miRNAs in CSF has not yet been elucidated, but in experimental models of intervertebral disc degeneration in humans, increased expression of miR-494 promotes apoptosis in nucleus pulposus cells.<sup>28</sup> In contrast, our NanoString data demonstrated that the expression levels of miR-494 are decreased in OA-CSM affected dogs. This finding is not surprising because OA-CSM is primarily an osseous disorder as compared to DA-CSM. This area would be interesting to study in Dobermans with DA-CSM because intervertebral disc degeneration plays a large role in the cervical spinal cord compression observed in DA-CSM.<sup>9</sup>

Recently, DA-CSM has been reported in conjunction with OA-CSM in up to 25% of cases (da Costa, Bonelli, 2018, p 2212-2213) and may explain why 2 Great Danes had higher expression of miR-494 on qRT-PCR. Both of these dogs had evidence of partial or complete disc degeneration either at the site or away from the site of compression on MRI. Further studies are required to evaluate miR-494 expression levels in the CSF of healthy dogs and in intervertebral disc tissue to understand the relevance of these findings and whether they relate to the pathogenesis of the disease.

The loss of miR-612 has been documented in a variety of cancers in humans,<sup>29-31</sup> and enforced expression of miR-612 in bladder cancer cell lines has been shown to have anticancer and antiproliferative effects<sup>30</sup> by suppressing cell growth, colony formation migration, and invasion. In our study, the expression of miR-612 was significantly decreased in the CSF of OA-CSM-affected dogs compared to clinically normal dogs. Because OA-CSM is mainly a disease of osseous proliferation and miR-612 appears to play an inhibitory role in various diseases, it is possible that this miRNA may play a role in the pathogenesis of OA-CSM. However, no information is currently available on the role of miR-612 in osteoblasts.

A challenging aspect of the clinical management of OA-CSM in dogs is predicting which subset of patients eventually will show clinical

deterioration and which will remain stable. One prognostic indicator in humans is the signal intensity of the spinal cord on MRI,<sup>32,33</sup> but no studies have correlated miRNA expression with spinal cord changes on MRI. We found no significant difference in the miRNA profile of Great Danes showing hyperintense intramedullary signal changes on T2-weighted images. All control and affected dogs underwent MRI of the cervical spine based upon their neurologic examination findings and neurolocalization (affected dogs). It is possible that miRNA expression may have been altered if these dogs had other concurrent CNS diseases because the brain and remainder of the spinal cord were not evaluated with advanced imaging. However, no historical or clinical evidence was present to support additional lesions.

Dogs with OA-CSM have severe canal stenosis secondary to proliferation of the vertebral arch, articular facets, pedicles, or some combination of these. The cause of compression appears to be multifactorial.<sup>9</sup> However, the degree of spinal cord compression does not always correlate with the severity of clinical signs.<sup>27</sup> In our study, upregulation of miR-1246 and miR-1322 was found to be associated with severity of clinical signs in OA-CSM-affected dogs. High expression of miR-1246 has been documented in mesenchymal cells, and data suggest that this miRNA may play a role in the regulation of pro-inflammatory factors such as interleukin-6, chemokine ligand 2 and chemokine ligand 5 in mesenchymal cells.<sup>34</sup> Studies have demonstrated that miR-1322 expression is increased in humans with intervertebral disc degeneration compared to those with spinal cord injury, but little is known regarding the function of miR-1322 in this disease process.<sup>35</sup> Such information may be important in determining why some dogs with OA-CSM do worse than others. MiR-1246 appears to upregulate certain inflammatory mediators in mesenchymal cells that have been associated with OA-CSM,<sup>16</sup> but no information is available regarding its role in other cells such as osteoblasts.

A limitation of our study is that paired serum or plasma samples were not available for this population of dogs. Evaluation of the circulating miRNA profile in these dogs would be of interest to determine whether CSF-associated miRNAs are detectable in blood and if such a finding could reflect a breakdown in the blood brain barrier. Because CSF sample collection is not routinely performed in dogs with OA-CSM, the ability to detect OA-CSM-associated miRNAs in readily accessible biological fluids such as serum or plasma may have potential clinical utility for diagnostic or prognostic purposes. Rodent models of cervical spondylotic myelopathy have shown that chronic spinal cord compression results in damage and breakdown of the blood-brain barrier.<sup>36</sup> This finding has not yet been confirmed in dogs. Dogs with OA-CSM have chronic extradural spinal cord compression secondary to osseous proliferation. It therefore is possible that the miRNAs identified in the CSF of dogs with OA-CSM are not involved in the osseous changes seen in this disease. By evaluating miRNA expression in the CSF alone, miRNAs playing a primary role in the osseous changes may not have been fully detected.

Further studies are necessary to evaluate if these dysregulated miRNAs reflect miRNA patterns in a larger cohort of patients and in diseased tissue of Great Danes with OA-CSM. The limited number of

samples analyzed in our study precludes the ability to draw major clinical associations.

## 5 | CONCLUSION

Ours is the first study to characterize the miRNA profile in the CSF of clinically normal and OA-CSM-affected Great Danes. We have identified a unique CSF miRNA expression profile, based on the NanoString nCounter assay results, that is associated with OA-CSM and determined that miRNAs are differentially expressed in the CSF of Great Danes clinically affected by OA-CSM compared to clinically normal Great Danes. These findings support the hypothesis that CSF miRNA expression is altered in dogs with OA-CSM. The specific role of these altered miRNAs remains to be determined.

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## CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

## OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the IACUC of The Ohio State University. Informed written consent was obtained from all owners.

## HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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