Evaluation of cell-free DNA as a diagnostic marker in cerebrospinal fluid of dogs

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OBJECTIVE
To determine whether cell-free DNA (cfDNA) was detectable in CSF samples from dogs, whether CSF sample volume impacted CSF cfDNA concentration measurement, and whether CSF cfDNA concentration was associated with CNS disease category or CSF RBC count (RBCC), nucleated cell count (NCC), or protein concentration, which could aid in the diagnosis of neurologic diseases in dogs.

SAMPLE
80 CSF samples collected from dogs with (n = 60) and without (20) clinical neurologic disease between February 2017 and May 2018.

PROCEDURES
Results for CSF RBCC, NCC, protein concentration, and cfDNA concentration were compared across CSF groups established on the basis of whether they were obtained from dogs with (case groups) or without (control group) clinical signs of neurologic disease In addition, 5 paired CSF samples representing large (3.0-mL) and small (0.5-mL) volumes, were used to evaluate whether sample volume impacted measurement of CSF cfDNA concentration.

RESULTS
cfDNA was detected in 76 of the 80 (95%) CSF samples used to evaluate parameters across disease categories and in all 5 of the paired samples used to evaluate whether sample volume impacted cfDNA quantification. There were no substantial differences in cfDNA concentrations identified between groups (on the basis of disease category or sample volume), and the CSF cfDNA concentration did not meaningfully correlate with CSF RBCC, NCC, or protein concentration.

CONCLUSIONS AND CLINICAL RELEVANCE
Although results indicated that the CSF cfDNA concentration could not be used to differentiate between categories of neurologic disease in dogs of the the present study, further investigation is warranted regarding the use of CSF analysis, including sequencing specific cfDNA mutations, for diagnosing and monitoring neurologic disease in dogs. (*Am J Vet Res* 2020;81:416–421)

Cerebrospinal fluid surrounds the entire CNS and provides a number of vital functions for the nervous tissue, including physical support, protection, nutrition, and transport.[1] Biopsy of the CNS is invasive and can be associated with patient morbidity and death, whereas sampling of the CSF is relatively routine and can provide a less invasive sampling that is reflective of changes in the CNS.[2] Cerebrospinal fluid contains valuable biochemical and cellular information that aids in the diagnosis and treatment of neurologic disease. In veterinary medicine, routinely evaluated CSF parameters include NCC, RBCC, and protein concentration.[3] However, changes in these parameters can be nonspecific for different neurologic diseases, and additional diagnostic tools are needed.

ABBREVIATIONS

cfDNA	Cell-free DNA
NCC	Nucleated cell count (WBC count)
RBCC	RBC count

Cell-free DNA is extracellular DNA released during cellular apoptosis or necrosis secondary to normal cell turnover or a variety of disease processes, and plasma cfDNA has been measured in human and veterinary patients.[4–16] A study[16] shows that cfDNA released from tumors in humans is predominantly the result of cellular necrosis, whereas cfDNA released secondary to nonneoplastic processes is predominately the result of apoptosis. Consequently, more malignant tumors result in a higher degree of necrosis and a corresponding greater plasma concentration of circulating cell-free tumor DNA.[16] A recent study[4] of dogs shows that the plasma concentration of cfDNA increased with disease severity in dogs and could be prognostically useful. Dogs that did not survive to hospital discharge had significantly higher cfDNA concentrations than did those discharged alive.[4] These studies[4,16] suggest that the plasma concentration of cfDNA may correlate with disease severity and prognosis.

Although plasma cfDNA concentration has been used in determining the diagnosis, treatment, and

prognosis of systemic diseases,[4–6,8,10,12–14] plasma cfDNA concentration may be of limited use for such purposes with CNS disease.[15,17,18] This is presumably because of the presence of the blood-brain barrier, which may prevent cfDNA released secondary to CNS disease from circulating systemically.[17] Because CSF surrounds the CNS, analysis of CSF cfDNA should provide a more accurate indication of the CNS environment. Concentrations of cfDNA in the CSF have been evaluated in human medicine, particularly in patients with neoplastic and inflammatory diseases.[17–21] Tumor-specific mutations in cfDNA have been identified in CSF samples from humans with various primary and metastatic brain tumors.[17] Additionally, a mutation specific for metastatic melanoma of the leptomeninges in a human patient was identified in CSF cfDNA but not plasma cfDNA, and the patient's CSF concentration of mutant alleles coincided with clinical symptoms in that as the patient's symptoms diminished with recovery, so did the CSF cfDNA concentration of mutant alleles.[18]

Although evaluation of CSF cfDNA has diagnostic and prognostic uses in human medicine, to our knowledge, there have been no investigations into whether CSF cfDNA is detectable or has diagnostic or prognostic use in dogs. Because the CSF volumes (≥ 5 mL) used in human studies[19,20] would not be practical in small dogs, it was also unknown whether smaller-volume CSF samples obtained from dogs would yield accurate quantitation of cfDNA.

The main objectives of study reported here were to determine whether cfDNA was detectable in CSF samples from dogs and whether CSF sample volume impacted cfDNA concentration measurement. If cfDNA was measurable in CSF samples from dogs, the second aim was to investigate whether CSF cfDNA concentration was associated with the CNS disease category or with the CSF NCC, RBCC, or protein concentration. We hypothesized that cfDNA would be detectable in CSF samples from healthy dogs and dogs with neurologic disease, the CSF sample volume would not meaningfully affect measurability of the CSF cfDNA concentration, and the CSF cfDNA concentration would be higher in dogs with (vs without) neurologic disease (neoplastic, infectious, or inflammatory).

Materials and Methods

Samples

Samples of CSF obtained from dogs with and without clinical neurologic disease between February 2017 and May 2018 were eligible for the study. Owner consent was obtained prior to sample collection from client-owned dogs, and all procedures performed in the study were approved by the institutional animal care and use committee.

Control group—The CSF samples from neurologically healthy dogs (control group [group 1]) were obtained from either research dogs undergoing terminal procedures not involving the CNS or client-owned dogs euthanized for nonneurologic reasons. The dogs from which control samples of CSF were obtained were examined by a veterinarian or veterinary neurologist and considered to be neurologically healthy prior to euthanasia. To minimize the effects of postmortem autolysis, the CSF samples from these dogs were collected within 10 minutes after euthanasia.

Case groups—Samples of CSF from dogs with clinical neurologic disease (case groups) were obtained as part of the dogs' diagnostic evaluation at the Colorado State University Veterinary Teaching Hospital, and cfDNA was quantified in superfluous CSF after routine cytologic and protein analyses. The CSF samples were grouped according to whether they were obtained from dogs with clinical signs of neurologic disease classified as primary or secondary neoplastic diseases affecting the CNS (group 2), seizure disorders (group 3), inflammatory but noninfectious CNS diseases (group 4), compressive myelopathies (group 5), or other neurologic diseases (eg, idiopathic vestibular syndrome, cranial polyneuropathy, and corticosteroid responsive tremor syndrome) not encompassed by a preceding group (group 6). More specifically, dogs from which CSF samples were assigned to group 2 had primary or secondary neoplastic diseases affecting the CNS diagnosed on the basis of findings from MRI (standard sequences on a 1.5T machine), postmortem examination, or cytologic evaluation of CSF (identification of neoplastic cells), alone or in combination. Dogs from which CSF samples were assigned to group 3 had suspected idiopathic epilepsy, with seizures as a clinical sign and CSF NCC ≤ 5 WBCs/μL. Group 4 samples consisted of those from dogs with MRI findings consistent with inflammation or with a CSF NCC > 5 WBCs/μL. Group 5 samples were from dogs with MRI evidence of an extradural compressive myelopathy (eg, intervertebral disk or bony compression).

CSF sample collection and initial assessments

For each dog, a 22-gauge, 1.5-inch spinal needle connected to a syringe was used to aseptically collect a CSF sample from the cisterna magna or lumbar cistern. Cytologic evaluation (including total and differential cell count) and protein concentration assessment were performed on all CSF samples. To prevent results from being impacted by cellular deterioration in samples, these evaluations were performed within 30 minutes after sample collection. A hemocytometer was used to obtain a manual NCC and RBCC of each CSF sample. Cytospin preparations for cytologic assessment were also prepared and evaluated by board-certified veterinary clinical pathologists who were not blinded to the health status of individual dogs. An automated system[a] was used to determine the protein concentration in each CSF sample.

Sample processing for cfDNA assessment

Within 2 hours after collection, CSF was transferred into thin-walled, clear PCR assay tubes and

centrifuged[b] at 1,000 × *g* for 10 minutes at 4°C. The supernatant was harvested, immediately frozen, and stored at −80°C until DNA purification.

DNA purification—The CSF samples were thawed to room temperature for 10 to 15 minutes, and DNA purification from CSF samples was performed with commercial kits[c] in accordance with the manufacturer's protocol. The recovered solution of purified DNA was used for measurement of cfDNA.

cfDNA measurement—Quantification of cfDNA was performed with a commercial assay kit[d] and fluorimeter[e] according to manufacturer's instructions. Double-stranded DNA binds a fluorescent dye, the intensity of which (as measured by the fluorimeter) correlates with cfDNA concentration.[22] The calibration, measurements, and calculations were performed according to the manufacturer's protocol, and the CSF cfDNA concentration was calculated from the fluorimeter reading by use of the conversion algorithm provided by the manufacturer. The cfDNA concentration in each sample of CSF was determined with 1 ng/mL as the lower limit of quantitation, and the mean cfDNA concentration of samples (tested in duplicate) was recorded.

Large- versus small-volume samples

To assess validity of cfDNA quantitation in large- versus small-volume samples of CSF, 5 paired samples of CSF from the originally collected 80 samples of CSF were used. These samples were each divided into aliquots of 3.0 mL (large-volume samples) and 0.5 mL (small-volume samples). These paired large- and small-volume samples were handled identically with respect to DNA purification and cfDNA measurement. Afterward, results for cfDNA concentrations in the paired samples were compared.

Statistical analysis

Results for concentration of CSF cfDNA were compared across disease categories, NCC, RBCC, and protein concentration. Continuous data were summarized as mean and SD. The nonparametric Kruskal-Wallis test was used to compare the cfDNA concentrations between disease categories. Pairwise comparisons were made between disease categories with least squares means and Bonferroni correction. The Spearman correlation was calculated when variables were continuous and not normally distributed. A Wilcoxon 2-sample test was used to compare results for cfDNA concentration in large- versus small-volume samples. Available software[d] was used for statistical analysis, and values of $P \leq 0.0\,5$ were considered significant.

Results

CSF cfDNA concentration by disease category

Cerebrospinal fluid samples obtained from 80 dogs with (n = 60) and without (20) neurologic disease between February 2017 and May 2018 were obtained from the cisterna magna (75) or lumbar cistern (5). Of the 80 samples of CSF, 76 (95%) had cfDNA extracted and quantified at concentrations ≥ 1 ng/mL. The remaining 4 samples yielded cfDNA concentrations too low (< 1 ng/mL) to be quantified, and the data for these 4 samples were not included in the statistical analysis. These 4 samples were from dogs with neurologic disease (group 2, 1 dog with a third ventricular tumor and secondary obstructive hydrocephalus and 1 dog with nephroblastoma; and group 3, 1 dog with suspected idiopathic epilepsy and 1 dog with immune-mediated meningomyelitis). Overall, the sample volume for each was between 0.75 and 1.0 mL

Of the 76 CSF samples from which cfDNA was extracted and quantified, 20 were from dogs without clinical signs of neurologic disease (group 1 [control group]), 13 were from dogs with primary or secondary neoplastic diseases affecting the CNS (group 2), 15 were from dogs with seizure disorders (group 3), 9

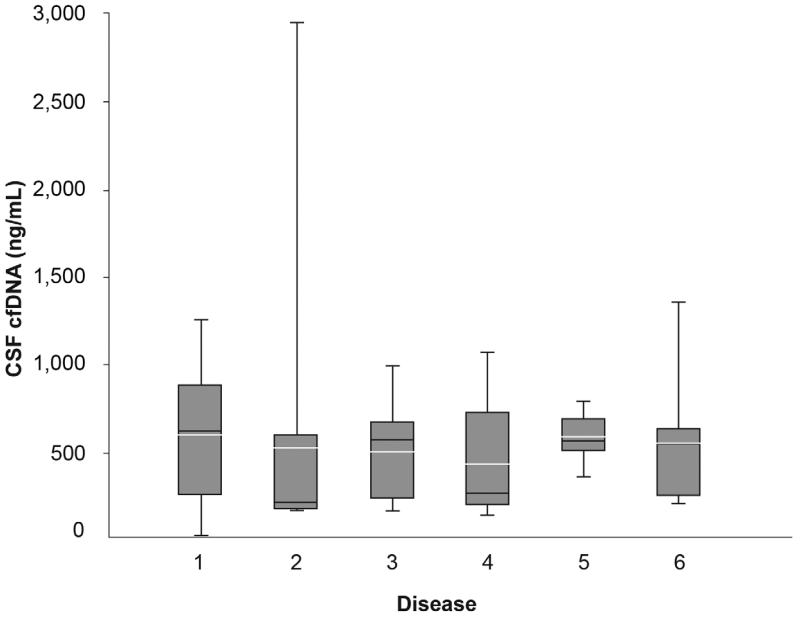

Figure 1—Box-and-whisker plots of cfDNA concentrations in samples of CSF obtained from dogs without (group 1 [control group]) or with clinical signs of neurologic disease classified as primary or secondary neoplastic diseases affecting the CNS (group 2), seizure disorders (group 3), inflammatory but noninfectious CNS diseases (group 4), compressive myelopathies (group 5), or other neurologic diseases not encompassed by a preceding group (group 6) between February 2017 and May 2018. In each plot, the central black line represents the median, the white line represents the mean, the upper and lower limits of the box represent the 95% confidence interval of the mean, and the whiskers represent the range.

Table 1—Results from the evaluation of CSF samples obtained from dogs without (group 1 [control group]) or with clinical signs of neurologic disease classified as primary or secondary neoplastic diseases affecting the CNS (group 2), seizure disorders (group 3), inflammatory but noninfectious CNS diseases (group 4), compressive myelopathies (group 5), or other neurologic diseases not encompassed by a preceding group (group 6) between February 2017 and May 2018.

					cfDNA (ng/mL)		
CSF sample group	No. of samples	NCC (WBCs/μL)*	RBCC (RBCs/μL)*	Protein (mg/dL)*	Mean ± SD	95% CI of the mean	Median (range)
1 (Control)	20	2 (0–5)	1 (0–1,200)	16.97 (13–22.92)	595.78 ± 352.79	430.67–760.89	621 (21–1,250.0)
2 (Neoplasia)	13	1 (0–354)	72 (1–26,259)	24.14 (8–192)	509.85 ± 757.78	51.93–967.77	200 (159–2,933)
3 (Seizures)	15	1 (0–3)	7.5 (0–2,945)	17.43 (11.47–61.51)	491.27 ± 242.96	356.72–625.81	552 (152–982)
4 (Noninfectious inflammation)	9	69 (1–2,460)	9 (0–6,650)	98.78 (10.94–1,800.00)	420.44 ± 366.42	138.79–702.10	248 (126–1,060)
5 (Myelopathies)	6	0.5 (0–5)	98.5 (0–367)	25.85 (15.64–73.97)	572.00 ± 140.21	424.86–719.14	557 (352–780)
6 (Other neurologic diseases)	13	1 (0–7)	2 (0–1,210)	19.45 (10.96–94)	530.63 ± 299.90	349.40–711.86	526 (194–1,340)

*Data reported as median and range.
CI = Confidence interval.

Table 2—Summary data for CSF cfDNA concentration detected in paired large-volume (3.0 mL) and small-volume (0.5 mL) samples of CSF obtained from dogs with (n = 2) and without (3) clinical signs of neurologic disease between February 2017 and May 2018.

		cfDNA (ng/mL)		
CSF sample volume (mL)	No. of CSF samples	Mean ± SD	95% CI of the mean	Median (range)
0.5	5	464.20 ± 242.42	163.20–765.20	442.0 (195.0–796.0)
3.0	5	440.04 ± 167.29	232.32–647.76	486.0 (194.2–594.0)

CI = Confidence interval.

were from dogs with inflammatory but noninfectious CNS diseases (group 4), 6 were from dogs with compressive myelopathies (group 5), and 13 were from dogs with other neurologic diseases (group 6; **Figure 1**; **Table 1**). Pairwise comparisons identified no substantial difference in median CSF cfDNA concentration between samples grouped on the basis of disease category. Results of Spearman correlation coefficient (ρ) analysis identified no significant correlation between median concentration of cfDNA and median CSF NCC (ρ = −0.137; P = 0.245), RBCC (ρ = −0.037; P = 0.752), or protein concentration (ρ = −0.011; P = 0.928) for all CSF samples.

CSF cfDNA concentration in large- versus small-volume samples

To assess validity of cfDNA quantitation in large- versus small-volume samples, 5 large-volume samples of CSF from the original sample pool were evaluated. Two of the samples were from dogs with neurologic disease, and 3 were from dogs without neurologic disease. These samples were each divided into paired aliquots of 3.0 and 0.5 mL and used to evaluate whether sample volume impacted measurement of CSF cfDNA concentration. The paired samples were handled identically with respect to DNA purification and cfDNA measurement. Results of the Wilcoxon 2-sample test indicated that the mean ± SD CSF cfDNA concentration did not differ significantly (P = 0.835) between small-volume samples (464.20 ± 242.42 ng/mL) and large-volume samples (440.04 ± 167.29 ng/mL; **Table 2**).

Discussion

In the present study, cfDNA was detected in most (76/80 [95%]) of the CSF samples obtained from dogs with (n = 60) and without (20) clinical signs of neurologic disease. This finding supported our hypothesis that cfDNA would be detectable in CSF samples from healthy dogs and dogs with neurologic disease.

The CSF samples in the present study were centrifuged to remove cellular debris within 2 hours of collection, consistent with previous studies.[4,16–18,23] In addition, this time frame was validated by a study[4] that shows the plasma cfDNA concentration in healthy research dogs was the most stable during the first 2 hours (vs 4, 6, 8, or 24 hours) after sample collection but increased over time, which probably reflects ex vivo breakdown of WBCs and subsequent DNA release.[4,23] Although CSF is far less cellular than plasma, CSF is also hyposmolar, which promotes cell lysis.[24,25] Further investigation is necessary to determine the ideal processing and handling conditions for CSF samples undergoing cfDNA analysis.

Our findings with pairwise comparisons indicated no meaningful difference in median CSF cfDNA concentration for the control group versus each individual case group. Therefore, absolute values of cfDNA concentration did not segregate the disease categories, and our hypothesis that CSF cfDNA concentration would be higher in dogs with versus without neurologic disease was not supported. Surprisingly, we detected a wide range of CSF cfDNA concentrations (median, 621 ng/mL; range, 21 to 1,250 ng/mL)

in the control group. It was possible that some of the dogs from which control samples had been collected had subclinical neurologic disease; yet, there was no history or clinical evidence of neurologic dysfunction. The CSF samples from this same group of dogs also had NCC and protein concentrations within reference limits; however, neurologically diseased animals may have normal values.[26,27] Further, because control samples were obtained from neurologically healthy research dogs that underwent terminal procedures not involving the CNS or from client-owned dogs euthanized for nonneurologic reasons, postmortem cell lysis could have falsely elevated CSF cfDNA concentration. However, to minimize this, we collected CSF samples from these dogs within 10 minutes after euthanasia.

The usefulness of CSF cfDNA assessment in human medicine is greatly enhanced by the sequencing of specific tumor mutations. The ability to detect tumor mutations in CSF cfDNA depends on the tumor type and location within the CNS.[17,19] For instance, a previous study[17] shows that tumor mutations were identified in CSF samples from 6 of the 7 people with solid brain tumors, and another study[19] indicates that tumor mutations were detected in CSF samples from 24 of the 28 (86%) people with CNS malignancies located adjacent to CSF reservoirs. Given that our findings indicated cfDNA could be extracted from CSF in dogs, future investigations of the usefulness of cfDNA sequencing in diagnosing, treating, and monitoring dogs with CNS tumors are warranted.

For the CSF samples of the present study, no meaningful correlations were detected between CSF cfDNA concentration and CSF NCC, RBCC, or protein concentration. High NCC and protein concentration often indicate CNS inflammation,[28–30] and we anticipated that CSF samples obtained from dogs with inflammatory CNS diseases and subsequently high NCC and protein concentrations would also have higher cfDNA concentration than would CSF samples from other dogs; however, we found no evidence of this. A study[31] of mitochondrial cfDNA concentration in CSF samples from people infected with HIV shows that patients with (vs without) lymphocytic pleocytosis had more inflammation and higher concentrations of mitochondrial cfDNA and that mitochondrial cfDNA concentration increased before the appearance of a lymphocytic pleocytosis, suggesting that mitochondrial cfDNA is a more sensitive marker of inflammation. Similarly, measurement of mitochondrial cfDNA in CSF of veterinary patients with inflammatory CNS disease may also prove useful.

Results of the present study indicated that 0.5 mL of CSF was sufficient to reliably measure cfDNA concentration in dogs and that there was no meaningful difference in CSF cfDNA concentration for large-volume samples (3.0 mL) versus small-volume samples (0.5 mL). This finding supported our hypothesis that CSF sample volume would not meaningfully affect measurability of CSF cfDNA concentration. This finding could have clinical impacts because although larger volumes (eg, 5 mL) of CSF have been used for analysis in human medicine, such a large-volume sample is rarely practical in dogs because the amount of CSF that can be sampled safely is approximately 1 mL CSF/5 kg of body weight.[29]

A limitation of the present study was the small sample size. More samples in each group may have helped in identifying differences in CSF cfDNA concentration between the study groups. However, even in our relatively small study, there was a wide range in CSF cfDNA concentrations in each group, and we therefore believe it was unlikely that CSF cfDNA concentrations will greatly contribute to differentiating among CNS disease types. Nonetheless, research is warranted into how we might optimize the value of CSF analysis, including sequencing of specific CSF cfDNA mutations, for diagnosing and monitoring neurologic disease in dogs.

Acknowledgments

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The authors declare that there were no conflicts of interest.

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Footnotes

a. Cobas c501 chemistry analyzer, Roche Diagnostics, Indianapolis, Ind.
b. Sorvall ST 16R centrifuge, Thermo Fisher Scientific Inc, Waltham, Mass.
c. QIAamp DNA mini kit, Qiagen, Hilden, Germany.
d. Qubit dsDNA HS assay kit, Thermo Fisher Scientific Inc, Waltham, Mass.
e. Qubit 2.0 Fluorimeter, Thermo Fisher Scientific Inc, Waltham, Mass.
f. SAS, version 9.4, SAS Institute Inc, Cary, NC.

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