

# Neurofilament light chain as a biomarker of meningoencephalitis of unknown etiology in dogs

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## Funding information

Ministry of Science and ICT, Grant/Award Number: 2021R1A2C1012058; Ministry of Education, Grant/Award Number: 2020R1A6A3A13077423

## Abstract

**Background:** Neurofilament light chain (NfL) is a neuron-specific cytoskeletal protein expressed in axons. Damaged axons of the central nervous system release NfLs into the cerebrospinal fluid (CSF) and the blood. In humans with neurologic diseases, NfL is used as a biomarker.

**Objectives:** To identify the potential of NfL as a supportive tool for the diagnosis, prognosis, and monitoring of meningoencephalitis of unknown etiology (MUE) in dogs.

**Animals:** Twenty-six client-owned healthy dogs, 10 normal Beagle dogs, and 38 client-owned MUE dogs.

**Methods:** Cohort study. The concentrations of NfL in serum and CSF were measured using single-molecule array technology.

**Results:** Median NfL concentration was significantly higher in MUE dogs (serum, 125 pg/mL; CSF, 14 700 pg/mL) than in healthy dogs (serum, 11.8 pg/mL,  $P < .0001$ ; CSF, 1410 pg/mL,  $P = .0002$ ). The areas under the receiver operating characteristic curves of serum and CSF NfL concentrations were 0.99 and 0.95, respectively. The cut-off values were 41.5 pg/mL (serum) and 4005 pg/mL (CSF) for differentiating between healthy and MUE dogs, with sensitivities of 89.19% and 90%, respectively, and specificities of 96.97% and 100%, respectively. The NfL concentration showed a significant decrease (pretreatment, 122 pg/mL; posttreatment, 36.6 pg/mL;  $P = .02$ ) in the good treatment-response group and a significant increase (pretreatment, 292.5 pg/mL; posttreatment, 1880 pg/mL,  $P = .03$ ) in the poor treatment-response group.

**Conclusions and Clinical importance:** Neurofilament light chain is a potential biomarker for diagnosing MUE and evaluating response to treatment.

## KEYWORDS

cerebrospinal fluid (CSF), MUE, MUO, NfL, serum, single-molecule array (Simoa)

**Abbreviations:** AUC, area under the receiver operator characteristic curve; CI, confidence interval; CNS, central nervous system; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; MS, multiple sclerosis; MUE, meningoencephalitis of unknown etiology; Nf, neurofilament; NfL, neurofilament light chain; ROC, receiver operator characteristic; Simoa, single-molecule array.

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## 1 | INTRODUCTION

Neurofilaments (Nfs) are neuron-specific cytoskeletal proteins that are highly expressed in axons. They play a pivotal role in supporting and maintaining axonal structure, size, shape, and caliber.<sup>1-3</sup> On the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis, Nfs were found to consist of 3 subunits: Nf-light (NfL; 68-86 kDa), Nf-medium (145-160 kDa), and Nf-heavy (200-220 kDa) chains.<sup>1,2</sup> When axonal damages in the central nervous system (CNS) occur because of inflammatory, neurodegenerative, vascular, and traumatic disorders, Nfs are released into the cerebrospinal fluid (CSF) and subsequently into the bloodstream. Recently, serum or plasma and CSF NfL concentrations have been shown to reflect neuroaxonal damage and to be potential prognostic biomarkers for disease activity, progression, and treatment response in human patients with neurological diseases including human immunodeficiency virus-associated dementia, amyotrophic lateral sclerosis, Creutzfeldt-Jakob disease, multiple sclerosis (MS), traumatic brain injury, normal pressure hydrocephalus, Alzheimer's disease, and Parkinson's disease.<sup>4</sup>

An important feature of a biomarker is the feasibility of objective evaluation and measurement as an indicator of normal physiologic and pathogenic processes or therapeutic responses. Many studies in humans with CNS disorders have investigated the potential role of biomarkers in diagnosis, evaluation of prognosis, and monitoring of treatment response.<sup>5-8</sup> However, studies on biomarkers associated with CNS diseases in veterinary medicine are rare.<sup>9</sup> Biomarkers suitable for incorporation in routine clinical practice must be easy to detect (in serum or plasma) and cost-effective. Therefore, NfL could be a potential CNS biomarker in veterinary medicine because its concentration can be easily measured in serum or plasma as well as in CSF.

To the best of our knowledge, no reports describe NfL as a biomarker of meningoencephalitis of unknown etiology (MUE) in dogs. We hypothesized that serum and CSF NfL concentrations would increase in dogs with MUE. Based on this hypothesis, we set out to establish the utility of serum and CSF NfL concentrations as accessible biomarkers for the diagnosis of MUE as well as for monitoring of disease severity and progression and treatment response.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

This retrospective cohort study included 26 client-owned healthy dogs, 10 normal Beagle dogs, and 38 client-owned dogs with MUE. Client-owned healthy and MUE dogs that visited our institution from January 2014 to October 2020 were evaluated. The healthy control group consisted of normal Beagle dogs and dogs that were presented for health examination and were considered clinically healthy. Meningoencephalitis of unknown etiology is clinically diagnosed based on the medical history, signalment, findings on neurologic examination, magnetic resonance imaging (MRI) findings, CSF analysis, exclusion of

infectious diseases, histopathological confirmation, or some combination of these. In the absence of histopathological findings, a presumptive diagnosis of MUE was made if all of the following criteria were satisfied: (a) dogs were >6 months of age, (b) single or multifocal neurologic signs were observed, (c) hyperintense lesions were observed on T2-weighted and fluid-attenuated inversion recovery images, (d) CSF abnormalities (increased protein concentration and pleocytosis with >50% mononuclear cells [monocytes and lymphocytes]) were noted, and (e) the presence of infectious diseases was ruled out.<sup>10</sup>

### 2.2 | Treatment protocol

Dogs with MUE were treated using a combination of prednisolone (1.5 mg/kg, PO, q12h; Solondo, Yuhan, Seoul, South Korea) and other immunomodulatory agents such as mycophenolate mofetil (20 mg/kg, PO, q12h; Roche, Basel, Switzerland) and cytosine arabinoside (50 mg/m<sup>2</sup>, SC, q12h for 48 hours, repeated every 3 weeks; Cytosar-U, Pfizer, New York, NY). Prednisolone generally was tapered using the following protocol: 1.5 mg/kg q12h for 3 weeks; 1.0 mg/kg q12h for 6 weeks; 0.5 mg/kg q12h for 3 weeks; 0.5 mg/kg q24h for 3 weeks; and 0.5 mg/kg q48h for maintenance.

### 2.3 | Serum and CSF collection

Blood samples were collected into serum-separating tubes from the jugular or a peripheral vein at the first visit and 6 months after the first treatment or before death or euthanasia. Serum was separated by centrifugation (2000g, 10 minutes) at room temperature and stored at -80°C within 2 hours of collection until further use. The CSF was obtained from the cerebellomedullary cistern using a 22-gauge spinal needle at the first visit. Whereas CSF of dogs with MUE was collected under general anesthesia administered for MRI, control CSF from normal Beagle dogs was obtained after inducing short-term anesthesia using alfaxalone (3 mg/kg, IV; Alfaxan, Jurox Pty Ltd, Rutherford, NSW, Australia). The CSF was collected in a plain tube without EDTA and stored at -80°C until further use.

### 2.4 | Measurement of NfL concentration

Serum and CSF concentrations of NfL were determined using single-molecule array (Simoa) technology (Quanterix, Billerica, Massachusetts). The assay was performed using a Simoa HD-1 Analyzer (Quanterix, Billerica, Massachusetts) with the NfL assay kit designed for humans using an anti-NfL monoclonal antibody (UmanDiagnostics, Umeå, Sweden). The NfL concentrations were measured in duplicate according to the manufacturer's instructions. Two control samples (high and low concentration) provided with the kit were analyzed in duplicate in each run for quality control. When the concentration of control samples was within the given range, the assay precision was considered to be satisfactory. For quality control samples with

concentrations of 5.21 and 192 pg/mL, the intra-assay coefficients of variation were 4.8% and 0.3%, respectively; the interassay coefficients of variation were <15%.

## 2.5 | Grouping and data analysis

The concentrations of NfL in both serum and CSF were measured at the first visit and compared between the healthy and MUE dogs to evaluate the differences at the onset of initial clinical signs. For serum NfL analysis, 26 client-owned and 7 normal Beagle dogs were included in the healthy control group ( $n = 33$ ), and 37 client-owned dogs were included in the MUE group ( $n = 37$ ). For CSF NfL analysis, 10 normal Beagle dogs and 10 client-owned dogs were included in the healthy control group ( $n = 10$ ) and the MUE group ( $n = 10$ ), respectively.

A receiver operator characteristic (ROC) curve was used to evaluate the cut-off value with sensitivity and specificity for the diagnosis of MUE.

Magnetic resonance imaging was performed using a 0.3-Tesla unit (Airis II, Hitachi, Japan) or 1.5-Tesla unit (Signa Creator, GE Healthcare, Milwaukee, Wisconsin). The T1-weighted (pre- and post-contrast), T2-weighted, and fluid-attenuated inversion recovery images were acquired in transverse, sagittal, and dorsal planes. To identify the correlation between lesion volume and NfL, the ratio of lesion volume to the entire brain volume was obtained. The volumes of the lesion and entire brain, which were defined using transverse fluid-attenuated inversion recovery images, were measured using a commercial image viewer (OsiriX MD v10.0, Pixmeo Sarl, Geneva, Switzerland) by adding all of the cross-sectional areas calculated on each transverse image. The ratio of the lesion volume was generated by dividing the lesional volume by the volume of the entire brain. For a more objective evaluation of the effect of lesion volume, dogs with spinal lesions were excluded because of the absence of complete MRI findings of the spinal cord. Thus, the serum group consisted of 32 dogs, and the CSF group consisted of 7 dogs.

To examine the effect of seizures on NfL, the MUE dogs were divided into 2 subgroups depending on the presence or absence of seizures: the seizure group (22 MUE dogs) and the nonseizure group (15 MUE dogs). The concentrations of NfL at the first visit in both the serum and CSF were measured and compared between the seizure and nonseizure groups to evaluate the utility of NfL as a biomarker for seizure in dogs with MUE.

Posttreatment or predeath (including pre-euthanasia) serum NfL concentrations were compared to those at the first visit to evaluate the utility of NfL as a biomarker for treatment response. For this analysis, the dogs with MUE were divided into 2 subgroups depending on the frequency and recurrence of neurologic signs. According to the therapeutic response, the good treatment-response group ( $n = 7$ ) included dogs with no neurologic signs until approximately 6 months posttreatment, and the poor treatment-response group ( $n = 6$ ) included the dogs that died or were euthanized because of periodic neurologic signs.

## 2.6 | Statistical analysis

Statistical analyses were performed using the Prism 9 software (Graphpad Software Inc, San Diego, California). The results are expressed as medians and interquartile ranges. A 2-sided  $P$ -value <.05 was considered statistically significant. The assessment of normal distribution was performed using the Shapiro-Wilk test, and a normal distribution was not identified. Therefore, nonparametric tests were used for the assessments. To compare the differences in the serum and CSF NfL concentrations between the healthy and MUE dogs and between the seizure and nonseizure groups, the Mann-Whitney  $U$  test was used. To determine the optimal cut-off value of serum and CSF NfL concentrations for differentiating between healthy dogs and dogs with MUE, the area under the ROC curve (AUC) was measured. The correlation between the ratio of lesion volume and NfL concentration was evaluated using Spearman's rank test. The Wilcoxon signed rank sum test was used to evaluate the change in the NfL concentration from before and after MUE treatment to identify treatment response.

## 3 | RESULTS

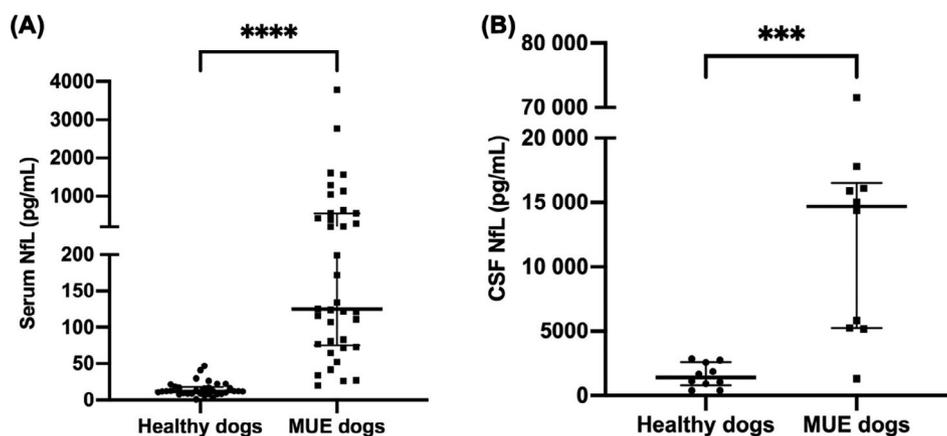
### 3.1 | Study population

Seventy-four dogs (healthy group, 36 dogs; MUE group, 38 dogs) were included in the study. The healthy group consisted of 10 Beagles, 1 Labrador Retriever, 4 mixed-breed dogs, 6 Toy or Miniature Poodles, 4 Bichon Frise, 1 Shiba Inu, 2 Welsh Corgis, 1 Spitz, 1 Yorkshire terrier, 2 Maltese, 2 Pomeranians, and 2 Miniature Schnauzers. The MUE group consisted of 21 Maltese, 2 Shih Tzus, 3 Yorkshire Terriers, 6 Chihuahuas, 7 Pomeranians, 1 Miniature Pinscher, 1 Toy

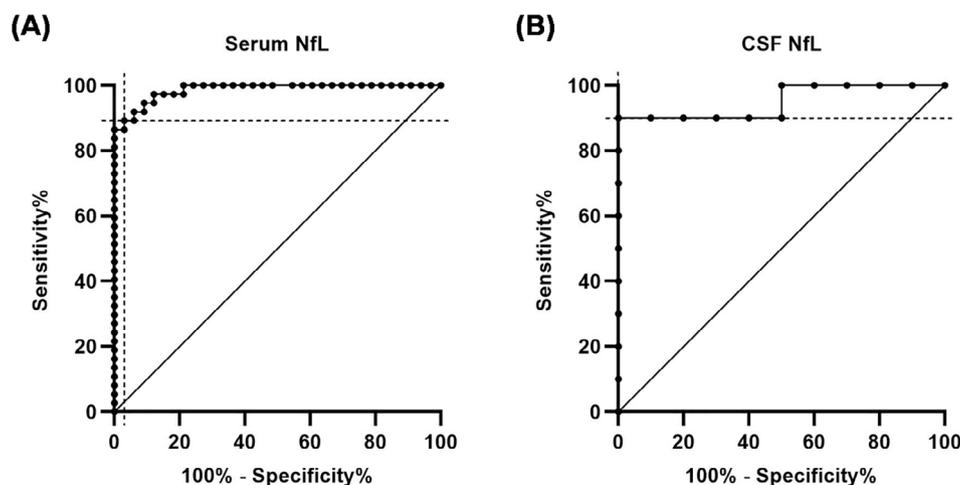
**TABLE 1** Characteristics of healthy dogs and dogs with MUE

	Healthy dogs (n = 36)	MUE dogs (n = 38)
Age (years)	3 (2-4.5)	5.3 (3-7.4)
Body weight (kg)	6.5 (4.5-8.1)	2.8 (2.2-4.2)
Sex (number)		
Male	15 (41.7%)	19 (50%)
Female	21 (58.3%)	19 (50%)
Seizure (number)		
Present	-	22 (59.5%)
Interval from last seizure to sampling	-	0 day (n = 17); 1 day (n = 1); 2 days (n = 1); 3 days (n = 1); 4 days (n = 1); no data (n = 1)
Absent	-	15 (40.5%)

Notes: The results are expressed as medians and interquartile ranges. Abbreviation: MUE, meningoencephalitis of unknown etiology.



**FIGURE 1** NfL concentrations in the serum (A) and CSF (B) samples of healthy dogs and dogs with MUE. A, There was a statistically significant difference in the serum NfL concentration between the healthy dogs ( $n = 33$ ) and dogs with MUE ( $n = 37$ ). B, There was a statistically significant difference in the CSF NfL concentration between the healthy dogs ( $n = 10$ ) and dogs with MUE ( $n = 10$ ). The horizontal bars show the medians and interquartile ranges from the first to the third quartile. The Mann-Whitney  $U$  test. \*\*\* $P < .001$ ; \*\*\*\* $P < .0001$ . CSF, cerebrospinal fluid; MUE, meningoencephalitis of unknown etiology; NfL, neurofilament light chain



**FIGURE 2** ROC curve predicting MUE based on the concentration of NfL in serum (A) and CSF (B) samples of dogs. The thick diagonal line shows a 50% chance. A, The AUC of the serum NfL concentration was 0.99 (95% CI = 0.9676-1.000). The point of intersection represents the optimal cut-off value of 41.5 pg/mL for the differentiation of healthy dogs from dogs with MUE, with sensitivity and specificity of 89.19% (95% CI = 75.29-95.71%) and 96.97% (95% CI = 84.68-99.84%), respectively. B, The AUC of the CSF NfL concentration was 0.95 (95% CI = 0.8477-1.000). The point of intersection represents the optimal cut-off value of 4005 pg/mL for the differentiation of healthy dogs from dogs with MUE, with sensitivity and specificity of 90% (95% CI = 59.58-99.49%) and 100% (95% CI = 72.25-100.0%), respectively. AUC, area under the receiver operating characteristic curve; CI, confidence interval; CSF, cerebrospinal fluid; MUE, meningoencephalitis of unknown etiology; NfL, neurofilament light chain; ROC, receiver operating characteristic

Poodle, 1 Japanese Chin, and 1 mixed-breed dog. Other demographic characteristics of the study dogs are presented in Table 1.

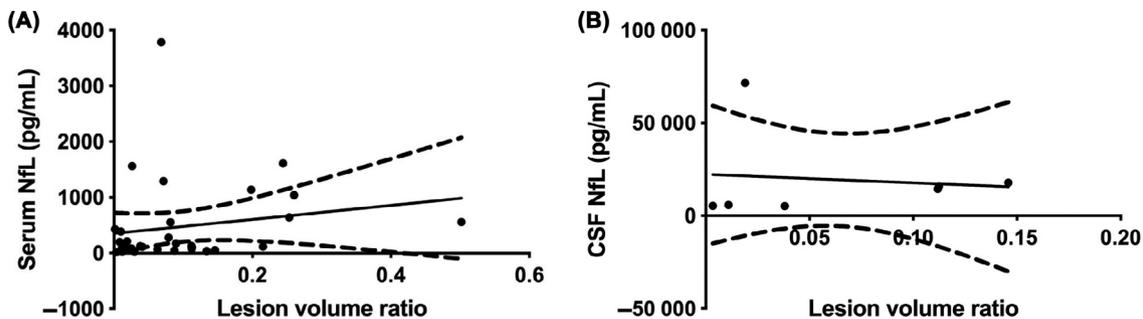
### 3.2 | NfL concentrations in healthy dogs and dogs with MUE

Concentrations of NfL in MUE dogs (serum [ $n = 37$ ]: 125 [76.7-554] pg/mL; CSF [ $n = 10$ ]: 14 700 [5405-16 050] pg/mL) were significantly higher than those in healthy dogs (serum [ $n = 33$ ]: 11.8 [8.7-16.9] pg/

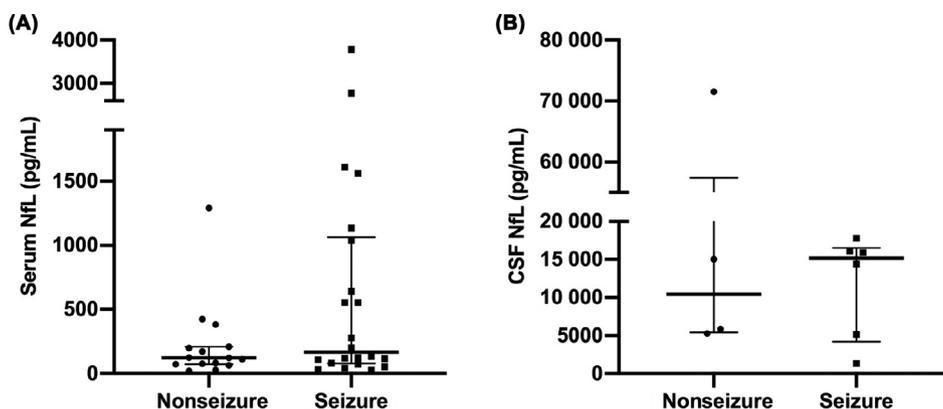
mL,  $P < .0001$ ; CSF [ $n = 10$ ]: 1410 [962-2388] pg/mL,  $P = .0002$ , Figure 1).

### 3.3 | AUC of NfL concentration in dogs with MUE

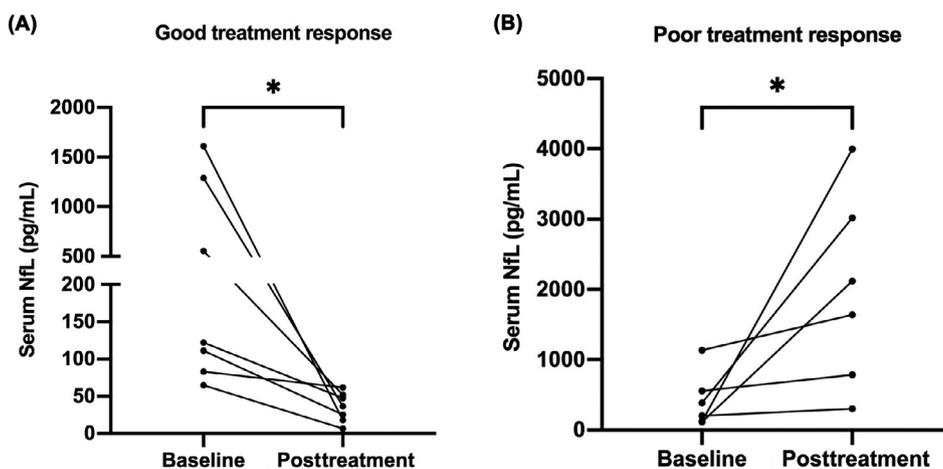
The AUCs of the serum and CSF NfL concentrations were 0.99 (95% confidence interval [CI], 0.9676-1.000) and 0.95 (95% CI, 0.8477-1.000), respectively (Figure 2). The corresponding optimal cut-off values to distinguish between healthy dogs and dogs with MUE



**FIGURE 3** Correlations between the NfL concentrations and the ratio of lesion volume to the entire brain volume in dogs with MUE (serum: A,  $n = 32$ ,  $P = .14$ ,  $r = 0.27$ ; CSF: B,  $n = 7$ ,  $P = .3$ ,  $r = 0.46$ ). Dotted lines represent the 95% confidence intervals. Spearman's rank test. CSF, cerebrospinal fluid; MUE, meningoencephalitis of unknown etiology; NfL, neurofilament light chain



**FIGURE 4** Serum (A) and CSF (B) NfL concentrations in dogs with MUE depending on the presence of seizure. A, The concentration of serum NfL in MUE dogs with seizure ( $n = 22$ ) was relatively higher than that in MUE dogs without seizure ( $n = 15$ ); however, there was no significant difference. B, The concentration of CSF NfL in MUE dogs with seizure ( $n = 6$ ) was relatively higher than that in MUE dogs without seizure ( $n = 4$ ); however, there was no significant difference. The horizontal bars show the medians and interquartile ranges from the first to the third quartile. Mann-Whitney  $U$  test. CSF, cerebrospinal fluid; MUE, meningoencephalitis of unknown etiology; NfL, neurofilament light chain



**FIGURE 5** Alterations in serum NfL concentration posttreatment in dogs with MUE according to therapeutic response. A, There was a significant decrease in NfL concentration in the good treatment-response group ( $n = 7$ ) at 6 months after commencement of treatment. B, There was a significant increase in NfL concentration in the poor treatment-response group ( $n = 6$ ) just before death or euthanasia. Wilcoxon-signed rank sum test.  $*P < .05$ . MUE, meningoencephalitis of unknown etiology; NfL, neurofilament light chain

were 41.5 pg/mL and 4005 pg/mL; sensitivity and specificity were 89.19% (95% CI, 75.29-95.71%) and 96.97% (95% CI, 84.68-99.84%), respectively, for the former and 90% (95% CI, 59.58-99.49%) and 100% (95% CI, 72.25-100.0%), respectively, for the latter.

### 3.4 | Correlation between NfL concentration and the ratio of lesion volume in dogs with MUE

Correlations of the serum and CSF NfL concentrations with the ratio of lesion volume were measured to identify the effect of lesion volume on NfL concentrations. The ratios of lesion volume in the serum and CSF groups were 0.07 (0.019-0.118) and 0.038 (0.015-0.112), respectively. There was no correlation between the ratio of lesion volume and NfL concentration in either serum ( $P = .14$ ,  $r = 0.27$ ) or CSF ( $P = .3$ ,  $r = 0.46$ ) group (Figure 3).

### 3.5 | Comparison of NfL concentration between MUE dogs with and without seizures

The concentrations of NfL in MUE dogs with seizures (serum [ $n = 22$ ]: 167.5 [87.4-940.3] pg/mL; CSF [ $n = 6$ ]: 15 150 [7477.5-16 050] pg/mL) were relatively higher than concentrations in MUE dogs without seizures (serum [ $n = 15$ ]: 122 [74.9-204.5] pg/mL; CSF [ $n = 4$ ]: 10 420 [5695-29 137.5] pg/mL), but no significant differences were identified (Figure 4).

### 3.6 | Treatment response

A significant decrease (pretreatment, 122 [97-922] pg/mL; posttreatment, 36.6 [21.4-49.7] pg/mL;  $P = .02$ ) in NfL concentration was observed in the good treatment-response group ( $n = 7$ ) at 6 months after commencement of treatment (Figure 5A). A significant increase (pretreatment, 292.5 [143.3-512.6] pg/mL; posttreatment, 1880 [998-2795] pg/mL;  $P = .03$ ) in NfL concentration was observed in the poor treatment-response group ( $n = 6$ ) just before death or euthanasia (Figure 5B). The median time interval between the first and last sampling of the poor treatment-response group was 46 days (range, 11-1021 days).

## 4 | DISCUSSION

Our study determined that serum and CSF NfL concentrations were significantly higher in dogs with MUE than in healthy dogs. Moreover, the concentrations of serum NfL decreased significantly 6 months after commencement of treatment in the good treatment-response group, and the concentration of serum NfL increased significantly just before death or euthanasia in the poor treatment-response group. Therefore, the concentration of NfL plays a supportive role in the diagnosis and evaluation of treatment response in dogs with MUE.

The Nfs are major structural proteins that are particularly abundant in myelinated axons. Because of neuroaxonal injuries associated

with neurodegeneration or inflammation, Nfs are released into the CSF and peripheral blood.<sup>11,12</sup> The concentration of NfL is increased in peripheral blood and CSF in various neurologic disorders in humans.<sup>4</sup> In a meta-analysis on MS, 1665 patients (mean serum NfL, 23.06 pg/mL; mean CSF NfL, 1207 pg/mL) showed significantly higher NfL concentrations than 986 healthy people (mean serum NfL, 13.1 pg/mL; mean CSF NfL, 187.5 pg/mL).<sup>13</sup> Our findings are consistent with the results of this meta-analysis on MS. Neurofilament light chain is related to the progression of brain damage, ongoing disease activity, and clinical signs including cognitive impairment,<sup>14</sup> which shows that the increase of NfL in neurologic diseases indicates ongoing axonal injury. Furthermore, the concentration of NfL could provide more information on ongoing neuroaxonal injury in focal T2 lesions and diffuse normal-appearing white matter lesions that are not accurately shown by conventional MRI.<sup>15</sup> Therefore, as a diagnostic biomarker, NfL, along with signalment, clinical signs, and MRI findings, improves the sensitivity and specificity of diagnosis.

Monitoring neuroaxonal damage remains an important challenge in the early diagnosis of MS. Numerous candidate diagnostic biomarkers for MS have been proposed.<sup>16</sup> Among them, NfL is a potential diagnostic biomarker that is sensitive and specific to neuroaxonal injury in patients with MS<sup>17,18</sup> and has a potential role to detect neuroaxonal damage in early MS.<sup>14</sup> Cut-off values of serum and CSF NfL concentrations to distinguish patients with MS from healthy individuals were 18.2 and 900 pg/mL, respectively, and AUCs (serum: 0.663; CSF: 0.774), sensitivities (serum: 45%; CSF: 67%), and specificities (serum: 80%; CSF: 75%) were moderate.<sup>18,19</sup> However, in our study, superior AUCs (serum: 0.99; CSF: 0.95), sensitivities (serum: 89.19%; CSF: 90%), and specificities (serum: 96.67%; CSF: 100%) were observed. Therefore, because it is also important to diagnose MUE in the early phase before the occurrence of irreversible necrotic lesions, the NfL concentration could be a useful diagnostic biomarker for MUE screening in dogs with neurologic signs.

Increased NfL concentrations in the blood and CSF of patients with MS are associated with lesion volume, brain atrophy (volume loss), and new or enlarging lesions.<sup>6,20,21</sup> Moreover, patients with contrast-enhancing lesions (62.5 pg/mL) had higher serum NfL concentrations than patients without gadolinium-enhancing lesions (29.6 pg/mL).<sup>22</sup> These observations indicate that patients with MS and increased NfL concentrations are at a higher risk of deterioration of brain atrophy (volume loss) and worsening of Expanded Disability Status Scale score in the long term. However, to date, none of the previous studies have investigated the correlation between lesion volume and biomarkers of MUE, including cytokines. Therefore, in our study, the ratio of lesion volume was determined to evaluate the correlation between lesion volume and NfL in MUE but no correlation was observed. The potential reason for this lack of correlation is that neuroaxonal damage probably is affected by the severity or activity of the lesion, and not the volume of the lesion.

The Nfs also are known to be associated with epilepsy, but their relationship with seizures has not been systematically studied. One study suggested that Nf heavy chain concentrations were significantly higher in patients with status epilepticus and repetitive generalized

tonic-clonic seizures without intracranial structural disease than in healthy controls.<sup>23</sup> This result indicated that seizure activity itself associated with status epilepticus or repetitive generalized tonic-clonic seizures affects neuronal cells, resulting in neuronal injury (necrosis or apoptosis).<sup>24,25</sup> In our study, although the concentrations of serum and CSF NfL in the seizure group were relatively higher than those in the nonseizure group, no significant difference was found between dogs with and without seizures. A potential reason for this result includes that neuronal injury associated with structural disease overwhelms the damage caused by the seizure itself. Furthermore, the type, duration, and frequency of seizures before sampling as well as the interval from the last seizure to the time of sampling also may have effects on the NfL concentrations.

The measurement of NfL concentrations in serum and CSF has been suggested for monitoring the response to drugs intended to decrease axonal injury. Regardless of the type of disease-modifying treatment, many studies have found an inverse correlation between NfL concentrations and treatment.<sup>6,18,26-28</sup> The concentration of NfL in patients with MS who were undergoing treatment was lower than that in patients who were not undergoing treatment.<sup>6,18,26-28</sup> Likewise, in our study on MUE, the concentration of NfL significantly decreased in dogs showing good treatment response at 6 months after commencement of treatment. This decrease occurred possibly because the treatment helps prevent brain damage and shows the role of NfL as a biomarker of neuroaxonal injury. In the poor treatment-response group, the concentration of NfL was significantly higher than the baseline NfL concentration, showing that an NfL increase despite treatment could reflect an inappropriate treatment response. Therefore, in combination with clinical and imaging monitoring, monitoring of NfL concentration could provide information on the therapeutic response, facilitating treatment decisions.

During the past decades, biomarkers have been widely studied in medicine.<sup>16,29</sup> The term biomarker refers to a quantifiable indicator that is measured as a variable of normal physiologic, pathogenic, or treatment responses to an intervention.<sup>30</sup> Therefore, biomarkers should be used for prediction (risk of developing a disease or establishing prognosis), diagnosis (or screening), and monitoring (disease activity and treatment response) for specific diseases.<sup>16,29,30</sup> Numerous candidates for various diseases have been studied, but only a few biomarkers have been thoroughly validated and are used clinically,<sup>16,29</sup> and it is very difficult for biomarker candidates to be considered for clinical practice. In our study, although the potential role of NfL as a biomarker in MUE was identified, the clinical use of NfL as a biomarker in MUE includes many challenges. First, in choosing biomarkers, the feasibility in the clinical environment where the biomarker will be used always should be taken into account.<sup>31</sup> After that, a biomarker could be used in clinical practice once it has analytical validation, such as sensitivity, accuracy, reproducibility, and practicality, and finally if it shows clinical utility.<sup>16</sup>

Because the antemortem histopathological diagnosis of MUE is challenging to obtain, it has been diagnosed based on clinical signs, MRI findings, and CSF results compatible with noninfectious inflammation. Magnetic resonance imaging is sensitive (94%) and specific

(95.5%) for detecting and classifying intracranial neoplastic and inflammatory lesions in dogs.<sup>32</sup> However, 7% (2/25) and 14% (5/36) of dogs with MUE had normal findings on T2-weighted MRI and computed tomography images, respectively.<sup>10</sup> Additionally, CSF cytology also was within normal limits in 16.7% (37/222) of dogs with MUE.<sup>10</sup> Therefore, an increase in NfL concentration, which reflects neuroaxonal damage, can support a diagnosis of MUE if observed on MRI and CSF in dogs with neurologic signs.

Information on prognostic factors for MUE is limited.<sup>33,34</sup> However, early detection of progression and therapeutic decision making may be important in the management of the disease and survival time. Although the acquisition of serial MRI is the best option for identifying the progression of MUE, it is not practical owing to the risk of anesthesia and the costs involved. In our study, the alterations in NfL concentrations were significant in the good and poor treatment-response groups. Therefore, NfL could be used with neurologic examination to evaluate the treatment response and to individualize therapeutic interventions.

Our study had some limitations. First, sample size was too small to generalize the relationship between MUE and NfL. Therefore, additional studies with a larger number of samples (especially, the CSF group and treatment group) are needed for an accurate evaluation of the relationship between MUE and NfL. Second, the healthy dogs were younger than the MUE dogs. The mean plasma concentration of NfL is proportional to normal aging, showing an age-dependent increase in healthy dogs: puppy/junior,  $4.55 \pm 1.70$  pg/mL; adult/mature,  $13.51 \pm 6.80$  pg/mL; and, senior/geriatric,  $47.1 \pm 12.68$  pg/mL.<sup>9</sup> Therefore, additional studies need to be performed using healthy and MUE dogs of similar ages. Third, the relationship between NfL and gadolinium-enhancing lesions was not investigated. The NfL concentration is known to be associated with the gadolinium-enhancing lesions.<sup>22</sup> However, because it was difficult to count the number of contrast-enhancing lesions and to identify the margins of lesions in low-resolution images in our study, we did not compare the correlation between the number and volume of contrast-enhancing lesion and NfL concentration.

In conclusion, our study shows that NfL is a very promising biomarker for neuroaxonal injury in MUE. Our results suggest that NfL has a potential role in the diagnosis and evaluation of therapeutic effects, but it does not correlate with the volume of the lesion and the presence of seizures. In the future, additional prospective investigations may identify other potential roles of NfL.

#### ACKNOWLEDGMENT

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2021R1A2C1012058) and the Ministry of Education (2020R1A6A3A13077423).

#### 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 (CBNUA-1466-20-01) of the Laboratory Animal Research Center of Chungbuk National University.

## HUMAN ETHICS APPROVAL DECLARATION

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

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**How to cite this article:** Yun T, Koo Y, Chae Y, et al. Neurofilament light chain as a biomarker of meningoencephalitis of unknown etiology in dogs. *J Vet Intern Med*. 2021;35(4):1865-1872. <https://doi.org/10.1111/jvim.16184>