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Neuroinflammatory diseases of domestic animals

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Neuroinflammatory diseases of domestic animals

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Editorial: Neuroinflammatory diseases of domestic animals

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Editorial on the Research Topic
Neuroinflammatory diseases of domestic animals

Inflammatory diseases account for a significant proportion of the nervous system disorders encountered in veterinary practice, are challenging to diagnose, and remain an important cause of morbidity and death in dogs and cats (1–3). Since in many of these diseases the etiopathogenesis and local and systemic immune reactions are poorly characterized, this Research Topic aimed to reduce this mechanistic knowledge gap. In this collection there are 10 papers providing new data on the epidemiology, neuropathologic features, immunopathogenesis, treatment and outcome associated with common or emerging inflammatory diseases affecting the nervous system of dogs and cats around the world.

Gonçalves et al. provide evidence that known or suspected immune-mediated conditions such as meningoencephalitis of unknown origin (MUO) and steroid-responsive meningitis-arteritis (SRMA) account for a substantially larger proportion of inflammatory nervous system diseases affecting dogs in the United Kingdom compared to infectious etiologies (3). In this study evaluating 1,140 dogs, they identified several risk factors associated with the signalment, history, and examination findings that aid in prioritizing infectious over immune-mediated differential diagnoses of inflammatory neurologic disease using multivariable and multinomial logistic regression.

The case series by Zdora et al. and Nessler et al. introduce variants of canine MUO with novel histological features, expanding the described repertoire of presumptively immune-mediated meningoencephalitides of dogs with distinct neuropathological phenotypes (3). Their collective findings highlight the heterogeneity of the conditions classified under the MUO umbrella that make conducting controlled and evidenced-based studies of this condition very difficult, reinforce the concept that MUO likely represents a spectrum of diseases in which the immune system reacts to different targets within the brain, and challenge conventional wisdom by suggesting the widely recognized necrotizing and granulomatous subtypes of MUO are perhaps representative of a disease continuum rather than distinct etiological entities.

In the continuing quest to elucidate autoimmune triggers of MUO, Barber et al. demonstrate that viral genomic material could not be recovered from the cerebrospinal

fluid (CSF) from 98% (168/172) of North American dogs with neurological dysfunction and inflammatory CSF. These data add to existing literature supporting that occult viral infections are either not a common cause of MUO in dogs or that the genomic screening techniques used to date are sufficiently insensitive to detect these pathogens (4). Another study contributed by Barber and Koos did not demonstrate a functional or survival benefit when cytosine arabinoside treatment was added at the time of diagnosis of MUO to dogs chronically treated with cyclosporine and prednisone.

Two studies in this collection offer initial insights into the immunopathogenesis of SRMA and MUO. The proof-of-concept study by Wohlsein et al. demonstrates the existence of neutrophil extracellular traps (ETs) in the meninges and perivascular tissues of dogs with SRMA. Given the evolving importance of ET's in other canine immune-mediated diseases, the discovery of ETs in SRMA provides justification to further explore their role in the etiopathogenesis of this common disorder (5). Barber and Barber provide data derived from dog blood further implicating the T helper type 17 signaling pathway in the pathogenesis of MUO (6). These promising results encourage the continued exploration of peripheral blood-based biomarkers to assist with the diagnosis and treatment of this syndrome (1).

Kleeb et al. provide a timely update on the clinical manifestations and outcomes in dogs associated with the emerging Eurasian zoonotic viral disease, tick-borne encephalitis (TBE). They show that TBE in dogs originating from an endemic region in Europe bears striking similarities to the disease in humans with respect to the temporal evolution of constitutional signs of illness, its ability to cause protean neurological presentations, and the chronic neurologic disability experienced by a significant proportion of dogs that survive the initial illness. While TBE remains a life-threatening disease in dogs, prompt hospitalization and symptomatic treatment may prevent long-term complications and 67% of dogs survived in this study, which represents a considerable improvement in outcome compared to previous reports (7).

In companion articles, van Renen et al. and Kolb et al. thoroughly annotate the clinical, electrophysiologic, and neuropathologic features of a large cohort of cats with presumed immune-mediated polyneuropathy, an increasingly recognized condition in Europe that recapitulates many clinicopathologic features of human chronic inflammatory demyelinating neuropathy. These studies provide important data regarding clinical variables that are significantly associated with neurological recovery, histopathological findings in nerve biopsies that may be predictive of recovery, and the overall favorable outcome experienced by nearly 80% of cats with this condition.

Although the data provided by the papers in this collection will undoubtedly assist clinicians and pathologists in the diagnosis and management of these diseases, this topic reinforces the many facets of neuroinflammatory diseases that require additional investigation in order to further improve our understanding of, and develop better treatments for, these disorders.

Author contributions

JR and AT contributed to drafting and proofing of this editorial. Both authors approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Canine Tick-Borne Encephalitis: Clinical Features, Survival Rate and Neurological Sequelae: A Retrospective Study of 54 Cases (1999–2016)

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Tick-borne encephalitis (TBE) is one of the most important infectious diseases of the central nervous system in dogs from endemic areas. While in humans survival rate and long-term outcomes are well described, these data are lacking in veterinary literature. The aim of the present paper is to characterize the clinical aspects of TBE and to investigate fatality rate, long-term outcome and the long-term neurological sequelae in a population of dogs infected with TBE. We performed a retrospective analysis of 54 dogs diagnosed with TBE at the veterinary hospital of the University of Zurich between 1999 and 2016. Medical data such as signalment, clinical presentation, results of diagnostic procedures, treatment and outcome were collected and analyzed. Statistical analysis including a cox proportional hazard model using a backward stepwise regression approach was performed. In 62% of the TBE cases unspecific signs were described before the onset of neurological signs, resembling a biphasic appearance that is well known in human TBE. Case fatality rate was 33% and all dogs died within the first 4 months after diagnosis. Long-term neurological sequalae were detected in 17% of the TBE cases. For each day of clinical signs before hospital entry the odds of sequalae increased by a factor of 1.88 (CI 1.04–3.15). Older dogs and dogs presented with seizure activity had an increased hazard risk of death (Hazard ration = 1.2, $p = 0.03$; and 9.38, $p = 0.001$, respectively). In conclusion, despite TBE being a life-threatening disease with severe clinical signs, the survival rate in our study was 67%. However, long-term sequalae can be of concern especially in dogs with longer clinical course.

Keywords: dog, TBE, meningoencephalomyelitis, outcome, cervical weakness, neurological sequelae

INTRODUCTION

Tick-borne encephalitis (TBE) is a viral disease, endemic in Europe, causing mainly nonspecific febrile illness followed by a remission phase in people. After this initial phase the disease may affect the nervous system in up to 50% of the cases. In approximately 30% to 80% of recovered patients, long-term neurological sequelae are observed (1–4). The disease is caused by the European subtype

of Flavivirus (TBEV), a single positive stranded RNA virus, that infects dogs, horses, domestic and wild ruminants, rodents, wild boars, and humans. In Eurasia, TBE has not only been associated with this subtype but also with the Siberian and the Far-Eastern subtypes that tends to show a mono-phasic pattern (1, 2). The TBEV is mainly inoculated by *Ixodes* spp. (4, 5) and *Dermacentor* spp. (6) ticks in Europe. The prevalence of TBEV within the tick population ranges from 0.1 to 5% in the European countries (7, 8). In Switzerland, the prevalence is around 0.46% even in previously considered safe regions such as the southern site of the Alps (8). Epidemiologically, TBE is endemic in 27 countries with a 400% increase of morbidity between 1974 and 2003 in Europe (1). In Switzerland, TBEV is causing over 100 reported human cases annually. In 2020, 455 cases were recorded (9).

The overall TBEV seroprevalence in canines varies but has been estimated in a recent metanalysis to range from 0 to 53.6% in dogs with neurological signs (10). Due to continuous climate changes and international transportation, TBEV should be included in the diagnostic workup of animals from previously unaffected areas such as Ticino (8) or United Kingdom where two autochthonous cases have been recently described (11). Despite canine TBEV infection having a calculated annual risk around 11.6% (12), dogs are rarely clinically affected (13, 14). TBEV was firstly isolated from the brain of a dog with neurological signs in 1972 (15). Since then various authors described TBEV as causes of central (13) and peripheral (16) neurological syndromes in dogs.

To clinically diagnose TBE in canines, we face the same challenges encountered in human medicine. In both species, diagnosis is based upon seasonal occurrence, known recent exposure to ticks, clinical and neurological signs, serological and cerebrospinal fluid (CSF) presence of IgM and/or IgG, and presence of magnetic resonance imaging findings suggestive of a viral encephalopathy (3, 17–20).

Causal treatment is not definitive in both canine and human patients, and acaricidal treatment might not necessary fully prevent infection (12). Therefore, preventive vaccination of the host is of paramount importance although at time of writing this is only available in humans (21).

In human TBE due to the European subtype, fatalities rate up to 2% (3, 19) and neurological sequelae are evident in about 10% of survivors (1, 3, 17–20). Up to date, data about survival and long-term neurological sequelae in dogs with TBE are scanty and not readily available. In previous publications mortality has been reported to be extremely high and TBE was considered to be a fatal neurological disease although based upon only five dogs (22). The aim of the present paper is to characterize the clinical aspects and to investigate case fatality rate, long-term outcome, and to describe long-term neurological sequelae in a clinical population of dogs with TBE presented at our hospital in the years 1999–2016.

Abbreviations: CI, Confidence Interval; CSF, Cerebrospinal fluid; CT, Computer tomography; ELISA, Enzyme-Linked ImmunoSorbent Assay; GI, GastroIntestinal; GLM, Generalized linear models; JAK-STAT, Janus kinase-signal transducer and activator of transcription; IFA, Indirect fluorescent antibody; IgM, Immunoglobulin M; IgG, Immunoglobulin G; MEUO, MeningoEncephalitis of Unknown Origin; MRI, Magnetic resonance imaging; RNA, Ribonucleic Acid; TBE, Tick-borne Encephalitis; TBEV, Tick-borne Encephalitis Virus; WBC, White Blood Cells.

MATERIALS AND METHODS

Study Population and Inclusion Criteria

This retrospective observational study was performed searching the database of the Vetsuisse Faculty, University of Zurich, for dogs with a diagnosis of TBE between the years 1999 and 2016. The inclusion criteria were as follows: (1) dogs presented with neurological signs suggestive of central nervous system disease and living in endemic areas for tick-borne encephalitis virus (or coming from an endemic area); (2) cerebrospinal fluid changes compatible with inflammatory disease; and (3) positive antibody titers in cerebrospinal fluid at time of admission (TBEV cerebrospinal fluid titer using ELISA from an external laboratory [Alomed Randolfzell–Böhringen, Deutschland] higher than 2 UI/L). Dogs that did not have positive CSF antibodies at initial presentation were excluded.

Neurological signs were grouped according to previous data on human cases of TBE on meningoencephalitis, encephalomeningomyelitis, meningomyelitis with or without poliomyelitic form, or meningitis (1). This allowed comparison with human TBE. Results of cerebrospinal fluid analysis (including total protein content, total nucleated cell count, and differential cell count) and results of enzyme-linked immunosorbent assay from CSF and/or histopathology in case of euthanasia were reviewed. TBEV detection via PCR of cerebrospinal fluid was performed at our laboratory using real time quantitative PCR (RT-qPCR) technique following the protocol previously published by Wicki and colleagues (23). Hematology and biochemistry results as well as imaging results (radiographs and advanced cross-sectional imaging) were reviewed, when available. Any short-term follow-up information (neurological follow-up examination, telephone follow-up) was included to document sequelae of TBE. A long-term follow-up was performed via structured phone interviews conducted by one of the first authors (CK) with the owners to determine if patients were still alive, to describe the cause of death or request for euthanasia, and if any long-term neurological sequelae were observed by the owners since the diagnosis of TBE. Specifically, gate and/or posture abnormalities, behavioral abnormalities compared to pre TBEV infection, time to fully recover from the neurological syndrome, and persistency of clinical symptoms were investigated.

Statistical Analysis

All analyses were undertaken in R including descriptive analysis of the demographic data (24). The association between the risk of death and demographic data and the occurrence of different variables (seizures, long-term sequelae, age of dog, TBEV titer, presence of vestibular signs, number of leucocytes in the cerebrospinal fluid, paresis/plegia and type of encephalitis) was assessed with a Cox proportional hazard model using a backward stepwise regression approach. This survival model was used to assess the association of these factors with survival time. The significance level considered was 5% ($P < 0.05$).

The hospitalization times of dogs and time taken until full recovery was analyzed by a gamma generalized linear models (GLM) as the dependent variable was not normally distributed and the gamma model gave the best fit. Analysis of residuals confirmed that the gamma GLM was an appropriate statistical model. For the analysis of time taken to full recovery, dogs that did not achieve recovery were removed from the data set. The risk of developing sequelae was analyzed by a binomial GLM (logistic regression).

RESULTS

Between 1999 and 2016, 57 dogs were diagnosed with TBE. In three cases, TBEV titer was negative at admission and they were therefore excluded. TBE was later confirmed in these cases, either by repetition of TBEV titers or histopathology.

The remaining 54 cases were presented between March and October and the owners recalled recent tick exposure in 23 of 54 dogs (42.5%). The most commonly affected breeds observed in this population were Newfoundland dogs (8/54), Rottweilers (8/54), and Labrador Retriever (5/54). These breeds accounted for 38.8% of the total population. The mean age at presentation was 5.61 (range 1–13 years). Sex and sexual physiological status were 28/54 males (intact/neutered 20/8) and 26/54 females (intact/neutered 10/16).

Clinical and Neurological Presentation

Mean duration of clinical signs before admission was 4 days (range: 0–14 days). In 34 of the 54 cases (62%), interviewed owners or first opinion veterinarians reported unspecific signs such as apathy, inappetence, gastrointestinal signs, and hyperthermia 1–10 days before hospital admission. Of these 34 dogs, 23 were reported to have had hyperthermia (23/34, 67.6%) and 15 gastrointestinal signs (15/34, 44.11%).

Physical examination at admission showed hyperthermia (mean 39.9 C; range: 39.1–41.3) in 36 dogs (66.6%). The most common neurological presenting signs at admission included ataxia/vestibular signs (28 dogs, 51.8%), cervical pain (21 dogs, 38.8%), plegia/paresis of one or more limbs (15 dogs, 27.7%), cranial nerve deficits (16 dog, 29.6%), proprioceptive ataxia (16 dogs, 29%), and cervical weakness (11 dogs, 20%). Isolated seizures were recorded in 8 dogs (14.8%) at time of admission whereas two dogs were admitted with a history of status epilepticus.

The clinical neurological syndrome at presentation was consistent with meningitis (3 dogs, 5%), meningoencephalitis (23 dogs, 42%), meningomyelitis (18 dogs, 33%), and meningoencephalo-myelitis (10 dogs/ 19%). Ten subjects showed seizure as part of initial presentation while seven dogs developed them during hospitalization (17 dogs; 31%). Of these 17 dogs, cluster seizures occurred in six (35%), four had focal seizures (23%), four had status epilepticus (23%), and three had isolated epileptic seizures (17%).

Laboratory Findings

Blood works results were abnormal in 48 cases [non-regenerative anemia (30/48, 62%), neutrophilic leukocytosis (24/48, 53%), and lymphopenia (17/48, 42%)]. The most frequently observed abnormalities in biochemical panel were elevated liver enzymes (24/48, 50%), elevated creatinine kinase (CK) (12/48, 25%), elevated urea (8/48,16%), hyperproteinemia (8/48, 16%), and hypoalbuminemia (6/48, 12%).

CSF analysis showed mean total nucleated cell count of 113.8 cells pro microliter (from 5.5 to 880 WBC per microliter, normal reference range < 4 WBC per microliter) and mean protein content of 68.7 mg/dL (ranging from 18 to 180 mg/dL, and a normal reference range <35 mg/dL). TBEV antibody titer in the cerebrospinal fluid was 82.1 UI/L (ranging from 8 to 173 UI/L). A TBEV real time quantitative PCR (RT-qPCR) was performed in 8 cases resulting in 7 negative (7/8, 88%) and 1 positive case (1/8, 12%). Other pathogens (Distemper, Toxoplasma spp., Neospora spp.) were tested by PCR and were negative in all cases. The differential cell count showed lymphocytic pleocytosis (36/54, 66.5%), mononuclear pleocytosis (10/54, 18.5%), or mixed pleocytosis (8/54, 14%).

Diagnostic Imaging

Radiological studies were obtained in 31/54 (57.4%) dogs. Thoracic radiographs were available in 18 (18/31, 58.06%) and abdominal radiographs in nine (9/31, 29.03%) dogs. A bronchial pattern was seen in three dogs, and atelectasis in two. Otherwise the studies were reported as normal.

In 22/54 (40.7%) cases imaging of the central nervous system was performed with computed tomography (CT) of the brain in 4/22 (18.18%) and MRI of the brain and/or spine in 18/22 (81.81%). MRI findings of 14 dogs have been previously described in Beckmann et al. (25) and Sievert et al. (26). Results of brain CT were within normal limits in all cases. Brain magnetic resonance imaging was carried out on 17 patients and 11 showed abnormal findings (64.7%). In six dogs the findings were confined to the gray matter of the thalamus (6/11, 54.54%) while in others, hippocampus (2/11, 18.18%), pons (1/11, 9.09%), brain stem (3/11, 27.27%), and basal nuclei (1/11, 9.09%) were also affected. The images were consistently similar, symmetric, and hyperintense lesions in T2-weighted sequences compared to white matter, iso- to hypointense in T1-weighted, non-enhancing post contrast administration, and had minimal or no mass effect or perilesional edema. Four of 17 (23.52%) dogs had meningeal enhancement. MRI of the spine performed in 11 cases (11/18, 61.11%) showed abnormal T2WI hyperintensity confined to the ventral horns of the gray matter in two patients (2/11, 18.18%) without post gadolinium administration T1WI contrast enhancement.

Hospitalization Time and Neurological Status/Outcome at Discharge

The mean hospitalization time was 8 days (range from 1 to 36 days). Tetraplegic cases were hospitalized for an average 10 days (range: 8–12 days); tetraparetic cases 13.7 days (range: 2–35 days); and paraparetic cases 8.7 days (range: 6–12 days).

Medical Management During Hospitalization

Phenobarbital was the first line medication in all patients with seizures. In 64% (11/17) seizure control was inadequate and Levetiracetam was introduced as a second antiepileptic drug. In those dogs with cluster seizures or status epilepticus constant rate infusions with midazolam was added to control the seizure activity.

In 49/54 cases (90%) anti-inflammatory doses of corticosteroids (dose ranging from 0.5 to 1 mg/Kg/q24h) for 2–5 days were administered, 45 (45/54, 83%) had antibiotics (clindamycin 11 mg/Kg/q12h) pending CSF results.

Fluid therapy, rehabilitation therapy, and nursing care were administered according to patient needs.

Mortality and Survival Rate

One-third (18/54, 33%) of the dogs in this study died or were euthanized because of complications associated with TBEV infection within 4 months after discharge.

Ten dogs did not survive until discharge or were euthanized within 3 days after discharge (10/54, 18.5%). Six dogs were euthanized on request of the owners because of absent improvement of tetraplegia/tetraparesis (6/10, 60%) during hospitalization (range: 3–18 days), one because of recurring seizures unresponsive to treatment (1/10, 10%), two died spontaneously due to cardiopulmonary arrest (2/10, 20%), and one was euthanized because of concurrent bone tumor (1/10, 10%).

Long-Term Outcome and Neurological Sequelae

Forty-one of 54 (81%) dogs were discharged from the hospital and survived more than 3 days after discharge. Of those 41 dogs, dogs with non-ambulatory paraparesis (4/41, 9%) were able to ambulate unassisted within 6 days (ranging from 2 to 12 days), whereas tetraplegic/paretic dogs (10/41, 24%) regained ambulation within 12 days (3–26 days). In nine dogs with a cervical weakness (poliomyelitic form), the weakness resolved in all patients within 2–71 days.

In total, three cases were lost for follow-up 30 days after discharge. Of these three dogs, one was neurologically normal (30 days), one had persisting thoracic limb lameness (28 days), and one had generalized proprioceptive deficits and not further well specified behavioral changes (21 days).

Further follow-up interviews were available for 38 cases, while 6 cases were lost for follow-up interviews. The interview was performed 4 months−12 years after hospital discharge.

Six of 38 (15%) dogs were euthanized due to incomplete neurological improvement and owner's perceived poor quality of life within an average of 43.4 days (range: 3–120 days), while 2 cases were euthanized due to unrelated disease within 120 days. Case fatality rate after discharge within the first 4 months was 21% (8/38). No dogs were euthanized afterward because of TBE sequalae. Fifteen dogs of 36 that survived showed complete neurological recovery (15/36, 41%), while 17/36 (59%) dogs had neurologic deficits at the time of writing. In 4 cases (4/38, 11%) no information regarding neurological recovery was available. Reported neurological sequalae included facial palsy (1/17, 6%), weakness in the hind limb (1/17, 6%), and rarely occurring fine head tremors (1/17, 6%). In 4 cases, owners did not report neurological sequalae but asthenia (4/17, 24%). In one case, late occurrence (3 years after discharge) of aggressive behavior was reported during the follow-up phone call but because of lacking neurological reevaluation and behavioral examination a direct correlation with the previous infection cannot be drawn.

Five of 17 dogs with seizures lived longer than 3 months after discharge (5/17, 29.4%). One dog started seizing 1 year after discharge; the owner refused additional workup to assess the possibility of a post infection encephalopathy.

Necropsy Findings

Necropsy was available in seven dogs. Macroscopic examination of the central nervous system showed meningeal hyperemia in one dog on seven examined. In two dogs a chronic meningitis was evident. Histologically the main finding was consistent with moderate to severe multifocal sterile lymphohystiocitic (4/7) or lymphoplasmacytic (3/7) meningoencephalitis also with spinal cord involvement (poliomyelitis) in 5/7 cases. Furthermore, neuronophagia (7/7), perivascular cuffs (2/7), activated microglia expressed as glial nodules in gray (7/7) and white matter (3/7), diffuse demyelination (2/7) and white matter oedema (1/7). The lesions were affecting the gray matter of the cerebral cortex (7/7), the hippocampus (7/7), the basal ganglia (6/7), the thalamus (7/7), the brainstem (7/7), the cerebellum (7/7). The spinal cord was affected by a poliomalacia in 5/7 cases, and in 4 of them the lesions were widespread along the entire neuroaxis, while in one the cervicothoracic region was mainly affected. In the latter, the ventral rootlets of the cervicothoracic intumescence were also investigated revealing presence of multifocal axonal degeneration and Schwann cells proliferation. In three cases immunohistochemistry for TBEV was performed confirming clinical diagnosis with positivity in glial nodules (3/3) and ventral horn axons (1/3).

Statistical Analysis

Six cases lost for follow-up after discharge were excluded from further survival analysis. Our statistical analysis of the reported data showed that dogs with increased temperature at admission (>39.0) dogs with tetraplegia or tetraparesis, and dogs showing signs of recovers survived longer (**Table 1**). In contrast dogs with seizures had a shorter survival time. Survival time also decreased with age.

Dogs presenting with fever or tetraplegia/tetraparesis due to meningoencephalomyelitis and meningomyelitis/poliomyelitic tended to have a longer hospitalization time. Dogs presenting with signs of tetraplegia/tetraparesis took 1.32 (CI 1.05–1.80) times longer compared to others, to regain normal ambulation. The odds of developing sequelae increased with the duration of clinical signs before admission. For each day of clinical signs before hospital entry the odds of sequalae increased by a factor of 1.88 (CI 1.04–3.15). Although neurological sequalae are various, the hazard model did not reveal any negative outcome associated with these sequalae (**Table 1**).

TABLE 1 | Factors influencing survival of dogs diagnosed with tick born encephalitis.

Factor	Hazard ratio*	*P*-value
Age^	1.2 (1.04–1.44)	0.013
Presence of seizures	9.38 (2.43–36.14)	0.001
Presence of fever	0.22 (0.06–0.78)	0.02
Presence of tetraplegia/tetraparesis	0.16 (0.03–0.71)	0.02
Recovery	0.005 (0.0004–0.05)	<0.0001

**Hazard ratio >1 indicates an increased risk of death in a time period and hence shorter survival. ^Age is a continuous variable, so hazard ratio is for each 1 year increase in age. Other variables are binomial and the hazard ratio is the ratio between the presence and absence of the factor.*

DISCUSSION

This paper presents a population-based study about overall clinical presentation, clinical course, fatality rate, and neurological sequelae of TBEV infection in large population of affected dogs. Previously, this information could be only inferred from different publications, encompassing case reports or small case series published from 1972 to 2014, ranging from one to a maximum 12 dogs affected by TBEV (22, 27, 28).

In humans, European TEBV infection shows typically a biphasic appearance (1–3), and during the initial consultation unspecific symptoms such as fever, malaise, headache, sickness, vomiting, and muscular pain are reported up to 10 days before neurological signs occur (1–3, 17). In dogs a biphasic disease-course has been debated controversially (27). In this study population 34 owners or referring veterinarians recalled fever, and unspecific GI signs occurring from 1 to 10 days before the admission at our referral center. This supports that "malaise" as first clinical signs of TBEV viremia does occur in dogs as well.

Hyperthermia was evident in two-thirds (36/54) of our population and interestingly dogs with hyperthermia had a significantly lower risk to die compared to those that did not show hyperthermia. Hyperthermia is a hallmark of active infection and quite common in acute viral encephalitis. Non-structural proteins of TBEV antagonize type 1 interferon signaling (29) suppressing the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (2). This inhibition of type 1 interferon is causing a reduced anti-viral immunity in the host. Possibly patients with higher flavivirus load have a stronger suppression of type 1 interferon signaling, and therefore are not showing fever which may possibly influence outcome negatively (29).

Clinical signs of TBE are variable in humans and dogs (1, 19). In humans, the predominant form of TBE is meningitis (1, 19) while in the dogs in our study meningitis only was a rare presentation. Possibly dogs with meningitis only, and therefore milder clinical signs, are less likely to be referred. In contrast to humans, meningomyelitis and meningoencephalo-myelitis (1, 19) are common in dogs. TBE, because of its predilection to affect large neurons, frequently causes poliomyelitis (3, 18). Poliomyelitis primarily presenting with neck weakness is uncommon in other etiologies of myelitis in dogs (28, 30), and mainly reported in viral myelitis (16, 31). Similar to human meningoencephalitis it is also common in dogs. While seizures are a sporadic event in human TBE, being reported in up to 3% of the cases only, in dogs seizures occurred in 31% of the cases.

The hematobiochemical changes are compatible with an acute systemic inflammation, and similar to those described in human medicine (17, 32), however it would be interesting in the future to investigate positive acute phase proteins in active acute TBE, especially since hypoalbuminemia and hyperglobulinemia were a common feature in our population.

Cross sectional imaging is of utmost importance for neurological diagnosis. While CT was not useful in detecting the cause of neurological signs in TBE (17), MRI showed abnormal findings supportive of TBEV infection in two-thirds of our population. As previously published in human and veterinary literature, these findings are also not pathognomonic for TBE (1, 16, 17, 25, 26).

Diagnosis of TBE, especially in cases with normal MRI, can be challenging (1, 3, 8, 17). We had to exclude 3 cases from our study because of negative antibody titers in CSF at presentation. In these cases, most likely the clinical signs developed so rapidly that CSF antibodies against TBEV were initially negative, despite showing clinical signs compatible with TBE. However, in all of these three dogs TBE was later confirmed, two being tested positive for TBEV in CSF 1 and 2 weeks after initial presentation, respectively, and in the third case by positive immunohistochemical staining of brain specimen, acquired during necroscopy. Also, in human medicine it is known that TBEV could sporadically determine hyperacute neurological syndromes without CSF alterations (33) or initial negative antibodies testing (1–3, 17). In these cases, the gold standard is seroneutralization, but this is an expensive test requiring live virus, and flavivirus can be handled only by specific laboratories. For diagnostic purpose, positivity in commercial IFA or ELISA kits in the CSF has been used in the present paper in accordance to human standards as diagnostic hallmark of TBEV infection in presence of consistent clinical signs (1, 3, 17, 19), even with normal CSF examination (33). In dogs it has been shown that commercial ELISA kits have a specificity of 98.2% thus performing well as a diagnostic kit for dogs from endemic areas, such as ours (34). In our population, the magnitude of positivity was not a predictor of a negative outcome.

A RT-qPCR evaluation of CSF for viral RNA has been tempted unsuccessfully in 88% of cases in this study, and it could be explained because viral clearance is a fast process in TBE; TBEV RNA could be detected only up to 3 days from neuroinvasion (19, 27). Moreover, our assay, in comparison to a nested RT-qPCR previously described by Hekrlová et al. (35), does not perform the second nested PCR amplification, thus possibly reducing sensitivity in detecting viral RNA but, on the other hand, it is less prone to false positive results due to possible contaminations during the two-step procedure.

Pathological findings were available for review in only seven patients. In our population, the main findings were a widespread meningoencephalopoliomyelitis in five out of seven patients and three dogs were also positive for TBEV at immunohistochemistry within glial nodules (3/3) and also within axons (1/3). In one

case, also, severe changes within ventral rootlets characterized by axonal degeneration and Schwann cells were observed, supporting the neurotropism of this virus mainly for large motoneurons (2, 13, 15, 27).

Treatmentwise, TBE in humans and in dogs require intense nursing care and only recently some antiviral agents have been used in humans without reaching a general consensus about efficacy (1, 3, 17). Corticosteroids have been used in our patients to decrease the brain inflammation, pending results about infectious disease testing. In our practice a short course of steroids has been routinely used in dogs diagnosed with inflammatory central nervous disease with the intention to limit severe neuroinflammation. However, because the lack of untreated controls we could not assess the benefit of a short course of corticosteroids on outcome. In human medicine, neither steroids nor osmoactive drugs, such as mannitol, have been advocated for use on TBE patients, whereas pharmacologic induced coma (deepening of analgosedation) followed as second line by therapeutic hypothermia, or decompressive craniotomies have been recommended (3, 17). Therefore, therapeutic strategies in dogs need to be further studied before robust treatment recommendations can be formulated.

In humans, survival of the neurological infection is not uncommon, however long-term sequelae should be expected in up to 10% of the patients ranging from cognitive domain (memory impairment) or psychiatric (depression) to motor and sensory deficits because of poliomyelopathy or polyradiculoneuropathy (3, 17–19, 36). In a recent paper, sequalae were observed more frequently with clinical signs compatible with meningoencephalomyelitis or meningoencephalitis, respectively (in 43 and 25% of patients); meningitis resulted in fewer sequelae (12.6%) (36).

Little is known about long-term sequelae in dogs affected by TBE. In the present study, long-term sequelae were identified in 17% of the cases. These should raise the awareness of long-term neurological sequelae in dogs. In human patients more severe clinical syndromes (meningoecephalitis, meningoencephalomyelitis/poliomyelitic) are more likely to results in long-term signs (1, 18, 19, 36). Dogs in our study had a significantly increased risk for sequelae when they had shown longer clinical signs before admission.

While a possible behavioral sequela was only reported in one of the dogs in our study, motor neurological sequelae were commonly reported. A refinement of neurobehavioural assessment in dogs could reveal an increased frequency of abnormalities.

Previously, a high mortality of TBEV affected dogs compared to humans was reported. In the previously reported cases, fatalities within first weeks of disease ranging from 16 to 50% (27) to 100% (22), in our population 33% of dogs died within 4 months from the diagnosis, without TBE related deaths were recorded after this time.

An increased risk of death was found in dogs with seizures. This is in contrast to dogs with meningoencephalitis of unknown origin in which survival was not affected by the occurrence of seizures (28, 37). Severe seizure types (cluster seizures, status epilepticus) were observed in a majority of dogs and seizures were poorly controlled following administration of first line medication, phenobarbital, with 61% of non-responders explaining the morbidity associated with their occurrence. Nevertheless, seizure as expression of post infection long term sequelae has affected only one of our cases rendering an association with TBE uncertain. Seizures have been recently also described as long-term sequelae in dogs suffering from MEUO (38).

Another aspect of interest highlighted in our population, and not previously reported, was that the hazard risk of death increased with the age of the patient. In other words, older animals were more at risk of death, similar to what has been observed in human patients with TBE (3). An explanatory mechanism is unknown in humans as well as in our patients. However, before corroborating this parallelism, further research about reasons for euthanasia in TBEV infected dogs should be undertaken. In fact, another aspect not yet investigated is also the economic burden that some owners might face. Leister et al. (39) in a study about tick paralysis in cats in Australia, reported that 250/2,077 cats were euthanized due to financial restriction to perform medical treatment. This financial factor has not been evaluated in this or any previous research about short- or long-term outcome in dogs with neurological disease that require long-term hospitalization, also involving intensive care units. Future research will also take this important factor in account. Furthermore, our study highlighted the role of hyperthermia, however due to its retrospective nature, true fever could not be differentiated readily from exercise-induced hyperthermia due to seizure activity. Therefore, we aim to further evaluate those prognostic factors in a prospective study.

Concluding, our study shows many similarities and few differences of TBE between dogs and humans. Despite case fatality rate could reach 33% of the affected population within initial 4 months, survival is commonly observed, but long-term sequalae might be encountered.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because of the retrospective nature of the study that is based on preexisting medical records filed to achieve full diagnosis. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LG and FS designed the study. CK collected the data. LG and CK wrote the first draft of the manuscript. PT performed statistical analysis and performed a proof screening for British English language. KB, LG, and FS reviewed and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Inflammatory Disease Affecting the Central Nervous System in Dogs: A Retrospective Study in England (2010–2019)

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The epidemiology of inflammatory diseases affecting the central nervous system (CNS) in dogs is largely unknown. We aimed to report the relative proportion of different causes of inflammatory disease affecting the CNS in dogs and identify predictors for infectious vs. immune-mediated conditions and predictors for the most common diseases affecting the brain and the spinal cord. This was a retrospective cohort study over a 10-year period in 2 referral institutions using multivariable and multinomial logistic regression for identification of risk factors. In total, 1,140 client-owned dogs diagnosed with inflammatory disease affecting the CNS were included. Fifteen different diagnoses were identified, with immune-mediated (83.6%) disease being more common than infectious conditions (16.4%). The most common immune-mediated conditions diagnosed were meningoencephalitis of unknown origin (47.5%) and steroid-responsive meningitis–arteritis (30.7%), and the most common infectious conditions were discospondylitis (9.3%) and otogenic intracranial infection (2.2%). Older age ($p < 0.001$, OR = 1.019, 95% CI: 1.014–1.024), higher body weight ($p < 0.001$, OR = 1.049, 95% CI: 1.025–1.074), male sex ($p = 0.009$, OR = 1.685, 95% CI: 1.141–2.488), longer duration of the clinical signs before presentation ($p < 0.001$, OR = 1.011, 95% CI: 1.006–1.017), progressive nature of the clinical signs ($p < 0.001$, OR = 2.295, 95% CI: 1.463–3.599), identification of a possibly associated preceding event ($p = 0.0012$, OR = 1.93, 95% CI: 1.159–3.213), and hyperesthesia on presentation ($p < 0.001$, OR = 2.303, 95% CI: 1.528–3.473) were associated with a diagnosis of infectious diseases. Our data shows that immune-mediated diseases are more common than infectious conditions as a cause for inflammatory CNS disease in dogs. The risk factors for the most common diagnoses were identified from signalment, history, and findings of the physical and neurological examinations to give valuable information that can guide clinicians with their investigations.

Keywords: canine, central nervous system (CNS), meningoencephalitis of unknown origin (MUO), SRMA, infection

INTRODUCTION

Inflammatory disease affecting the central nervous system (CNS) in dogs can be due to infectious or immune-mediated causes (1–3). Infectious conditions include diseases caused by bacterial, viral, protozoal, rickettsial, fungal, parasitic, and algal agents (1–6). On the other hand, several idiopathic non-infectious meningoencephalomyelitides (NIMEs) have been described, including steroid-responsive meningitis–arteritis (SRMA) (7, 8), eosinophilic meningoencephalitis (9), granulomatous meningoencephalomyelitis (GME) (10–12), necrotizing meningoencephalomyelitis (NME) (10–14), necrotizing leucoencephalitis (NLE) (15, 16), idiopathic generalized tremor syndrome (IGTS) (17, 18), and idiopathic hypertrophic pachymeningitis (19). Due to the difficulty of distinguishing between GME, NME, and NLE without histopathological confirmation (which is rarely sought *in vivo*), the term meningoencephalomyelitis of unknown origin (MUO) is often used to describe cases for which any of these three conditions is suspected (20).

A definitive diagnosis, in most cases, would require a histopathological examination of the brain and/or spinal cord tissue, and so in clinical practice, the diagnosis of inflammatory CNS diseases relies on a cautious review of the clinical data. Clinical diagnosis is based on a combination of signalment, neurological deficits detected on examination, magnetic resonance imaging (MRI) findings, and results of cerebrospinal fluid (CSF) analysis and of infectious disease titers and antigen tests (20, 21). Treatment of infectious conditions relies on the use of specific medications against the infectious agent identified and, when dealing with bacterial diseases, preferably guided by culture and susceptibility results (2, 3). Conversely, treatment of immune-mediated conditions consists of immunosuppression, generally using corticosteroids, and often other immunosuppressive drugs (22).

It is currently suspected that NIMEs are more common than infectious causes, although the actual prevalence of any of these conditions is still undetermined in dogs (21). Our aim was therefore to report the proportion of the different types of inflammatory diseases affecting the CNS in England and to identify predictors for infectious and immune-mediated causes. As the causes for intracranial and spinal cord CNS inflammatory diseases are very different, we also aimed to identify predictors for the most common conditions affecting the brain and the spinal cord individually.

MATERIALS AND METHODS

Dogs diagnosed with any form of inflammatory CNS disease at the Small Animal Teaching Hospital (SATH) of the University of Liverpool and the Queen Mother Hospital for Animals (QMHA) of the Royal Veterinary College between January 2010 and December 2019 were identified through their respective hospital databases. Ethical approval for use of data was granted by the Ethics Committee of the University of Liverpool (VREC906).

Inclusion required that all clinical information required for statistical analysis was available for review and that historical findings, clinical signs, and clinicopathological and imaging findings allowed for a highly probable diagnosis; histopathological confirmation was used where available. A diagnosis of MUO required that dogs had to be older than 6 months of age, showed multiple, single, or diffuse intra-axial hyperintensities on T2-weighted MR images, had mononuclear pleocytosis on CSF analysis [more than 5 total nucleated cell count (TNCC)/μl, with more than 50% mononuclear cells], and with negative *Toxoplasma gondii* and *Neospora caninum* titers (20). Dogs were excluded if no pleocytosis was found on CSF analysis, with the exception of dogs with signs of raised intracranial pressure (ICP) on imaging studies, in which case CSF collection was not performed.

The following data were extracted from the medical records of the animals: age, sex, neuter status, breed, year of presentation, season of the year, onset, duration and progression of the clinical signs, description of any previous episode with similar clinical signs, identification of a possibly associated preceding event, presence of hyperthermia ($>39.5°C$) on presentation, presence of hyperesthesia on presentation, neurological deficits identified on presentation, and survival to discharge. Onset of the clinical signs was categorized as acute (<24 h), subacute (between 24 and 72 h), and chronic (more than 72 h).

MRI was performed in 958 cases using a 1.5-T scanner (Philips Ingenia CX, Philips Healthcare) and in 182 cases using a 1-T scanner (Siemens Magnetom, Siemens Healthcare), and the following information was recorded: if there were signs of raised intracranial pressure (identification of foramen magnum herniation, transtentorial herniation, and/or effacement of cerebral sulci) and whether there was one or multiple lesions seen. When cerebrospinal fluid analysis was performed, the following information was recorded: TNCC, protein concentration, and differential cell count. The results of the CSF analysis were considered abnormal when the protein concentration was >0.30 g/L (cerebellomedullary cistern collection) or >0.45 g/L (lumbar collection) or when the TNCC was >5 cells/μl. Additional test results, including synovial fluid analysis, infectious disease titers, and CSF culture results, were recorded when available.

Information regarding survival to hospital discharge was recorded for all cases, and data on long-term survival was collected from the records when available; the referring veterinary surgeons and pet owners were not contacted for the purposes of this study.

Statistical Analysis

Statistical analysis was performed using the software SPSS 22.0 (SPSS Inc., Chicago, Illinois, USA). Continuous data were assessed for normality using the Shapiro–Wilk test. Descriptive statistics are reported for continuous variables using mean (standard deviation) for approximately normally distributed variables and median (interquartile range) for variables with skewed distributions, and frequencies [with 95% confidence intervals (CI) where appropriate] are reported for categorical variables.

Univariable binary logistic regression was performed to identify clinical variables associated with either an underlying immune-mediated or infectious cause among all cases. Any independent variable demonstrating some association on preliminary univariable analysis ($P < 0.2$) was considered for inclusion in a multivariable model. Multivariable models were constructed with a manual backwards stepwise removal approach; variables with $P < 0.05$ were retained as statistically significant. The association between the underlying immune-mediated or infectious cause and survival to discharge was evaluated using the chi-square test.

Multinomial logistic regression was then used to further identify clinical variables associated with the three largest groups of diseases affecting the brain and the spinal cord separately. Inflammatory conditions affecting the brain were grouped in 3 categories: MUO (including the focal, disseminated, and optic forms), immune-mediated causes other than MUO (including idiopathic generalized tremor syndrome, eosinophilic meningoencephalitis of unknown origin, and idiopathic hypertrophic meningitis), and all infectious diseases. Inflammatory conditions affecting the spinal cord were grouped in 3 categories: SRMA, meningomyelitis of unknown origin, and all infectious causes. Similarly, univariable multinomial logistic regression was performed initially to identify clinical variables associated with the different brain and spinal cord disease categories, using MUO as the reference category for intracranial disease and SRMA as the reference category for spinal disease. Any independent variable demonstrating some association on preliminary univariable analysis ($P < 0.2$) was considered for inclusion in a multivariable model. Multivariable models were constructed with a manual backwards stepwise removal approach; variables with $P < 0.05$ were retained as statistically significant.

Variables included in the univariable analysis included age (months), body weight (kg), sex, neuter status, breed category, year of presentation, season of the year, center, onset, duration (in days) and progression of the clinical signs (defined as the development of new signs), description of a previous episode with similar clinical signs, identification of a possibly associated preceding event, presence of hyperthermia on presentation, presence of hyperesthesia on presentation, neurolocalization [categorized as forebrain, brainstem, cerebellum, central vestibular (used when there were insufficient signs to localize as brainstem or cerebellum), optic nerves/chiasm, C1–C5, C6–T2, T3–L3, and L4–S3 spinal cord segments, multifocal or no neurological deficits when only pain was identified], MRI abnormalities (one or multiple lesions identified), and CSF analysis findings (TNCC and protein concentration). Due to the significant number of cases in which MRI and/or CSF analysis had not been performed, these variables were not included in the multivariable analysis. For statistical analysis, the breeds were categorized according to the American Kennel Club guidelines (organized by the original type of work each breed was developed to do) into the following groups: sporting, hound, working, terrier, toy, herding, and non-sporting.

TABLE 1 | Proportion of the causes of inflammatory disease affecting the central nervous system in 1,140 dogs.

Suspected diagnosis	Frequency	Percentage (%)	95% CI
Meningoencephalomyelitis of unknown origin (MUO)[a]	541	47.5	44.7–50.4
Steroid responsive meningitis-arteritis (SRMA)[b]	350	30.7	28–33.2
Discospondylitis	106	9.3	7.7–11.1
Idiopathic generalized tremor syndrome	39	3.4	2.5–4.5
Otogenic intracranial infection	25	2.2	1.4–3.1
Empyema[c]	16	1.4	0.7–2.1
Eosinophilic meningoencephalitis	13	1.1	0.6–1.8
Bacterial meningoencephalitis	12	1.1	0.5–1.7
Neosporosis	12	1.1	0.5–1.7
Idiopathic hypertrophic meningitis	11	1	0.4–1.6
Angiostrongylosis	5	0.4	0.1–0.8
Fungal encephalitis	3	0.3	0–0.6
Distemper	3	0.3	0–0.6
Toxoplasmosis	2	0.2	0–0.4
Rickettsial meningoencephalitis	2	0.2	0–0.4

CI, confidence interval.
[a] *Within the MUO category, 73 dogs had meningomyelitis and 16 dogs had optic neuritis.*
[b] *SRMA was associated with concurrent immune-mediated polyarthritis in 31 cases.*
[c] *Empyema affected the brain in 12 cases and the spine in 4 cases.*

RESULTS

A total of 1,140 client-owned dogs diagnosed with inflammatory disease affecting the CNS were included in the analysis, with 450 dogs presented to the SATH and 690 presented to the QMHA. A total of 36 dogs that had been initially identified with a suspected diagnosis of MUO were excluded due to having normal TNCC on CSF analysis; 18 of these dogs were from breeds with reported necrotizing encephalitis and long duration of clinical signs.

A total of 15 different diagnoses were identified (**Table 1**), with immune-mediated diseases (83.6%) being considerably more common than infectious conditions (16.4%). In 604 dogs, the clinical signs suggested an intracranial neurolocalization (**Table 2**), while 536 dogs presented signs of spinal cord diseases (**Table 3**). An immune-mediated disease was diagnosed in 88.2% of dogs with intracranial neurolocalization, and 78.8% of dogs were diagnosed with spinal neurolocalization.

The cases presented evenly across the 10 years of study (between 7.7 and 12.7% each year) and throughout the seasons, with 23.3% presenting in winter, 25.3% in spring, 25.2% in summer, and 25.8% in autumn. Preceding events that may be associated with a condition were identified in 152 (13.3%) cases. The most commonly reported preceding events included a recent surgical procedure (63/152, 41.5%; the surgical procedure was neutering in 38 cases and spinal surgery in 5 cases), an infection focus (such as urinary tract infection or skin abscess) identified prior to the development of clinical signs (33/152, 21.7%), development of gastrointestinal signs prior to the onset of neurological deficits (27/152, 17.8%), vaccination within 2 weeks

TABLE 2 | Descriptive statistics for the 604 dogs with inflammatory disease affecting the brain.

	MUO (*n* = 468)	Other immune-mediated causes (*n* = 63)	Infectious causes (*n* = 73)
Median age (IQR)	48 months (51)	27 months (39)	58 months (61)
Median body weight (IQR)	8 kg (7)	9.9 kg (11.3)	15.6 kg (17.8)
Sex			
Female	259 (55.4%)	36 (57.1%)	24 (32.9%)
Male	209 (44.6%)	27 (42.9%)	49 (67.1%)
Neuter status			
Intact	183 (39.1%)	22 (34.9%)	31 (42.5%)
Neutered	285 (60.9%)	41 (65.1%)	42 (57.5%)
Breed categories			
Crossbreeds	66 (14.2%)	15 (23.8%)	6 (8.2%)
Sporting	48 (10.3%)	8 (12.7%)	19 (26%)
Hound	8 (1.7%)	17 (27%)	8 (11%)
Working	14 (3%)	1 (1.6%)	12 (16.5%)
Terrier	95 (20.3%)	13 (20.6%)	5 (6.8%)
Toy	185 (39.6%)	7 (11.1%)	12 (16.5%)
Herding	7 (1.5%)	1 (1.6%)	2 (2.7%)
Non-sporting	44 (9.4%)	1(1.6%)	9 (12.3%)
Previous similar episode	31 (6.6%)	1 (1.6%)	9 (12.3%)
Possibly associated preceding event	31 (6.6%)	13 (20.6%)	7 (9.6%)
Onset of clinical signs			
Acute	156 (33.4%)	15 (23.8%)	26 (35.6%)
Subacute	110 (23.6%)	17 (27%)	14 (19.2%)
Chronic	201 (43%)	31 (49.2%)	33 (45.2%)
Median duration of clinical signs (IQR)	7 days (12)	7 days (10)	6 days (19)
Progression of clinical signs	388 (82.9%)	54 (85.7%)	65 (89%)
Hyperthermia on presentation	22 (4.7%)	16 (25.4%)	16 (22%)
Hyperesthesia on presentation	152 (32.5%)	10 (15.9%)	27 (37%)
Median CSF TNCC (IQR)	36 cells/μl (208)	15 cells/μl (63)	201 cells/μl (2,409)
Median CSF protein concentration (IQR)	0.47 g/L (0.73)	0.21 g/L (0.24)	0.69 g/L (1.54)
Survival to discharge	381 (81.4%)	62 (98.4%)	62 (84.9%)

CSF, cerebrospinal fluid; IQR, interquartile range; MUO, meningoencephalomyelitis of unknown origin; TNCC, total nucleated cell count.

TABLE 3 | Descriptive statistics for the 536 dogs with inflammatory disease affecting the spinal cord.

	SRMA (*n* = 350)	MUO (*n* = 73)	Infectious causes (*n* = 113)
Median age (IQR)	11 months (8)	52 months (59)	76 months (91)
Median body weight (IQR)	14.1 kg (11.1)	9.8 kg (14.6)	22 kg (15.3)
Sex			
Female	146 (41.8%)	41 (56.2%)	42 (35.7%)
Male	203 (58.2%)	32 (43.8%)	71 (64.3%)
Neuter status			
Intact	203 (57.5%)	26 (35.6%)	33 (37.2%)
Neutered	150 (42.5%)	47 (64.4%)	51 (62.8%)
Breed categories			
Crossbreeds	68 (19.6%)	13 (18.1%)	16 (14.2%)
Sporting	77 (22.2%)	5 (7%)	32 (28.3%)
Hound	86 (24.8%)	4 (5.6%)	6 (5.3%)
Working	38 (10.9%)	7 (9.7%)	24 (21.2%)
Terrier	23 (6.6%)	7 (9.6%)	11 (9.7%)
Toy	28 (8.1%)	27 (37.5%)	3 (2.7%)
Herding	24 (6.9%)	1 (1.4%)	2 (1.8%)
Non-sporting	3 (0.9%)	8 (11.1%)	19 (16.8%)
Previous similar episode	81 (23.2%)	4 (5.4%)	14 (12.4%)
Possibly associated preceding event	68 (19.4%)	9 (12.3%)	25 (22.1%)
Onset of clinical signs			
Acute	89 (25.4%)	17 (23.3%)	17 (15%)
Subacute	135 (38.6%)	16 (21.9%)	22 (19.5%)
Chronic	126 (36%)	40 (54.8%)	74 (65.5%)
Median duration of clinical signs (IQR)	4 days (5)	7 days (18)	13 days (27)
Progression of clinical signs	95 (27.1%)	68 (93.2%)	79 (69.9%)
Hyperthermia on presentation	289 (82.6%)	9 (12.3%)	28 (24.8%)
Hyperesthesia on presentation	341 (97.4%)	46 (60.3%)	104 (92%)
Median CSF TNCC (IQR)	249 cells/μl (1,035)	174 cells/μl (835)	3 cells/μl (7)
Median CSF protein concentration (IQR)	0.65 g/L (1.16)	1.27 g/L (2.89)	0.55 g/L (0.8)
Survival to discharge	348 (99.4%)	67 (91.7%)	105 (92.9%)

CSF, cerebrospinal fluid; IQR, interquartile range; MUO, meningoencephalomyelitis of unknown origin; SRMA, steroid-responsive meningitis–arteritis; TNCC, total nucleated cell count.

from the onset of clinical signs (20/152, 13.2%), and recent estrus or phantom pregnancy (10/152, 6.6%).

Magnetic resonance imaging was performed in 796 cases (in 576 dogs, MRI of the brain was performed; in 202 dogs, it was MRI of the spinal cord; and in 18 dogs, it was MRI of both the brain and spinal cord) and was abnormal in 692 cases. The cases that did not undergo MRI mostly comprised dogs diagnosed with SRMA and discospondylitis (which, in some cases, had undergone radiography and/or computed tomography); radiography was performed in 78 dogs and computed tomography in 63 dogs. In dogs with intracranial disease, 189/590 (32%) showed signs on MRI suggestive of raised intracranial pressure, and 371/590 (62.9%) had multiple lesions identified, while in dogs with a spinal disease, only 60/206 (29.1%) had multiple lesions identified. Cerebrospinal fluid analysis was performed in 903 cases, with identification of signs of suspected raised intracranial pressure on MRI precluding

CSF collection in 102 dogs (**Tables 2**, **3**). CSF was collected from the cerebellomedullary cistern in 713 cases and from the lumbar cistern in 106; in 84 dogs, CSF was collected from both sites. Serum titers of *Toxoplasma gondii* (IgG and IgM) and *Neospora caninum* (IgG) were performed in 408 cases and revealed positive titers for *T. gondii* in 23 cases and *N. caninum* in 12 cases; in 21 cases, they were suggestive of exposure, and the repeat titers were supportive of this. Polymerase chain reaction to identify different infectious agents (most commonly canine distemper virus—CDV, *T. gondii* and *N. caninum*) was performed on CSF from 195 dogs and confirmed CDV in 3 dogs, *N. caninum* in 5 dogs, and *Ehrlichia canis* in 2 dogs. Cerebrospinal fluid culture was performed in 70 cases, and culture of other tissues (blood, urine, and/or wounds) was performed in 165 cases. The bacterial cultures were positive in 78 dogs: 22 on urine culture, 19 on blood culture, 6 on intervertebral disc culture, 4 on CSF culture, 4 on wound culture, 1 on post-mortem tissue culture, and 22 on surgical sample culture (12 from ventral bulla osteotomy, 6 from spinal surgery, and 4 from intracranial surgery). Arthrocentesis and synovial fluid analysis were performed in 52 cases and were supportive of immune-mediated polyarthritis in 31 cases (all these dogs were also diagnosed with SRMA). Histopathological confirmation of diagnosis was achieved in 44 cases through post-mortem examination and in 7 cases through surgical biopsies.

One thousand twenty-four dogs (89.8%) survived to discharge. Long-term follow-up information at 1 year following diagnosis was available for 744 dogs, and at that time, 554 dogs (65.3%) were still alive.

Binary Logistic Regression Results

On univariable analysis, age, body weight, sex, breed category, study center, year of presentation, onset, duration and progression of the clinical signs, identification of a possibly associated preceding event, presence of pyrexia on presentation, and presence of hyperesthesia on presentation showed some evidence of association ($P < 0.2$) with a diagnosis of an underlying immune-mediated or infectious cause. On multivariable analysis, older age, longer duration of the clinical signs before presentation, being male, progressive nature of the clinical signs, and identification of a preceding event were associated with a diagnosis of infectious diseases (**Table 4**). In breed categories, sporting and non-sporting breeds (with the French and English bulldogs as the most common breeds represented in the latter group) were associated with infectious causes. There was no association between survival to discharge nor survival at 12 months after diagnosis with a diagnosis of an underlying immune-mediated or infectious cause ($p = 0.729$ and $p = 0.69$, respectively).

TABLE 4 | Final multivariable logistic regression model for risk factors associated with infectious vs. immune-mediated causes among all cases.

	OR	95% CI	*P*-value
Age (months)	1.019	1.014–1.024	<0.001[a]
Body weight (kg)	1.049	1.025–1.074	<0.001[a]
Sex	1.685	1.141–2.488	0.009[a]
Duration of clinical signs (days)	1.011	1.006–1.017	<0.001[a]
Progression of clinical signs	2.295	1.463–3.599	<0.001[a]
Possibly associated preceding event	1.93	1.159–3.213	0.0012[a]
Hyperesthesia on presentation	2.303	1.528–3.473	<0.001[a]
Breed categories			
Crossbreeds	Ref	–	–
Sporting	2.198	1.143–4.229	0.018[a]
Hound	0.913	0.405–2.06	0.826
Working	2.043	0.911–4.579	0.083
Terrier	0.69	0.311–1.529	0.36
Toy	0.7	0.312–1.568	0.386
Herding	0.788	0.231–3.043	0.788
Non-sporting	6.255	2.981–13.126	<0.001[a]

CI, confidence interval; OR, odds ratio.
[a] *Statistically significant.*

Multinomial Logistic Regression Results for Intracranial Disease

On univariable analysis, age, body weight, sex, breed category, identification of a possibly associated preceding event, duration of signs, description of a previous episode with similar clinical signs, neurolocalization, presence of pyrexia on presentation, and presence of hyperesthesia on presentation showed some evidence of association ($P < 0.2$) with a diagnosis of MUO, immune-mediated causes other than MUO, or infectious diseases in dogs presenting with intracranial disease.

Identification of multiple lesions on MRI was negatively associated both with immune-mediated causes other than MUO ($p < 0.001$, OR = 0.118, 95% CI: 0.05–0.277) and with infectious causes compared to dogs with MUO ($p < 0.001$, OR = 0.161, 95% CI: 0.092–0.282).

On multivariable analysis (**Table 5**), a previous episode with similar clinical signs reported, male sex, and working, hound, and sporting breeds showed a significant association with a diagnosis of infectious causes compared to MUO. The hound breeds were positively associated, while the non-sporting breeds were negatively associated with a diagnosis of immune-mediated causes other than MUO compared to MUO. Hyperthermia was associated both with a diagnosis of immune-mediated causes other than MUO and with infectious causes compared to dogs with MUO. A cerebellar neurolocalization was associated with a diagnosis of immune-mediated causes other than MUO compared to MUO, and a central vestibular neurolocalization was associated with a diagnosis of infectious causes compared to MUO.

Multinomial Logistic Regression Results for Spinal Cord Disease

On univariable analysis, age, sex, neuter status, breed category, description of a previous episode with similar clinical signs, onset, duration, and progression of the clinical signs neurolocalization, presence of hyperthermia on presentation, and presence of hyperesthesia on presentation ($P < 0.2$) was associated with a diagnosis of SRMA, meningomyelitis of

TABLE 5 | Multinomial logistic regression results evaluating associations between different variables and a diagnosis of MUO, other immune-mediated causes, or infectious causes among cases with intracranial disease.

	Final diagnosis	OR (95%CI)	*P*-value
Sex	MUO	Ref	–
	Other IM causes	0.805 (0.386–1.681)	0.564
	Infectious causes	2.379 (1.248–4.408)	0.006[a]
Breed categories			
Crossbreeds	Ref	–	–
Sporting	MUO	Ref	–
	Other IM causes	0.798 (0.234–2.723)	0.718
	Infectious causes	5.571 (1.836–16.907)	0.002[a]
Hound	MUO	Ref	–
	Other IM causes	19.075 (5.408–67.286)	<0.001[a]
	Infectious causes	19.381 (4.762–78.875)	<0.001[a]
Working	MUO	Ref	–
	Other IM causes	0.742 (0.074–7.429)	0.8
	Infectious causes	18.136 (4.928–66.748)	<0.001[a]
Terrier	MUO	Ref	–
	Other IM causes	0.583 (0.193–1.765)	0.34
	Infectious causes	0.521 (0.129–2.108)	0.361
Toy	MUO	Ref	–
	Other IM causes	0.283 (0.088–0.911)	0.034[a]
	Infectious causes	0.873 (0.276–2.763)	0.818
Herding	MUO	Ref	–
	Other IM causes	0.921 (0.069–12.346)	0.95
	Infectious causes	5.911 (0.854–40.927)	0.072
Non-sporting	MUO	Ref	–
	Other IM causes	0.102 (0.01–1.043)	0.054
	Infectious causes	2.011 (0.553–7.312)	0.289
Previous similar episode	MUO	Ref	–
	Other IM causes	0.322 (0.032–3.293)	0.339
	Infectious causes	3.084 (1.13–8.417)	0.028[a]
Duration of clinical signs before presentation (days)	MUO	Ref	–
	Other IM causes	1.013 (1.001–1.024)	0.031[a]
	Infectious causes	1.014 (1.005–1.024)	0.003[a]
Hyperthermia on presentation	MUO	Ref	
	Other IM causes	3.1 (1.105–8.696)	0.032[a]
	Infectious causes	5.157 (2.164–12.29)	<0.001[a]
Neurolocalization			
No neurological deficits	Ref	–	–
Forebrain	MUO	Ref	–
	Other IM causes	1.588 (0.263–9.579)	0.614
	Infectious causes	1.047 (0.31–3.539)	0.941
Brainstem	MUO	Ref	–
	Other IM causes	1.292 (0.133–12.545)	0.825
	Infectious causes	1.519 (0.347–6.646)	0.579
Cerebellum	MUO	Ref	–
	Other IM causes	67.907 (11.75–392.18)	<0.001[a]
	Infectious causes	3.221 (0.674–915.338)	0.143
Central vestibular	MUO	Ref	–
	Other IM causes	1.403 (0.161–12.225)	0.759
	Infectious causes	5.369 (1.564–18.433)	0.008[a]
Optic nerves/chiasm	MUO	Ref	–
	Other IM causes	3.611 (0.424–30.75)	0.24
	Infectious causes	0.404 (0.038–4.261)	0.451
Multifocal	MUO	Ref	–
	Other IM causes	1.585 (0.284–8.841)	0.599
	Infectious causes	0.893 (0.277–2.885)	0.85

CI, confidence interval; IM, immune-mediated; MUO, meningoencephalomyelitis of unknown origin; OR, odds ratio; ref, reference category.
[a]*Statistically significant.*

unknown origin, or infectious causes in dogs presenting with a spinal disease.

On multivariable analysis (**Table 6**), older age was positively associated with a diagnosis of both MUO and infectious causes compared to SRMA. Increased body weight was associated with a diagnosis of infectious causes compared to SRMA. Sporting breeds were negatively associated with a diagnosis of MUO compared to SRMA. Hound breeds were negatively associated with a diagnosis of both MUO and infectious causes compared to SRMA. Non-sporting breeds were associated with a diagnosis of infectious causes compared to SRMA. Presence of hyperesthesia on presentation was negatively associated with a diagnosis of MUO compared to SRMA, while hyperthermia was negatively associated with a diagnosis of both MUO and infectious causes compared to SRMA. A chronic onset of the clinical signs was associated with a diagnosis of both MUO and infectious causes compared to SRMA. All spinal neurolocalizations were positively associated with a diagnosis of MUO compared to SRMA, and the T3–L3 and L4–S3 spinal segments were also associated with a diagnosis of infectious causes compared to SRMA.

DISCUSSION

Identifying the most common types of inflammatory disease affecting the CNS in dogs and their associated risk factors is important so that clinicians can develop appropriate differential diagnosis lists and be guided in diagnostic investigations. This study showed that immune-mediated conditions are considerably more common than infectious conditions, comprising over 80% of all inflammatory diseases affecting the CNS in a large population of dogs presenting to referral practices. The most common immune-mediated conditions diagnosed were MUO and SRMA (representing a combined total of 78.2% of all inflammatory diseases affecting the CNS in dogs), and the most common infectious conditions were discospondylitis and otogenic intracranial infection (accounting for a combined total of 11.5%). Older age, longer duration of the clinical signs

TABLE 6 | Multinomial logistic regression results evaluating associations between different variables and a diagnosis of SRMA, meningomyelitis of unknown origin (MUO) or infectious causes amongst cases with spinal disease using SRMA as the reference category.

	Final diagnosis	OR (95% CI)	*P*-value
Age (months)	SRMA	Ref	–
	MUO	1.029 (1.01–1.049)	0.003[a]
	Infectious causes	1.053 (1.036–1.071)	<0.001[a]
Body weight (kg)	SRMA	Ref	
	MUO	0.994 (0.918–1.077)	0.88
	Infectious causes	1.063 (1.006–1.123)	0.031[a]
Breed categories			
Crossbreeds	Ref	–	–
Sporting	SRMA	Ref	–
	MUO	0.061 (0.008–0.475)	0.008[a]
	Infectious causes	0.969 (0.263–3.573)	0.963
Hound	SRMA	Ref	–
	MUO	0.032 (0.002–0.469)	0.012[a]
	Infectious causes	0.05 (0.005–0.504)	0.011[a]
Working	SRMA	Ref	–
	MUO	0.234 (0.024–2.316)	0.214
	Infectious causes	0.782 (0.142–4.302)	0.777
Terrier	SRMA	Ref	–
	MUO	1.087 (0.132–8.944)	0.938
	Infectious causes	1.839 (0.292–11.576)	0.516
Toy	SRMA	Ref	–
	MUO	0.442 (0.048–4.043)	0.47
	Infectious causes	0.122 (0.011–1.411)	0.092
Herding	SRMA	Ref	–
	MUO	0.085 (0–44.842)	0.441
	Infectious causes	0.048 (0–15.36)	0.302
Non-sporting	SRMA	Ref	–
	MUO	0.552 (0.047–6.533)	0.638
	Infectious causes	14.159 (2.027–98.914)	0.008[a]
Hyperthermia on presentation	SRMA	Ref	–
	MUO	0.09 (0.022–0.363)	0.001[a]
	Infectious causes	0.139 (0.053–0.367)	<0.001[a]
Hyperesthesia on presentation	SRMA	Ref	–
	MUO	0.047 (0.005–0.44)	0.007[a]
	Infectious causes	0.352 (0.044–2.828)	0.326
Onset of the clinical signs			
Subacute	Ref	–	–
Acute	SRMA	Ref	–
	MUO	2.039 (0.299–13.912)	0.467
	Infectious causes	0.695 (0.137–3.518)	0.66
Chronic	SRMA	Ref	–
	MUO	7.983 (1.642–38.813)	0.01[a]
	Infectious causes	4.169 (1.13–15.388)	0.032[a]
Progression of the clinical signs	SRMA	Ref	–
	MUO	11.348 (2.526–30.97)	0.002[a]
	Infectious causes	2.931 (1.061–8.098)	0.038[a]
Neurolocalization			
No neurological deficits	Ref	–	–
C1–C5	SRMA	Ref	–
	MUO	362.291 (36.51–3,595.059)	<0.001[a]
	Infectious causes	2.064 (0.151–28.24)	0.587
C6–T2	SRMA	Ref	–
	MUO	31.459 (2.55–388.053)	0.001[a]
	Infectious causes	4.426 (0.419–46.736)	0.216
T3–L3	SRMA	Ref	–
	MUO	486.444 (41.354–5,721.931)	<0.001[a]
	Infectious causes	59.701 (5.871–607.11)	0.001[a]
L4–S3	SRMA	Ref	–
	MUO	574.41 (13.08–25,225.188)	0.001[a]
	Infectious causes	127.273 (3.723–4,350.853)	0.007[a]
Multifocal	SRMA	Ref	–
	MUO	404.147 (1.626–99,290.213)	0.033[a]
	Infectious causes	7.229 (0.032–1,624.233)	0.474

CI, confidence interval; MUO, meningoencephalomyelitis of unknown origin; OR, odds ratio; ref, reference category; SRMA, steroid-responsive meningitis–arteritis.
[a]*Statistically significant.*

before presentation, being male, progressive nature of the clinical signs, identification of a possibly associated preceding event, and presence of hyperesthesia on presentation were associated with a diagnosis of infectious diseases. Signalment, clinical history, and findings of the physical and neurological examinations were identified as different risk factors for the most common diagnoses of intracranial and spinal inflammatory conditions. Interestingly, there was no significant difference in short- or long-term survival between immune-mediated and infectious conditions.

In humans with encephalitis and meningitis, infectious causes have been reported as significantly more common than non-infectious ones, although between 20 and 60% of cases remain with an unknown diagnosis (23–25). Viral etiologies (most commonly enterovirus) are most common, followed by bacterial and sporadically fungal agents (23–25). Despite this, autoimmune encephalitis is being increasingly recognized, and it is thought that there are likely unidentified antibodies or other immune mechanisms explaining why many cases have an antibody-negative status (26). The etiology of MUO remains undetermined and likely has a multifactorial pathogenesis involving a combination of genetic factors and environmental triggers. Multiple attempts have been made to identify different infectious agents as possible triggers for MUO in dogs, but

these have so far been unsuccessful (27–29). In contrast, myelitis in humans is significantly more likely to be non-infectious, with multiple sclerosis as the most common cause, while infectious causes account for 1–12% of cases (30, 31). In veterinary medicine, only one previous study has investigated an inflammatory disease affecting the CNS, and its findings were very different from those in this study, as infectious causes accounted for ∼75% of the cases (1). In this study, canine distemper encephalitis (CDV) was the most common diagnosis (40%), followed by unclassified viral encephalitis (a group with non-suppurative meningoencephalomyelitis with no known virus detected on immunocytochemistry). This markedly contrasts with our findings as CDV was extremely rare in our population which was mostly vaccinated against CDV. It can also be speculated that some of the dogs diagnosed with unclassified viral encephalitis indeed had MUO, a less well-described entity at that time. In the previous study, most cases (over 80%) had post-mortem examination, likely affecting the proportion of dogs with less severe disease. Lastly, there is a 20-year time interval between both studies, so the difference in the results may also reflect temporal changes in the prevalence of the different diseases.

Our findings provide support for signalment being very useful in guiding the differential diagnosis list, with age, sex, body weight, and breed all showing significant associations with various diagnoses. Younger age was associated with SRMA, which was not unexpected as this condition has an age of onset typically between 6 and 18 months (3, 7, 32–35). Male sex was associated with a diagnosis of infectious conditions compared to MUO in dogs presenting with intracranial disease and also overall. A female predisposition in MUO has been reported in previous studies (10, 11, 14), but more recently, this finding has been disputed (21). In most immune-mediated diseases, no clear sex predisposition has been reported (18, 32–34), although a possibly slight male predisposition has been recorded in a cohort of dogs diagnosed with SRMA (35). In contrast, a male predisposition has been reported in discospondylitis (3, 36, 37) and possibly also in bacterial meningoencephalitis (4) and otogenic intracranial infection (38). In dogs presenting with intracranial disease, working, hound, and sporting breeds showed a statistically significant association with a diagnosis of infectious causes compared to MUO. It is well-known that small-breed dogs, particularly of toy and terrier breeds, are predisposed to MUO (10, 11, 21), so it was not surprising that these breeds were less likely to be diagnosed with infectious causes. Hound breeds were also positively associated with a diagnosis of immune-mediated causes other than MUO compared to MUO. This most likely reflects that sighthounds (a major representative of this breed group) might be predisposed to idiopathic hypertrophic meningitis (19), while similar breeds are affected by MUO and IGTS (18). In dogs presenting with a spinal cord disease, sporting and hound breeds were negatively associated with a diagnosis of MUO compared to SRMA, which was expected as these groups are predisposed to SRMA (7, 32–35). Interestingly, non-sporting breeds (the group mostly composed of English and French bulldogs) were positively associated with a diagnosis of infectious causes (most commonly discospondylitis) compared to SRMA; this predisposition has not been reported before.

Our results support that clinical history is very useful in guiding the clinician, as longer duration of the clinical signs before presentation, progressive nature of the clinical signs, and identification of a possibly associated preceding event were associated with infectious causes. The longer duration of the clinical signs before presentation in dogs with infectious causes is likely associated with the slower progression of some of the most common infectious conditions seen, namely, discospondylitis and otogenic intracranial infections, compared to dogs with MUO or SRMA (38, 39). The most commonly reported preceding events were surgical interventions and an infectious focus, and these have been associated with discospondylitis and bacterial meningoencephalitis (3, 4). In dogs presenting with a spinal disease, progression of the clinical signs was positively associated with a diagnosis of MUO and infectious causes compared to SRMA. In SRMA, two forms are reported, with a chronic protracted form possibly occurring following relapse of the acute disease and/or inadequate treatment (3, 7). Episodic and recurrent clinical signs are usually reported, and only rarely do neurological deficits develop in the chronic form, likely explaining the lack of progression of the clinical signs reported in the dogs with SRMA seen in this study.

The findings of the physical and neurological examinations also demonstrated usefulness in distinguishing between the different causes of inflammatory CNS diseases. Hyperthermia was positively associated with a diagnosis of immune-mediated causes other than MUO and with infectious causes compared to MUO in dogs presenting an intracranial disease. Hyperthermia was negatively associated with a diagnosis of both MUO and infectious causes compared to SRMA in dogs presenting with a spinal disease. This is not surprising as dogs with SRMA (7, 8), IGTS (18), and many infectious conditions affecting the CNS (3, 4, 38) are frequently reported with hyperthermia. As expected, different neurolocalizations were associated with different conditions. A cerebellar neurolocalization was significantly associated with a diagnosis of immune-mediated causes other than MUO compared to MUO, most likely related to the typical signs of cerebellar dysfunction seen in dogs with IGTS (particularly tremors and ataxia) (18). A central vestibular neurolocalization was significantly associated with a diagnosis of infectious causes compared to MUO in dogs presenting with an intracranial disease. It would seem most likely that this is related to many of the infectious causes of intracranial diseases being dogs diagnosed with otogenic intracranial infection, in which involvement of the cerebellomedullary region of the brainstem is most common (38). All spinal neurolocalizations were positively associated with a diagnosis of MUO compared to SRMA, and the T3–L3 and L4–S3 spinal segments were associated with a diagnosis of infectious causes compared to SRMA. This is an unsurprising finding as neurological deficits are not common with SRMA (7) but expected in MUO (21), and discospondylitis most commonly affects the lumbosacral and thoracolumbar regions (3, 36, 37, 37).

Only a small number of dogs with a suspected diagnosis of angiostrongylosis was identified. It is uncertain in these cases

if the cause of the abnormalities detected on MRI was related to a direct effect of the migrating *Angiostrongylus vasorum* larvae or more likely due to the resulting coagulopathy seen with the infection, as both have been reported previously (40, 41). If it was the latter, these cases should not be considered true CNS infection, as the clinical signs would be associated with a vascular etiology rather than inflammatory. The authors felt that, without a histopathological evaluation, it would be impossible to rule out larvae migration, and even in the event of an indirect effect, when an infectious underlying cause would be present, dogs diagnosed with angiostrongylosis would be included. This may have introduced a small bias, but we felt that the information was relevant to the study.

The MRI findings were mostly non-specific, but the identification of multiple lesions on MRI was associated with MUO as expected (21). There were no seasonal, geographical (with one of the centers located in the north and the other in the south of England), or temporal variations over the decade studied identified in our data. It is unknown if our findings would be reproducible in other areas of the world with a higher prevalence of infectious agents, and so further studies evaluating regional differences are warranted.

There are several limitations to this study. The main limitation is the lack of a histopathological confirmation in the majority of cases, which makes misdiagnosis possible, and therefore the relative proportion of the diseases may be inaccurate. Most dogs (89.5%) survived to discharge, with many still alive at 12 months later. Thus, including a histopathological examination as part of the inclusion criteria would have reduced the sample size considerably and risked introducing much more significant bias to the results. Due to the lack of a histopathological confirmation, it was also not possible to distinguish between the subtypes of MUO, and therefore it was not possible to assess if more specific diagnoses would have shown different associations or impacted survival. Because of its retrospective nature, not all dogs underwent the same diagnostic investigations, and not all epidemiologic data were available for review. Dogs that did not undergo CSF analysis due to the identification of raised ICP on MRI were not excluded from the analysis, as this would have likely biased the data by excluding the most severely affected cases. Nonetheless, this may also have added bias if those dogs were misdiagnosed from the lack of that data. Cases with suspected MUO but with normal CSF analysis and/or normal MRI were excluded, and this may have falsely reduced the frequency of this disease, as normal findings in either of these modalities can be seen in MUO (10, 20, 42). It should also be considered that the presumptive diagnosis of many of these conditions is often based on a combination of factors, including signalment and clinical and imaging findings, and some of these were subsequently identified as associated with specific conditions; this was also likely a source of bias. Lack of data on MRI or CSF analysis in some cases raised difficulties in the application of multivariable statistical analysis—the reason is related to the fact that the different diseases diagnosed require different diagnostic tests, and so not all results are available for all animals. Imputation approaches would not be appropriate for the missing information, so the authors felt that it was more appropriate to exclude the MRI and CSF variables from the multivariable analyses.

Our data confirms that immune-mediated disease is more common than infectious conditions as a cause for an inflammatory CNS disease in dogs as previously suspected. Signalment, clinical history, and findings of the physical and neurological examinations were identified as different risk factors for the most common diagnoses, presenting valuable information which can help guide clinicians. Close to 90% of all dogs survived to discharge, with no significant difference in short- or long-term survival between immune-mediated and infectious conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the University of Liverpool. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

RG and GW were responsible for the conception of the study. Data acquisition was done by RG, SD, and SB. Statistical analysis, data analysis, and manuscript writing were performed by RG. TM provided statistical advice. GW, SD, and TM supervised the data collection and manuscript editing. All authors contributed to the article and approved the submitted version.

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Screening for Viral Nucleic Acids in the Cerebrospinal Fluid of Dogs With Central Nervous System Inflammation

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Central nervous system (CNS) inflammation is a common cause of neurological dysfunction in dogs. Most dogs with CNS inflammation are diagnosed with presumptive autoimmune disease. A smaller number are diagnosed with an infectious etiology. Additionally, at necropsy, a subset of dogs with CNS inflammation do not fit previously described patterns of autoimmune disease and an infectious cause is not readily identifiable. Because viral infection is a common cause of meningoencephalitis in people, we hypothesize that a subset of dogs presented with CNS inflammation have an occult viral infection either as a direct cause of CNS inflammation or a trigger for autoimmunity. The goal of this research was to screen cerebrospinal fluid from a large number dogs with CNS inflammation for occult viral infection. One hundred seventy-two dogs with neurological dysfunction and cerebrospinal fluid (CSF) pleocytosis were identified. Of these, 42 had meningoencephalitis of unknown origin, six had steroid-responsive meningitis-arteritis, one had eosinophilic meningoencephalitis, five had documented infection, 21 had and undetermined diagnosis, and 97 had a diagnosis not consistent with primary inflammatory disease of the CNS (e.g., neoplasia). CSF samples were subsequently screened with broadly reactive PCR for eight viral groups: adenovirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus, and parechovirus. No viral nucleic acids were detected from 168 cases screened for eight viral groups, which does not support occult viral infection as a cause of CNS inflammation in dogs. La Crosse virus (LACV) nucleic acids were detected from four cases in Georgia. Subclinical infection was supported in two of these cases but LACV could not be ruled-out as a cause of infection in the other two cases, suggesting further research is warranted to determine if LACV is an occult cause of CNS inflammation in dogs.

Keywords: canine, central nervous system (CNS), inflammation, meningoencephalitis of unknown origin (MUO), virus, polymerase chain reaction (PCR), bunyavirus, La Crosse virus (LACV)

INTRODUCTION

Central nervous system (CNS) inflammation is a common cause of neurological dysfunction in dogs. Currently, at tertiary referral hospitals, the majority of dogs with CNS inflammation are diagnosed with presumptive autoimmune disease, or meningoencephalomyelitis of unknown origin (MUO) (1). The remainder are diagnosed with a variety of infectious etiologies, with bacterial, viral, protozoal, and fungal being the most common (1, 2). There is also a subset of cases that after histological evaluation of CNS tissue, do not fit previously described patterns of autoimmune disease and lack a readily identifiable infectious cause despite extensive searching with traditional histological stains, bacterial and viral culture, and PCR for previously described infectious organisms.

Several research groups have looked for occult infectious causes of CNS inflammation in dogs. Most notably, researchers have long hypothesized that MUO has an infectious cause, either directly leading to stimulation of an immune response or triggering autoimmunity (3). However, to date, an infectious etiology for MUO has not been identified (4–10). Large numbers of dogs with neurological dysfunction also have been screened for specific infectious agents such as *Anaplasma phagocytophilum*, *Borrelia burgdorferi sensu lato* (5), and *Bartonella* spp. (8) with negative results.

We hypothesize that a subset of dogs presenting for primary CNS inflammation have an occult viral infection. Viral infections are a common cause of meningoencephalitis in people (11), and there is strong support for virus-triggered CNS autoimmunity derived from animal models (12). Additionally, over the past several decades, there have been sporadic reports of uncommon viral infections leading to CNS disease in dogs (13–20).

Diagnosis of viral meningoencephalitis in people is often accomplished by PCR of cerebrospinal fluid (CSF) (11), and broadly reactive PCR methodologies have repeatedly proven useful in viral discovery (21, 22). Based on this, we utilized broadly reactive PCR assays to interrogate cerebrospinal fluid (CSF) from dogs with evidence of CNS inflammation for detection of pathogens in 8 viral groups (adenovirus, bunyavirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus, and parechovirus), all of which have been documented as causative agents of meningoencephalitis in people (23–30).

MATERIALS AND METHODS

Case Samples

CSF was collected in accordance with Institutional Animal Care and Use guidelines in routine fashion from the cerebellomedullary or lumbar cistern from dogs that presented with neurological signs to the University of Georgia College of Veterinary Medicine (UGA-CVM), Texas A&M University College of Veterinary Medicine and Biomedical Sciences (TAMU-CVM), and The Royal Veterinary College (RVC), University of London between 2003 and 2008. Cytologic analysis and protein quantification were performed by a board-certified clinical pathologist. A separate aliquot of excess CSF, that was not subjected to clinical pathological testing and therefore still contained cells, was stored at −80°C. All samples were analyzed from 2008 to 2010.

Cases were included in the study if the dogs had neck or back pain and/or neurological deficits referrable to the CNS with concurrent CSF pleocytosis (defined as >5 white blood cells (WBC)/μl and <4,000 red blood cells/μl) (31). Clinical information, including age, gender status, breed, clinical history, neurological signs, neuroanatomic localization, magnetic resonance imaging (MRI) or computed tomography (CT) findings, clinical pathology results, presumptive diagnosis, treatment, response to treatment, and necropsy findings were recorded from medical records. Based on diagnostic results, dogs were categorized into one of three groups: (1) a diagnosis of primary inflammatory disease of the CNS, (2) a diagnosis not consistent with primary inflammatory disease of the CNS (e.g., neoplasia), and (3) a lack of a definitive diagnosis after MRI and CSF analysis and/or necropsy (**Supplementary Table 1**). Where possible, a necropsy diagnosis was utilized. When not possible, previously described clinical diagnostic criteria were utilized to diagnose non-infectious inflammatory disease (32–37), and infection was diagnosed based on culture, serology, PCR, and/or CSF cytology.

Nucleic Acid Extraction and PCR Quality Control

Total nucleic acids were extracted from CSF (Qiagen MinElute Virus Spin Kit, Qiagen) and stored as single-use aliquots at −80°C. A 215 base pair (bp) fragment of the canine histone 3.3 gene (38) or 191 bp fragment of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (39) was amplified from all samples to confirm DNA integrity. RNA integrity was confirmed in all samples by reverse transcription PCR (RT-PCR) amplification of superoxide dismutase (SOD) (expected product size 440 bp) (40). To avoid contamination, nucleic acid extraction, PCR preparation (pre-amplification), PCR, and sequencing were carried out in different rooms. Negative controls containing no DNA or RNA template were run in parallel with all PCR reactions. Additionally, mock nucleic acid extraction of sterile water was performed in parallel with all clinical cases and utilized as a negative control for all PCR reactions.

Broadly Reactive Pan-Viral Group PCR

Consensus, degenerate, or consensus-degenerate hybrid primers were used for broadly reactive viral PCR (**Table 1**). Adenovirus PCR (Platinum *Taq* DNA Polymerase kit, Invitrogen); bunyavirus RT-PCR and coronavirus, flavivirus, and paramyxovirus semi-nested RT-PCR (SuperScript III One-Step RT-PCR System, Invitrogen); herpesvirus semi-nested PCR (snPCR) (HotStar*Taq* DNA Polymerase kit, Qiagen); and parechovirus and enterovirus real-time RT-PCR (rRT-PCR) (SuperScript III One-Step Quantitative RT-PCR System, Invitrogen) were performed according to manufacturer's instructions with a final volume of 50 μl and final primer concentration of 1 μM unless otherwise noted. RT-PCR reactions contained 20 U RNase inhibitor (Roche Diagnostics) and PCR and snPCR reactions used 200 μM (each) of deoxynucleotide triphospates (dNPTs) (Roche Diagnostics). Initial reactions were

TABLE 1 | Sequences of viral oligonucleotide primers use for polymerase chain reaction.

	Primer sequence (5′ to 3′)	Amplicon (bp)
Adenovirus ADVE2B F	TCMAAYGCHYTVTAYGGBTCDTTTGC	450
Adenovirus ADVE2B R	CCAYTCHSWSAYRAADGCBCKVGTCCA	
Adenovirus ADVhexon F	AARGAYTGGTTYYTGRTNCARATG	400
Adenovirus ADVhexon R	CCVAGRTCNGTBARDGYSCCCAT	
Bunyavirus BCS82C	ATGACTGAGTTGGAGTTTCATGATGTCGC	251
Bunyavirus BCS332V	TGTTCCTGTTGCCAGGAAAAT	
Coronavirus F2	ATGGGITGGGAYTATCCWAARTGTG	440
Coronavirus R3A	AATTATARCAIACAACISYRTCRTCA	
Coronavirus R3B	TATTATARCAIACIACRCCATCRTC	
Coronavirus R2A8	CTAGTICCACCIGGYTTWANRTA	199
Coronavirus R2B8	CTGGTICCACCI GGYTTNACRTA	
Flavivirus cFD2	GTGTCCCAGCCGGCGGTGTCATCAGC	250
Flavivirus MAMD	AACATGATGGGRAARAGRGARAA	
Flavivirus FS778	AARGGHAGYMCDGCHATHTGGT	214
Herpesvirus DFASA	GTTCGACTTYGCNAGYYTNTAYCC	500
Herpesvirus GDTD1B	GCATGCGACAAACACGGAGTCNGTRTCNCCRTA	
Herpesvirus VYGA	GTGCAACGCGGTGTAYGGNKTNACNGG	236
Paramyxovirus PAR-F1	GAAGGITATTGTCAIAARNTNTGGAC	650
Paramyxovirus PAR-F2	GTTGCTTCAATGGTTCARGGNGAYAA	
Paramyxovirus PAR-R	GCTGAAGTTACIGGITCICCDATRTTNC	563
Enterovirus AN350	GGCCCTGAATGCGGCTAATCC	145
Enterovirus AN 351	GCGATTGTCACCATWAGCAGYCA	
Enterovirus probe AN234	FAM-CCGACTACTTTGGGWGTCCGTGT-BHQ-1	
Parechovirus AN345	GTAACASWWGCCTCTGGGSCCAAAAG	194
Parechovirus AN344	GGCCCCWGRTCAGATCCAYAGT	
Parechvirus probe AN257	YY-CCTRYGGGTACCTYCWGGGCATCCTTC-BHQ-1	

performed with 5 μl template DNA or RNA, and semi-nested reactions were performed with 2 μl of template from the initial reaction.

Generic adenovirus primers previously designed (41) to an ∼450 bp region of the DNA polymerase gene (AdVE2B F and AdVE2B R) and 400 bp region of the hexon gene (AdVhexon F and AdVhexon R) were used for PCR as previously described (4). Canine adenovirus (CAV)-1 and CAV-2 DNA extracted from purified virus-infected tissue culture supernatant was used as a positive control.

Generic bunyavirus primers BCS82C and BCS332V previously designed to an ∼251 bp region of the small segment were used for RT-PCR (42). After initial reactions at 60°C for 1 min, 45°C for 30 min, and 94°C for 2 min, RT-PCR cycled 40 times at 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s, followed by a final elongation step at 72°C for 7 min. RNA from a mutated clone of La Crosse virus (LACV) was used as a positive control.

Pan-coronavirus primers previously designed to an ∼440 bp region of the highly conserved polymerase 1b open reading frame were used for snRT-PCR (21). Primers F2, R3A (0.5 μM), and R3B (0.5 μM) were used for the initial reaction and F2, R2A8, and R2B8 were used for the semi-nested reaction. Reverse transcription began at 60°C for 1 min, 45°C for 30 min, and 94°C for 2 min, followed by 40 cycles at 94°C for 15 s, 50°C for 30 s, and 72°C for 30 s with a final elongation step at 72°C for 7 min. RNA from human coronavirus OC43 was used as a positive control (expected product size 199 bp).

Pan-flavivirus primers previously designed to an ∼250 bp conserved region of the non-structural protein NS5 gene were utilized for snRT-PCR (25). Primers cFD2 and MAMD were used for the initial reaction and cFD2 and FS778 were used for the semi-nested reaction. Reverse transcription began at 60°C for 1 min, 42°C for 30 min, and 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min with a final elongation step at 72°C for 7 min. RNA from a mutated clone of St. Louis encephalitis (SLE) virus was used as a positive control (expected product size 214 bp).

Pan-herpesvirus primers previously designed to an ∼500 bp region of the DNA polymerase gene were used for snPCR (43). Primers DFASA and GDTD1B were used for the initial reaction and VYGA and GDTD1B were used for the semi-nested reaction. Both reactions began with an initial hot-start at 94°C for 2.5 min, followed by 50 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final elongation step at 72°C for 10 min. DNA from canine herpesvirus type 1 was used as a positive control (expected product size 236 bp).

Pan-paramyxovirus primers PAR-F1, PAR-F2, and PAR-R previously designed to an ∼650 bp region of the polymerase L gene were used for snRT-PCR as previously described (44). Template RNA from human

parainfluenza virus 2 was used as a positive control (expected product size 563 bp).

Previously designed enterovirus and parechovirus primers (0.4 μM each) and probes (0.2 μM each) (TaqMan, Applied Biosystems) previously designed based on highly conserved 5′ non-translated regions were used for rRT-PCR (45, 46). After initial reactions at 50°C for 30 min and 95°C for 10 min, rRT-PCR cycled 50 times with the following parameters: 95°C for 15 s, 58°C for 30 s, and 72°C for 10 s, with probe detection during the 58°C annealing step (Roche LightCycler, Roche Diagnostics). Threshold cycle values were determined using commercially available software (Roche LightCycler, Roche Diagnostics). Template DNA from echovirus 30 and human parechovirus 1 (Harris strain) were used as positive controls for enterovirus and parechovirus rRT-PCR, respectively.

Sequencing

PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining under ultraviolet exposure, and amplicons were purified (MinElute PCR Purification Kit and QIAquick Gel Extraction Kit, Qiagen) for sequencing. Purified amplicons or plasmids were sequenced (BigDye Terminators v3.1 and ABI 3730xl, Applied Biosystems) using the corresponding amplification primers. Viral species were defined by comparison of DNA sequences with GenBank database entries using the Basic Local Alignment Search Tool (BLAST 2.0).

RESULTS

Cerebrospinal fluid from 172 pure or mixed breed dogs was tested by all PCR methodologies, including 92 from UGA-CVM, 26 from TAMU-CVM, and 54 from RVC. Primary CNS disease was diagnosed in 54 dogs: 42 with MUO, 6 with steroid-responsive meningitis-arteritis (SRMA), 1 with idiopathic eosinophilic meningoencephalitis, and 5 with infection (two with Rocky Mountain spotted fever, one with *Bartonella vinsonii*, one with Zygomycetes encephalitis, and one with encephalitis secondary to bacterial abscesses). Non-inflammatory CNS disease was diagnosed in 97 dogs. A diagnosis was not reached in 21 dogs.

All positive controls produced the expected PCR results; all negative controls were free of viral amplicons. Histone or GAPDH and SOD were amplified successfully from all cases. Nucleic acids from pan-viral group PCR for adenoviruses, coronaviruses, enteroviruses, flaviviruses, herpesviruses, paramyxoviruses, and parechoviruses were not detected in the 172 samples evaluated by PCR. Amplicons of the expected size (251 bp) (42) were detected by generic pan-bunyavirus RT-PCR in one case with MUO, two dogs with non-inflammatory CNS disease, and one dog with an open diagnosis. Direct sequencing of the amplicons from all cases demonstrated >95% sequence identity to the nucleoprotein gene of several LACV strains, including 65/OH-M (GenBank accession GU206123.1), 97/NC-M (GenBank accession GU206126.1), 93/MO-H (GenBank accession GU206138.1), 74/NY-M (GenBank accession GU206141.1), 00/WV-M (GenBank accession GU206147.1), and 00/NC-M (GenBank accession GU206111.1).

All LACV-positive cases were evaluated by the neurology service at the UGA-CVM. The positive MUO case was a 3-year-old male intact Boston terrier dog that was presented for evaluation of acute-onset blindness and an abnormal gait. The general physical exam, serum biochemistry, complete blood count, and thoracic radiographs were normal aside from a mild leukocytosis characterized by a mature neutrophilia. Neurological exam was consistent with multifocal CNS disease. No abnormalities were identified on magnetic resonance imaging (MRI) of the brain and cervical spinal cord. CSF from the cerebellomedullary cistern revealed 69 RBC/μl, 20 WBC/μl, characterized by a lymphocytic pleocytosis, and a total protein concentration of 16.5 mg/dl. There was complete resolution of clinical signs after treatment with prednisone and cytosine arabinoside. The other cases included an 11-year-old Boxer dog with seizures secondary to an insulinoma, a 12-year-old Weimaraner with seizures secondary to a nasal adenocarcinoma invading the olfactory bulb and frontal lobe of the brain, and an 11-year-old Shih Tzu with epilepsy and a normal MRI.

DISCUSSION

We tested a large number of dogs with CSF pleocytosis for occult viral infections using broadly reactive PCR. No viral nucleic acids were detected in CSF for seven of the viral groups evaluated: adenovirus, coronavirus, enterovirus, flavivirus, herpesvirus, paramyxovirus, parechovirus, which does not support occult viral infection as a cause of CNS inflammation in dogs. A small number of cases from the University of Georgia were positive for LACV. Subclinical infection was supported in two of these cases but LACV could not be ruled-out as a cause of infection in the other two cases, suggesting further research is warranted to determine if LACV is an occult cause of CNS inflammation in dogs in endemic regions of the United States.

The findings shared here are consistent with previous research reports. In recent research studies, no occult infectious organisms have been identified in dogs with MUO (7, 9, 10) or CNS inflammation (5, 8). However, this report expands on published research in several ways. First, CSF from a larger number of cases than has been previously reported was evaluated using broadly reactive molecular methodologies (i.e., interrogation for a large number of viral groups by broadly reactive PCR intended to identify all species within each group). Also, we included cases with CSF pleocytosis that did not have a readily identifiable diagnosis (and as such may have been more likely to have an occult viral infection).

The predominately negative results presented here could be due to study limitations. It is possible that screening a larger number of cases from a more geographically diverse population would yield positive results. There also were only a small number of dogs <1 year of age included in the final analysis. This was likely a reflection of the referral populations included in the study but may have precluded

identification of viruses that would be expected to affect younger patients (13, 47). Additionally, a large number of cases screened ($n = 97$) had a diagnosis not consistent with primary CNS inflammation. These cases were included as a control population that might have blood-CSF barrier breakdown and thus be susceptible to contamination from bloodborne agents.

Panviral group (family and genera) PCR was chosen for this study due to its sensitivity in viral discovery (21, 22). Although next generation sequencing is ideal to identify a broad range of microorganisms, including those not yet known, the depth of sequencing may limit sensitivity and necessitate enrichment of viral nucleic acids to improve outcomes (48–52). However, despite the sensitivity of PCR, false negative results are still possible in this study for a number of reasons. For example, it is possible that viruses targeting CNS parenchyma could be missed in CSF. Using CSF with an elevated cell count and total protein has been shown to be important in humans when searching for viruses. For herpes simplex virus, screening of CSF samples requires a minimum of 5 cells/μl and >50 mg/dl protein to increase yield (53). Although all cases in our study had pleocytosis, we did not use elevated protein as an inclusion criterion.

Future studies could include larger numbers of dogs with CNS inflammation and assessment of brain parenchyma concurrent with CSF using a combination of methodologies such as next generation sequencing and panviral PCR to improve sensitivity. Clinicians and pathologists should work together to save fresh frozen brain tissue from all potential inflammatory cases. This would allow researchers to thorough molecular interrogation of larger numbers of dogs with MUO (9) and allow additional evaluation of the subset of dogs that have CNS inflammation but no definitive diagnosis upon completion of necropsy.

The LACV-positive findings in this report could represent false positive PCR results, evidence of subclinical infection, or suggest that LACV is a more common cause of CNS inflammation in dogs than previously recognized. LACV is an arbovirus known to cause disease in a variety of mammals in the Midwestern, mid-Atlantic, and southeastern United States (54). Although the majority of infections in people are suspected to be asymptomatic or result in mild, flu-like illness (54–59), true incidence is hard to quantify because diagnostic testing for LACV is not performed in these cases. In the United States, 50–150 cases of more severe LACV infection are reported each year (Centers for Disease Control and Prevention)[1], with >90% representing neuroinvasive disease in children under age 16 (54, 60–62). Neuroinvasive disease has also been reported in numerous species (54), with necrotizing meningoencephalitis secondary to LACV infection documented in in five puppies (13) and one adult dog (63), all from Georgia.

False positive PCR results are considered unlikely in this study for several reasons. First, sequencing of PCR products confirmed specific amplification of LACV. Second, all PCR was conducted in a PCR-dedicated laboratory where PCR preparation and sample handling were physically separated from analysis of PCR products. Additionally, all PCRs were run in a laboratory that had not previously amplified or worked with LACV, and each PCR was run with a negative control to ensure no contamination was present (64, 65).

Subclinical infection is considered likely in the two cases that had neoplasia (invasive nasal adenocarcinoma and insulinoma) as a diagnosis for the cause of seizures and also may be possible the other two positive cases (one with MUO and one with seizures but a normal MRI). As dicussed, false positives are considered unlikely and asymptomatic infection has been documented in people. Although seroprevalence studies do not exist for dogs or people in Georgia, seroprevalence in people has been documented in neighboring states. It has been reported as 9.7% (66) and 11.3% (56) in the general population of North Carolina and 0.5% in the general population of Tennessee (67). Additionally, 22.7% of park employees tested in North Carolina and Tennessee were seropositive (68, 69).

Unfortunately, given the nature of this study, it is not possible to determine if LACV was a causative agent of disease in the dog diagnosed with MUO or the dog with late onset epilepsy of unknown cause. The dog with MUO improved with immunosuppressive therapy and the dog with epilepsy responded to treatment with anti-seizure medications. In the previous reports of canine LACV meningoencephalitis, all animals were severely affected, resulting in spontaneous death with pathology that demonstrated a severe, necrotizing meningoencephalitis (13, 63). However, five of these reported cases were puppies <2 weeks of age that lacked developed immune systems (13), and the adult dog described did initially respond to treatment with corticosteroids before relapsing 2 weeks later (63). Additionally, a spectrum of neuroinvasive disease has been documented in children. Reported signs on hospital admission include headache, vomiting, disorientation, seizures, but not all cases require hospitalization and the majority of cases have normal cross sectional imaging (70).

Further research into LACV as a possible cause of CNS inflammation in dogs should be considered. This research could be 2-fold. First, to document LACV seropositivity in the general population of dogs in endemic states. Second, to prospectively test dogs with CNS inflammation for LACV, allowing for long-term monitoring of these patients as well as additional LACV-specific diagnostic tests where indicated. This research will be hampered by the fact that there are no commercially available serological tests for LACV in dogs, leaving PCR and virus isolation as possibilities for clinical cases. A previously developed neutralization assay may aid in further serological evaluation of dogs for LACV antibodies (71).

Ultimately, this study limited evidence for previously unrecognized viral infections as a cause of CNS inflammation in dogs. However, the finding of LACV positive cases warrants further research in endemic areas. Moreover, considering that new, more sensitive technologies are constantly emerging and proving useful in viral discovery, researchers should

[1]Centers for Disease Control and Prevention, C. *La Crosse Encephalitis: Epidemiology and Geographic Distribution*. Available online at: https://www.cdc.gov/lac/tech/epi.html (accessed February 12, 2022).

continue to collect and preserve tissues from these cases for future analysis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Georgia Clinical Research Committee.

AUTHOR CONTRIBUTIONS

SS contributed to conception and design of the study. ST contributed PCR methodology. RB, JL, GL, PK, and SS obtained clinical samples. RB, QL, and SR performed PCR studies. RB, ST, and SS supervised performance and analysis of PCR studies. RB wrote the first draft of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.850510/full#supplementary-material

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Detection of Extracellular Traps in Canine Steroid-Responsive Meningitis-Arteritis

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Extracellular traps (ETs) are DNA networks formed by immune cells to fight infectious diseases by catching and attacking pathogenic microorganisms. Uncontrolled ET formation or impaired ET clearance can cause tissue and organ damage. Steroid-responsive meningitis-arteritis (SRMA) represents an immune-mediated, presumably non-infectious, purulent leptomeningitis and fibrinoid-necrotizing arteritis and periarteritis of young-adult dogs. Chronic and recurrent cases of SRMA are characterized by lymphohistiocytic inflammatory cell infiltration in the meninges and perivascular tissue. This study aimed to identify extracellular traps in dogs with SRMA, a model for immune-mediated diseases in the central nervous system (CNS). Hematoxylin and eosin-stained samples of two young dogs with chronic, recurrent SRMA were examined by light microscopy for characteristic lesions and consecutive slices of affected tissues were stained for detection of ETs by immunofluorescence microscopy using antibodies against DNA–histone-1 complexes, myeloperoxidase, and citrullinated histone H3. Histology revealed purulent and lymphohistiocytic leptomeningitis (n = 2/2) with meningeal periarteritis (n = 2/2) and periadrenal located lymphohistiocytic periarteritis (n = 1). Extracellular DNA networks and inflammatory cell infiltrates of macrophages, neutrophil granulocytes, and lymphocytes were detected in the subarachnoid space of the leptomeninx (n = 2/2) and perivascularly in meningeal (n = 2/2) as well as periadrenal vessels (n = 1/1). In summary, extracellular DNA fibers and attached ET markers are detectable in affected perivascular and meningeal tissues of dogs suffering from SRMA. The proof of principle could be confirmed that ETs are present in canine, inflammatory, and non-infectious CNS diseases and possibly play a role in the pathogenesis of SRMA.

Keywords: extracellular traps (ETs), steroid-responsive meningitis-arteritis (SRMA), vasculitis, meningitis, immunofluorescence microscopy, non-infectious, citrullinated histone H3 (H3Cit)

INTRODUCTION

Several immune cells of the innate immune system, including neutrophils, eosinophils, monocytes, mast cells, and basophils, are capable of producing extracellular DNA traps (ETs) (1–5).

Extracellular trap formation can be differentiated in three pathways: suicidal, vital, and mitochondrial mechanisms (1, 2, 6–11). The term ETosis was created to define this distinct cell death apart from necrosis and apoptosis describing the suicidal pathway of ET formation (6, 12). This particular cell death involves the resolution of nuclear membrane, decondensation of chromatin, and mixing with granule components followed by release of ETs after permeabilization of the cell membrane (1, 6). ET-related proteins and components such as myeloperoxidase (MPO), citrullinated histone H3 (H3Cit), and DNA–histone complexes were used with antibody-based techniques to co-stain these specific ET markers (13).

The view on ETosis and suicidal ET formation had to be renewed after a groundbreaking discovery in 2012. Pilsczek et al. (7) described viable neutrophils performing phagocytosis and migration after the release of ETs during an acute infection with *Staphylococcus aureus*. The term ET formation was expanded and divided up in suicidal and vital way of ET release (7, 10, 11). Suicidal ET formation is reactive oxygen dependent and pursues after 3–8 h, whereas vital ET formation is reactive oxygen independent and performed in 5–60 min (6, 7, 14). The third way of ET formation due to mitochondrial DNA release by viable cells is not entirely understood (9). In this study, the term ET formation is used to resume all the different ways of creating extracellular DNA traps regardless of the cell origin and type of ET metabolism.

These extracellular DNA formations are composed of a scaffold of decondensed chromatin fibers equipped with granule proteins [e.g., myeloperoxidase (MPO) and neutrophil elastase (NE)], nuclear proteins [e.g., citrullinated histone H3 (H3Cit)], and antimicrobial enzymes forming web-like structures (1, 13, 15, 16). Beyond phagocytosis, degranulation, and creation of reactive oxygen species, ET formation is a genuine extracellular strategy especially of neutrophils to kill, disarm, and entrap invading pathogens (1, 17, 18).

Recently, study shifted from infectious to non-infectious diseases investigating potential impact and therapeutic opportunities of extracellular trap release and degradation. On one hand, ET formation is another effective antimicrobial mechanism of the innate immune system combating different pathogens; on the other hand, excessive ET expression, unregulated ET release, and insufficient ET clearance can cause detrimental effects and lead to or are associated with ET-related pathologies ["ETopathies" (19, 20)]: endothelial or epithelial tissue damage (21–23), pancreatitis (24, 25), autoimmune diseases (26, 27), thrombosis (16), vasculitis (28), and cancer (29, 30).

In dogs, neutrophil extracellular traps (NETs) were recently described in infectious diseases such as parasitic infections with *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Dirofilaria immitis* (31–33) and bacterial infections such as pyometra caused by *Escherichia coli* and *Streptococcus species* (34) and NETs were isolated from pleural and abdominal effusions in septic dogs (35). However, the influence on the pathogenesis and prognosis of ET formation in canine non-infectious diseases, especially in the CNS, still has to be elucidated. NETs have an impact on the immune system in canine immune-mediated hemolytic anemia (36–38), on clot formation and canine immunothrombosis (16, 39).

Steroid-responsive meningitis-arteritis (SRMA) is an immune-mediated, systemic, inflammatory, and presumably non-infectious disorder predominantly in young-adult and medium-to-large-sized dogs (40). The disease affects typically 6 to 18 month-old dogs with a possible range of 3 months to 9 years (40–42). SRMA can occur in any dog breed, but is overrepresented in Bernese mountain dogs, Boxers, Beagles (43, 44), Nova Scotia Duck Tolling Retrievers, Weimaraners, and Petit Basset Griffon Vendéens (45). A German study showed a sex predisposition for male individuals (46), but other studies do not show significant difference in sex distribution of this disease (47, 48). The assembly of signalment, clinical signs, and laboratory findings of CSF and blood analysis associated with a quick clinical improvement after application of immunosuppressive therapy with glucocorticosteroids and an exclusion of an infectious etiology lead to the antemortem diagnosis of SRMA (40, 48–50).

The typical, acute form of SRMA is characterized by recurrent fever, cervical hyperesthesia, neck rigidity, stiff gait, reluctance to move, and depression. Laboratory findings of the acute form include a moderate-to-severe, non-degenerative neutrophilic pleocytosis of the cerebrospinal fluid (CSF) and blood profiles show a neutrophilic leukocytosis with left shift (44, 51). Furthermore, elevated immunoglobulin A levels in serum and CSF serve as diagnostic tool (44, 52). Levels of acute phase proteins such as C-reactive protein, serum amyloid A, haptoglobin (53), or neutrophil gelatinase-associated lipocalin (54) are elevated in the acute disease episode compared to non-inflammatory neurological diseases. Especially, CRP is used as a remission and therapy monitoring marker (55). Pathohistologically, the acute form is represented by a multifocal to generalized fibrinoid-necrotizing vasculitis with thrombosis and purulent leptomeningitis preferentially in the meninges of the cervical spinal cord (40, 43, 56–58).

The atypical, chronic, and protracted form of SRMA is observed primarily due to relapses and inadequate, immunosuppressive treatment. CSF analysis predominantly reveals mononuclear cells (44) and non-suppurative, mononuclear cell infiltrates in the meninges and perivascular tissue dominate pathohistological findings (58).

Steroid-responsive meningitis-arteritis offers ideal circumstances for the possible detection of ETs for the first time in canine central nervous system (CNS) tissue representing an immune-mediated, inflammatory, and non-infectious neuronal disorder mainly driven by a neutrophil immune response (40, 44). ET detection in the acute phase of Kawasaki disease of children, which causes a comparable vasculitis with consecutive inducing tissue damage-like SRMA, was successful (59–61). Consequently, we hypothesized that ETs take part in the etiopathogenesis of SRMA and a successful detection of ETs

in the commonly affected tissues of meninges and vessels seems promising. This study should be a proof of principle that ETs can be detected in histologically confirmed cases of SRMA. Based on confirmation of extracellular DNA traps, corresponding clinical studies, new diagnostic, and treatment strategies could be developed.

MATERIALS AND METHODS

Sample Collection

From the archive of the Department of Pathology, two dogs were selected for this study. The inclusion criteria were signalment, reported clinical signs, pathohistological findings such as purulent or lymphohistiocytic leptomeningitis with associated arteritis or periarteritis, and no detectable pathogens with special staining such as periodic acid–Schiff-reaction, Gram's staining, Ziehl–Neelsen's staining, or Grocott's silver impregnation method. Retrospective study revealed that no further microbiological or virologic examination was conducted on serum or CSF samples of these dogs to exclude a pathogenetic etiology.

Histological Evaluation

Routinely processed formalin-fixed and paraffin-embedded (FFPE) tissues of the affected dogs were selected from the block archive of the Department of Pathology for further histopathologic and immunofluorescent examination.

Regions of affected tissue of the cervical spinal cord with associated leptomeningeal vessels and peripheral, particularly periadrenal vessels were embedded in paraffin and cut at 2–4 μm for H&E and immunofluorescence staining. H&E staining of the affected tissue was performed by automated dying in Leica ST4040 (Leica, Wetzlar, Germany) with 0.1% hematoxylin (Roth, Karlsruhe, Germany) and 1% eosin (Roth, Karlsruhe, Germany). The presence of vascular and meningeal lesions was evaluated qualitatively by a board certified veterinary pathologist [the European College of Veterinary Pathologists (ECVP)] with special emphasis on neutrophilic and inflammatory cell invasion in the vascular walls or meninges. The H&E slides were examined microscopically on an Olympus BX53 (Olympus, Tokyo, Japan) light microscope. Pictures were edited with ImageJ software (version 1.53, National Institutes of Health, USA).

Extracellular Trap Examination

For ET detection, unstained, native paraffin slides of affected tissues of the spinal cord, brain, and periadrenal arteries were analyzed. Co-staining of DNA–histone-1 complexes and MPO or H3Cit was performed according to the following protocol as previously described (62, 63) with the following changes.

After permeabilization for 10 min (0.1% Triton X-100) and blocking for 20 min (blocking buffer for co-staining of DNA–histone-1 complexes and MPO: 5% bovine serum albumin, 5% goat serum, 2% cold water fish gelatin, 0.05% Tween-20, and 0.05% Triton X-100; blocking buffer for co-staining of DNA–histone-1 complexes and H3Cit: 10% fetal calf serum, 2% bovine serum albumin, 0.05% Tween-20, and 0.1% Triton X-100), samples were incubated overnight at 4°C using the following first antibodies, diluted in respective blocking buffer: mouse monoclonal IgG2a anti-DNA/histone (Millipore MAB3864, Billerica, Massachusetts, USA; 0.55 mg/ml; 1:100) and rabbit antihuman myeloperoxidase (Dako, A0398, 3.3 mg/ml, 1:300) or rabbit antihuman H3Cit (citrulline R2 + R8 + R17) antibody (Abcam, ab5103, Cambridge, UK, 1 mg/ml, 1:31.6). For isotype control, murine IgG2a (from murine myeloma M5409, Sigma Aldrich, Munich, Germany, 0.2 mg/ml, 1:36.4) and rabbit immunoglobulin G (IgG) (from rabbit serum I5006, Sigma Aldrich, Munich, Germany, 1.16 mg/ml, 1:108.75 for staining of DNA–histone-1 complex and MPO 1:36.7 for staining of DNA–histone-1 complex and H3Cit) were used. The secondary staining was performed for 1 h in the dark at room temperature using a goat anti-rabbit Alexa 633-conjugated antibody (Invitrogen, Carlsbad, California, USA, 2 mg/ml, 1:500) and a goat anti-mouse Alexa 488-conjugated antibody (Invitrogen, Carlsbad, California, USA, 2 mg/ml, 1:500). Counterstaining of DNA was performed with aqueous Hoechst 33342 (Sigma B-2261, St. Louis, Missouri, USA, 0.5 mg/ml, 1:1,000) for 10 min. At the end, all the samples were processed with the TrueVIEW Autofluorescence Quenching Kit (Vector laboratories, San Francisco, California, USA) following the manufacturer's instructions and covered with Mounting Medium of the TrueVIEW Autofluorescence Quenching Kit (Vector laboratories, San Francisco, California, USA).

Serial cuts of histopathologically altered tissues were stained and analyzed, whether ET formation or ET markers were detectable. Neutrophils and macrophages infiltrating the subarachnoid space, meningeal arteries, and extraneural perivascular tissue of SRMA-affected dogs are capable of releasing extracellular DNA fibers consisting of ET-markers such as DNA–histone-1 complexes, attached MPO, or H3Cit, which is a typical and strong evidence of ET formation (1, 64–66).

Extracellular trap formation was semiquantitatively analyzed in the meninges of the spinal cord and affected vessels. The amount of ET formation was counted in five 400 μm × 400 μm fields (0.16 μm^2) of affected tissues of each dog and compared to each other (**Table 1**).

Immunofluorescence Microscopy

The stained samples were examined microscopically on a Leica TCS SP5 AOBS confocal inverted-base fluorescence microscope with HCX PL APO 40 × 0.75–1.25 oil immersion objectives with an Argon 405 and 633 nm laser (Leica, Wetzlar, Germany). The settings were adjusted using isotype control antibodies in separate preparations. Pictures were edited with ImageJ software (version 1.53, National Institutes of Health, USA).

RESULTS

Signalment, History, Macroscopic, and Histopathological Findings

Two dogs were included with histological lesions indicative of acute and chronic SRMA. The first dog was an 11 months old, male Bernese mountain dog. Anamnestically, this dog had recurrent episodes of pyrexia up to 41°C and lameness. Laboratory findings revealed moderate leukocytosis of 25,000/μl

TABLE 1 | Semiquantitative analysis of extracellular trap (ET) events in five representative immunofluorescent pictures.

Localization		Bernese mountain dog		Petit Basset Griffon Vendéen	
		ET-events/ 0.16 μm^2	Average	ET-events/ 0.16 μm^2	Average
Vessels	intraluminal	4, 1	2, 5	1, 1	1
	intramural	0, 0	0	0, 0	0
	perivascular	16, 4	10	11, 7	9, 5
Meninges		13, 10, 14	12, 3	25, 20, 22	22, 3

*ET events were semiquantitatively analyzed by counting matching extracellular MPO or H3Cit and DNA-histone-1-complex singals in affected tissues in a square of 400 x 400 μm. Meninges and Vessels divided in intraluminal, intramural and perivascular events were seperately screened for ETs for each dog. Intraluminal there was no event detectable. Perivascularly and intraluminally there was no big difference in the counted ET-events. Meninges of the Petit Basset Griffon Vendéen were histopathologically (**Figures 3A**, **4A**) and semiquantitatively more affected than the meninges of the Bernese mountain dog (**Figure 1A**).*

and elevated protein content of the CSF [positive Pandy-reaktion (+)]. Initial treatment was started with doxycycline and prednisolone for an unknown period and clinical signs were ameliorating. 3 weeks after terminating the medication, the dog relapsed and showed similar clinical signs with episodes of fever and leukocytosis of 28,000/μl. Another treatment with prednisolone was initiated and the dog was anesthetized for further diagnostics, but developed cardiorespiratory arrest. After unsuccessful resuscitation, it was sent to the Department of Pathology for necropsy. Necropsy revealed only agonal gross changes. Pathohistological evaluation of the cervical spinal cord revealed infiltration of neutrophils, macrophages, lymphocytes, and plasma cells resulting in a moderate diffuse, purulent, and lymphohistiocytic leptomeningitis (**Figure 1A**). Extraneural findings showed moderate infiltrations of lymphocytes, macrophages, and plasma cells causing a subacute to chronic, diffuse lymphohistiocytic, periadrenal periarteritis (**Figure 2A**).

The second dog was a 5-month-old, female, Petit Basset Griffon Vendéen, which was euthanized because of a pleural effusion causing dyspnea and additional acute kidney injury (urea in the aqueous humor: 180 mg/dl). The history revealed undulating fever episodes of unknown origin and relapsing episodes of forelimb lameness. No treatment strategies were attached to the submission report of this dog. Anamnestically, another littermate was affected with comparable clinical signs. Necropsy revealed diffuse subdural hemorrhage expanding from the cerebellum throughout the dural tube. A circumferential dark red mass was located in the precardiac mediastinum (4 cm × 4 cm × 3 cm). In the thoracic cavity, there was a hemothorax, consisting of partially clotted 300 ml in the left and 100 ml in the right pleural cavity. Pathohistologically the leptomeninx of the cervical spinal cord was moderately-to-severely infiltrated with neutrophils, macrophages, lymphocytes, and plasma cells showing a severe, subacute, multifocal, lymphohistiocytic leptomeningitis accompanied with severe subarachnoid hemorrhage with erythrophagocytosis (**Figure 3A**). Cervical meningeal arteries revealed mild periarterial infiltration of lymphocytes, macrophages, and few neutrophils resulting in a moderate, acute, diffuse, lymphohistiocytic periarteritis with an intraluminal thrombus formation and meningeal hemorrhage (**Figure 4A**).

Both the dogs showed features of chronic-active and acute neural and extraneural histopathologic lesions that are characteristically observed in undulating clinical courses of SRMA (44, 67). The severity of meningeal inflammatory infiltration of the Bernese mountain dog was lower than of the Petit Basset Griffon Vendéen.

Immunofluorescence Findings in Meninges and Arteries of Canine Steroid-Responsive Meningitis-Arteritis

As a next step, we used fluorescence microscopy to visualize formation of ETs in the biopsies. Since the major backbone of ETs is DNA, DNA intercalating dyes are widely used to stain ETs based on the electrostatic interactions of these dyes, e.g., 4′, 6-diamidino-2-phenylindole (DAPI) with DNA (13). However, this method cannot discriminate between DNA derived from ET-releasing cells vs. necrosis. Furthermore, it has to be considered that some granule components such as antimicrobial peptides block the visualization of ETs by DNA-intercalating dyes (68). Therefore, antibody-based techniques that stain ET-specific markers such as DNA–histone complexes in combination with cell-specific proteins that are frequently found associated with ETs such as myeloperoxidase are needed to confirm release of ETs by immunofluorescence microscopy (13, 68–70).

Using this technique, the lymphohistiocytic, periadrenal periarteritis of the Bernese mountain dog showed mild presence of DNA–histone-1 complex positive web-like structures and moderate, extracellular MPO signal surrounding infiltrating macrophages. ET formation appeared perivascularly and intraluminally, but was not to be found in the vascular walls (**Figures 2B,C**; **Table 1**). These ET detections were similar to histopathological findings, which showed only perivascular inflammatory cell infiltration. Meningeal lesions occurred in contrast to perivascular lesions with mild infiltration of neutrophils next to macrophages and lymphocytes. Mild-to-moderate detection of extracellular DNA–histone-1 complexes and MPO as ET markers could visualized in the meninges of the spinal cord around infiltrating neutrophils (**Figures 1B,C**). To sum up pathohistological as well as immunofluorescent findings, ET formation could present in the meninges and extraneural arteries of this dog.

Extracellular trap markers in terms of extracellular DNA–histone-1 complexes and MPO were also positive in the meninges of the Petit Basset Griffon Vendéen. Infiltrating neutrophils, macrophages, and lymphocytes causing a purulent to lymphohistiocytic leptomeningitis are forming ETs proven by DNA–histone-1 complex, MPO, and H3Cit signals in this affected area (**Figures 3B,C**, **4B,C**). Summarizing pathohistological and immunofluorescent findings of this dog, ETs were present at the time of death in damaged meninges and meningeal arteries. Respective isotype control images did not

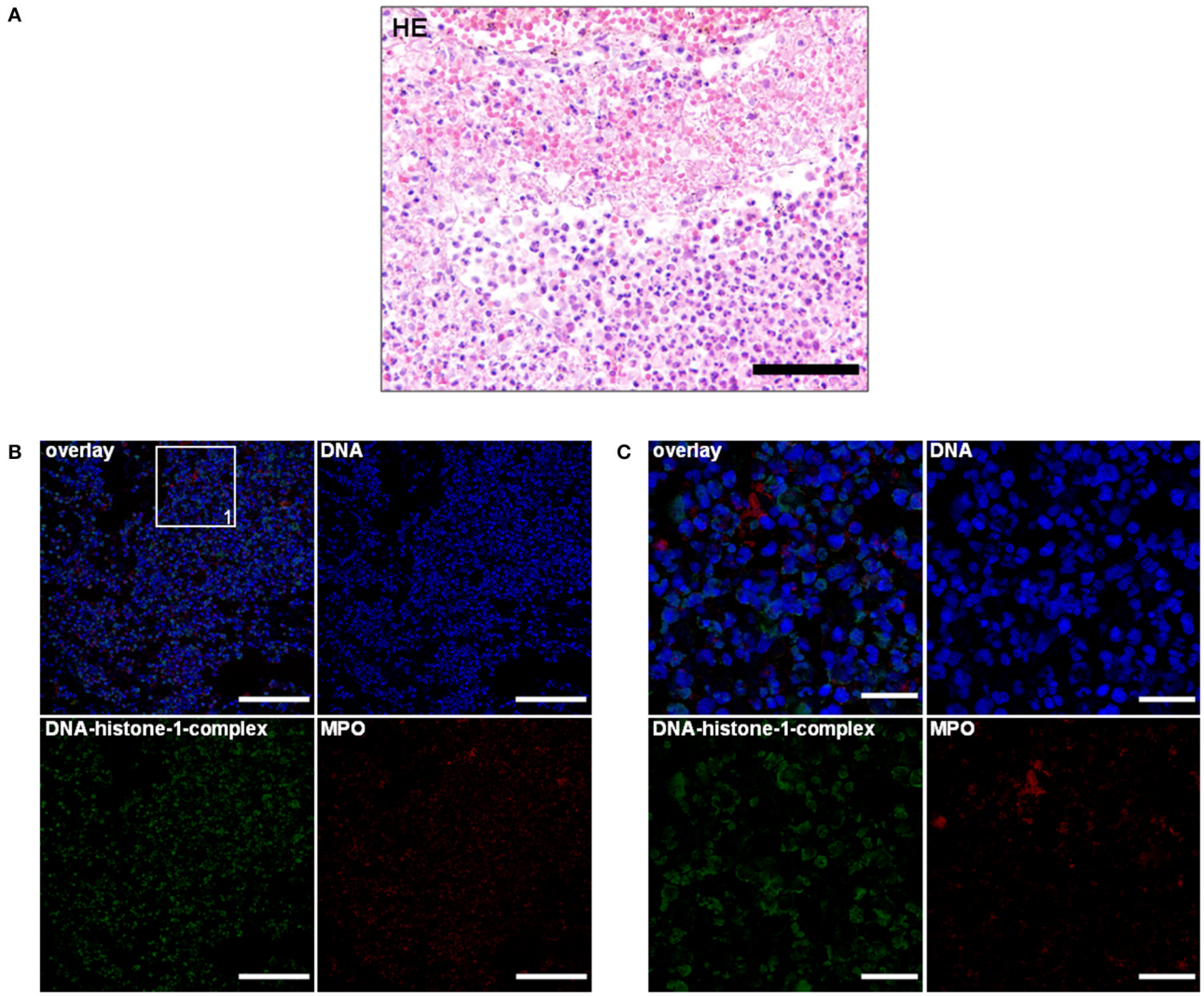

FIGURE 1 | (A) An 11-month-old Bernese mountain dog. Histopathology. Cervical spinal cord: predominant infiltration of neutrophils and macrophages resulting in a moderate diffuse, purulent leptomeningitis. H&E. Scale bar = 100 μm. **(B)** Serial cuts of the concurrent formalin-fixed and paraffin-embedded (FFPE) block. Extracellular traps (ETs) as extracellular DNA fibers with associated myeloperoxidase (MPO) were present in the purulent leptomeningitis of the spinal cord. Settings of the immunofluorescence images were adjusted to a respective isotype control. Representative images are shown. IF. Blue, counterstaining of DNA (Hoechst), green, DNA–histone-1 complexes (ETs), and red, myeloperoxidase (MPO). Scale bar = 100 μm. **(C)** Zoom picture of area 1. IF. Scale bar = 20 μm.

show any signal intensity at DNA–histone-1 complex, MPO, or H3Cit settings.

DISCUSSION

In this study, we could proof our hypothesis that ET formation and ET markers of two representative dogs suffering from SRMA are detectable and visualizable in typical affected tissues such as meninges of the cervical spinal cord and neural, as well as extraneural vessels. To the best of the author's knowledge, this is the first study confirming the presence of extracellular DNA formations composed of DNA–histone-1 complexes, MPO, or H3Cit in the CNS of dogs and especially affected with SRMA. ET formation was present in acute and chronic-active lesions of recurrent, waxing-waning disease periods of both the dogs, implicating that this mechanism of the immune system seems to play a certain role in the pathogenesis of SRMA.

As hallmark of the pathogenesis of SRMA, neutrophils conquer the subarachnoid space causing a neutrophil pleocytosis of the CSF (44). The detailed mechanism of this immune compartmentalization is not fully understood. Neutrophil recruitment to vascular wall adhesion is mediated by CD11a upregulation (51) and a possible factor of the blood–brain barrier disruption is caused by releasing matrix metalloproteinases-2 and−9 (MMP-2/-9) (71). Khandpur et al. (27) positively correlate the amount of netting neutrophils and production of interleukin-17 (IL-17). These findings could be supported by

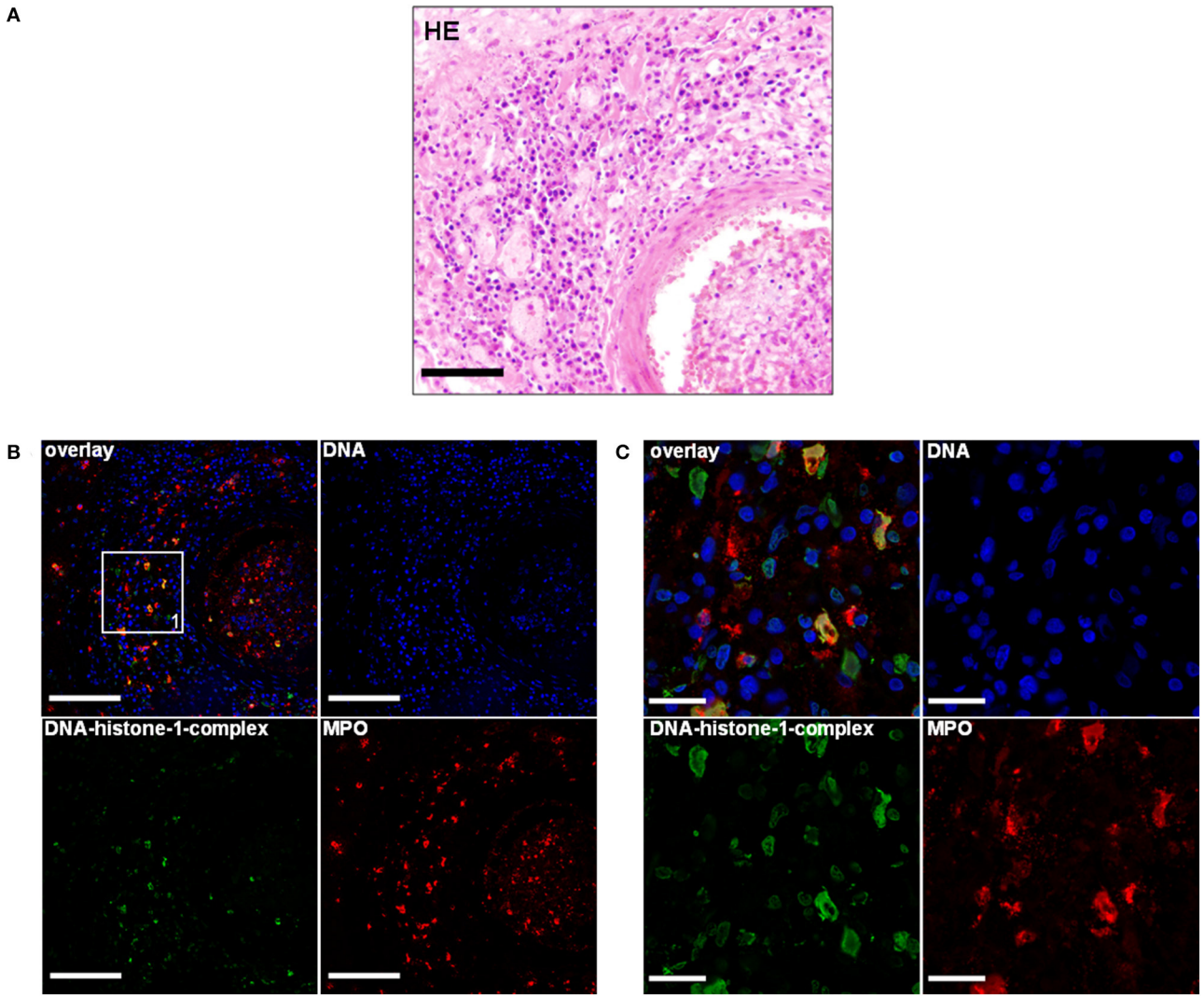

FIGURE 2 | (A) An 11-month-old-Bernese mountain dog. Histopathology. Periadrenal artery: Moderate infiltration of lymphocytes, macrophages, and plasma cells causing a moderate, subacute to chronic, diffuse lymphohistiocytic, periadrenal periarteritis. H&E. Scale bar = 100 μm. **(B)** Serial of the concurrent FFPE block. Extracellular traps (ETs) as extracellular DNA fibers were detected in the perivascular region of a periadrenal artery. Settings of the immunofluorescence images were adjusted to a respective isotype control. Representative images are shown. Blue, counterstaining of DNA (Hoechst), green, DNA–histone-1 complexes (ETs), and red, myeloperoxidase (MPO). IF. Scale bar = 100 μm. **(C)** Zoom picture of area 1 is presented. IF. Scale bar = 20 μm.

Freundt-Revilla et al. (72) that production of IL-17 ensures neutrophil granulocyte recruitment in the CNS compartment and disruption of the blood–brain barrier in dogs with SRMA. IL-17 production in dogs with SRMA can lead to increased NET formation and may facilitate the leukocyte extravasation of neutrophils by disrupting the blood–brain barrier.

We hypothesize that ET formation interdigitates with the current detailed knowledge of immunologic dysregulation causing SRMA (43). Recently, it was shown that ET formation promotes vasculopathies (73) and externalization of ET-associated proteins such as histones and MPO leads to vascular barrier injury (23, 74, 75). Especially, histones are described in small-vascular angiopathies to drive vascular damage and vascular wall necrosis (23, 74, 75). The positive evidence of H3Cit in the meningeal arteries could be another explanation to the invasion and compartmentalization of neutrophils in the subarachnoid space with associated hemorrhage and frequently detected fibrinoid-necrotizing arteritis in SRMA (43).

Furthermore, histones as major proinflammatory components of extracellular released DNA traps may drive and perpetuate the innate immune response and maintain persistent sterile inflammation in SRMA through interaction with Toll-like receptor (TLR) 4 (76–78). Being part of pattern recognition receptors (PRRs), TLRs play a crucial role of the innate immune system and stimulating the adaptive immune response (79). They are able to recognize foreign, pathogen-associated molecular patterns (PAMPs) in infectious diseases, as well as host-derived damage-associated molecular patterns (DAMPs)

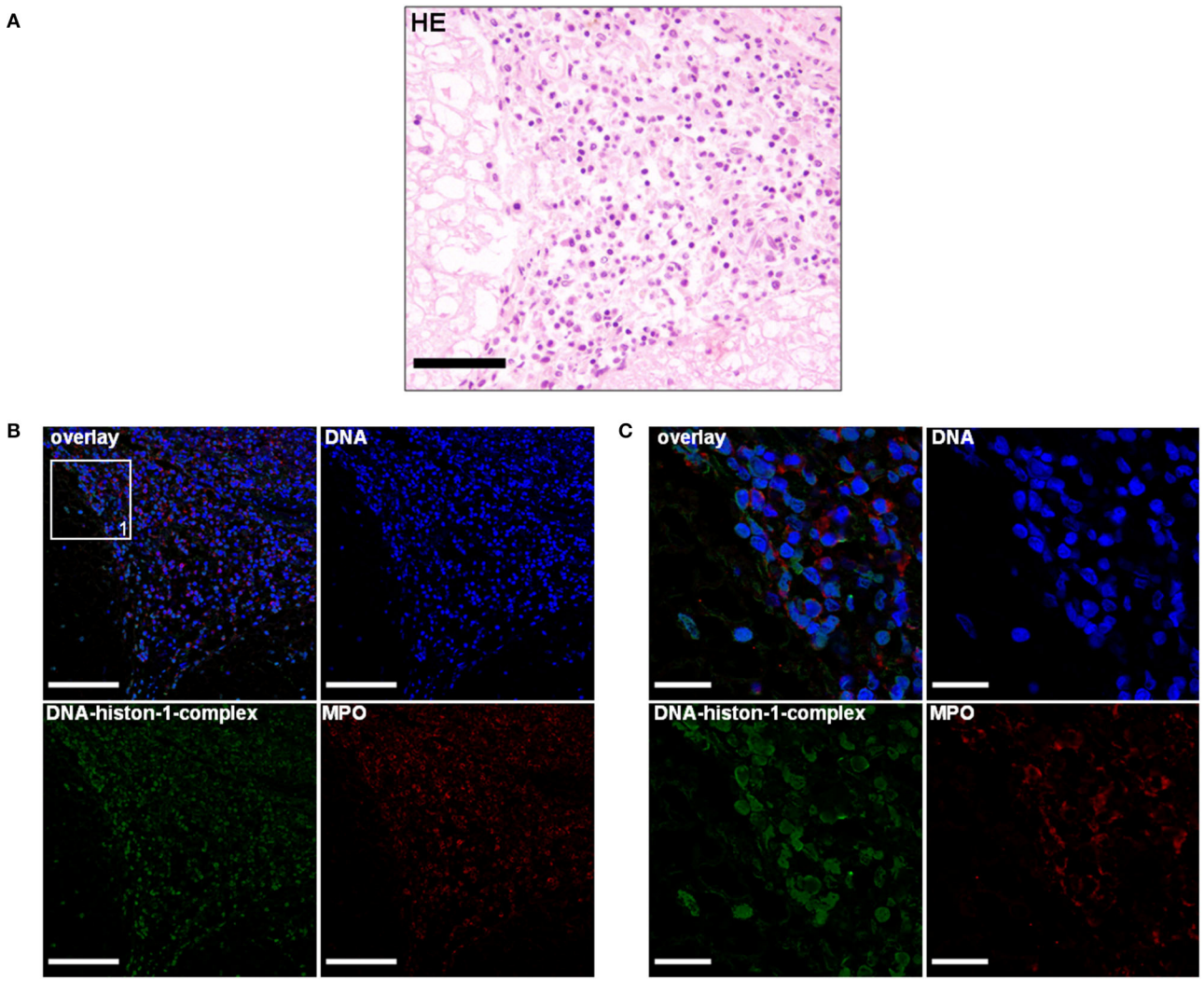

FIGURE 3 | (A) A 5-month-old Petit Basset Griffon Vendéen. Histopathology. Cervical spinal cord: Moderate-to-severe infiltration of neutrophils, macrophages, lymphocytes, and plasma cells causing a severe, subacute, multifocal, lymphohistiocytic leptomeningitis H&E. Scale bar = 100 μm. **(B)** Serial cuts of the concurrent FFPE block. Extracellular traps (ETs) as extracellular DNA fibers and ETs markers were detected in the subarachnoid space. Settings of the immunofluorescence images were adjusted to a respective isotype control. Representative images are shown. IF. Blue, counterstaining of DNA (Hoechst), green, DNA–histone-1 complexes (ETs), and red, myeloperoxidase (MPO). Scale bar = 100 μm. **(C)** Zoom picture of area 1 is presented. Scale bar = 20 μm.

produced by tissue damage or cell death (80). Maiolini et al. (81) described higher expression of TLR-4 and TLR-9 on polymorphonuclear cells and monocytes in the acute stage of SRMA. The upregulation of this PRR on immune cells mediating the pathogenesis of SRMA such as neutrophils and macrophages illuminates chronic inflammation and autoimmunity and may be attributed due to higher levels of H3Cit (82, 83).

In addition, higher concentration of intrathecal produced extracellular heat shock protein 70 (eHSP70) as representative example of the DAMP family may interact with TLR4 (84). Continuous activation of neutrophils releasing their ETs due to interaction with DAMPs such as citrullinated histones or other host-derived self-antigens such as eHSP70 can lead to a vicious circle of autoimmunity and supporting the theory of existing autoantigenic triggers (14, 85). Based on these findings, the hypothesis of an existing self-antigen or environmental trigger acting according to the hit-and-run principle must be requested.

Furthermore, the externalization of ETs is the source of major autoantigens for autoantibody formation and is supposed to be pathogenic in several autoimmune-derived diseases (86). Until now, autoantibodies against endogenous CNS tissue only serve as "epiphenomenon" of SRMA (40, 87). If the presence of these major autoantigenetic in terms of ET-associated structures drive and maintain immunologic processes in meninges and vessels of these dogs, the complex pathogenesis of SRMA could be well explained.

Histopathological and immunofluorescent findings (**Figures 1**–**4**) of acute and chronic lesions in both the dogs

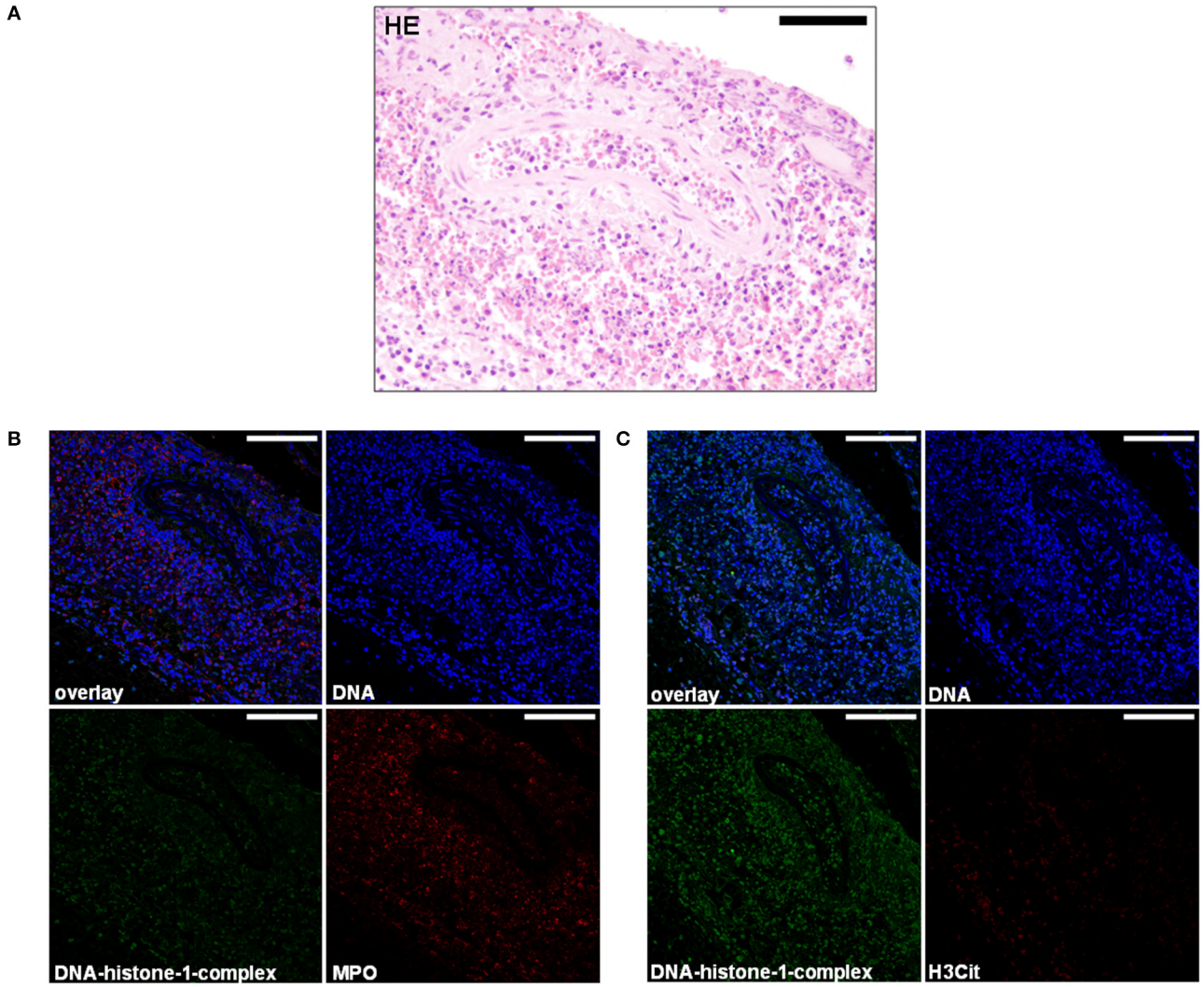

FIGURE 4 | (A) A 5-month-old Petit Basset Griffon Vendéen. Histopathology. Cervical meningeal arteries revealed moderate perivascular infiltration of lymphocytes, macrophages, and a few neutrophils resulting in a moderate, acute, diffuse, purulent, lymphohistiocytic periarteritis. H&E. Scale bar = 100 μm. **(B)** Serial cuts of the concurrent FFPE block. Extracellular traps (ETs) as extracellular DNA fibers were detected in the subarachnoid space and perivascular. Settings of the immunofluorescence images were adjusted to a respective isotype control. Representative images are shown. IF. Blue, counterstaining of DNA (Hoechst), green, DNA–histone-1 complexes (ETs), and red, myeloperoxidase (MPO). Scale bars = 100 μm. **(C)** Extracellular traps (ETs) as extracellular DNA fibers were detected in the subarachnoid space and perivascular. Settings of the immunofluorescence images were adjusted to a respective isotype control. Representative images are shown. IF. Blue, counterstaining of DNA (Hoechst), green, DNA–histone-1 complexes (ETs), and red, citrullinated histone H3 (H3Cit). Scale bars = 100 μm.

represented by mainly lymphohistiocytic, as well as neutrophils invading meninges or perivascular spaces could be explained with prolonged activation of macrophages and neutrophils generating ETs or an impaired self-clearance of ETs. Remnants of ETs could serve as constant trigger in terms of DAMPs for immune cells maintaining CNS and vascular inflammation resulting in continuous invasion of neutrophils in this already chronic process. Both the dogs showed similar pathohistologic lesions of chronic active inflammation at the time of death with different amount of ET formation (**Table 1**). In general, the meninges of the Petit Basset Griffon Vendéen were infiltrated more severely than in the Bernese mountain dog. This could be explained by a more acute and severe clinical course of the Petit Basset Griffon Vendéen in contrast to the Bernese mountain dog. Another explanation could be the different pharmacological influence of variably administered anti-inflammatory drugs. Primarily perivascular detection of ETs was present in affected arteries.

Generating histological samples in the future will be very unlikely because clinical diagnosis, treatment management, and awareness of this disease reduced the mortality of SRMA in the last decades (44, 46, 88). Therefore, prospective clinical studies could confirm antemortem evidence of ETs in dogs measuring ET markers and correlating ET inducers in clinical accessible

samples of serum and CSF such as H3Cit and cell-free DNA. Visualization or stimulation assays of ETs released by isolated nucleated cells in CSF samples in acute diseased, treated, and relapsed dogs with SRMA offer another possibility supporting the results of this study. Comparing ET markers to dogs with other inflammatory as well as non-inflammatory CNS diseases of infectious and non-infectious origin is necessary to probably underline and distinguish the final role of ETs in the pathogenesis of SRMA.

Treatment of autoimmune and immune-mediated diseases in veterinary medicine is lacking of specific therapeutic options such as the usage of recombinant monoclonal antibodies, intracellular pathway modulating, or receptor-targeting drugs (89). Also, steroid therapy is associated with many undesirable side effects and 30% of human patients are identified as "non-responders" or resistant to glucocorticoid application (88, 89). Specific ET-targeting therapeutics options with fewer side effects such as DNases (90) exist in human medicine and urge to be tested in veterinary medicine. SRMA represents an ideal large animal model of suppurative, non-infectious meningitis with proven ET formation to evaluate and develop new therapeutics in future research studies in a translational context (81). Based on this pilot study of ET formation in the CNS of dogs, clinical studies will be performed investigating the influence on canine neuropathies.

In conclusion, ETs are detectable in tissue samples of necropsied SRMA cases. This study represents the first trial to proof of principle of ET visualization in canine central nervous system tissue. The detection of ETs in SRMA gains new possibilities to explore the existence and etiopathogenetic influence of this host mechanism of immune cells in infectious and non-infectious canine neuropathies. To give an outlook, a magnitude of study is required concerning clinical importance of innovative diagnostics tools as remission and therapy marker and the development of specific, ET-targeting therapeutic options with fewer side effects than conventional glucocorticoid therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AT, WB, MvK-B, and JN conceived, designed, and supervised the study. JW and MM performed all the experiments, analyzed the data, and performed immunofluorescence staining and microscopy. JW drafted the manuscript. PW performed histopathological examination. All authors contributed to the revision of the manuscript and have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.863579/full#supplementary-material

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Clinical Course and Diagnostic Findings of Biopsy Controlled Presumed Immune-Mediated Polyneuropathy in 70 European Cats

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There is a paucity of information on the clinical course and outcome of young cats with polyneuropathy. The aim of the study was to describe the clinical features, diagnostic investigations, and outcome of a large cohort of cats with inflammatory polyneuropathy from several European countries. Seventy cats with inflammatory infiltrates in intramuscular nerves and/or peripheral nerve biopsies were retrospectively included. Information from medical records and follow up were acquired via questionnaires filled by veterinary neurologists who had submitted muscle and nerve biopsies (2011–2019). Median age at onset was 10 months (range: 4–120 months). The most common breed was British short hair (25.7%), followed by Domestic short hair (24.3%), Bengal cat (11.4%), Maine Coon (8.6%) and Persian cat (5.7%), and 14 other breeds. Male cats were predominantly affected (64.3%). Clinical signs were weakness (98.6%) and tetraparesis (75.7%) in association with decreased withdrawal reflexes (83.6%) and, less commonly, cranial nerve signs (17.1%), spinal pain/hyperesthesia (12.9%), and micturition/defecation problems (14.3%). Onset was sudden (30.1%) or insidious (69.1%), and an initial progressive phase was reported in 74.3%. Characteristic findings on electrodiagnostic examination were presence of generalized spontaneous electric

muscle activity (89.6%), decreased motor nerve conduction velocity (52.3%), abnormal F-wave studies (72.4%), pattern of temporal dispersion (26.1%) and unremarkable sensory tests. The clinical course was mainly described as remittent (49.2%) or remittent-relapsing (34.9%), while stagnation, progressive course or waxing and waning were less frequently reported. Relapses were common and occurred in 35.7% of the cats' population. An overall favorable outcome was reported in 79.4% of patients. In conclusion, young age at the time of diagnosis and sudden onset of clinical signs were significantly associated with recovery ($p < 0.05$). Clinical and electrodiagnostic features and the remittent-relapsing clinical course resembles juvenile chronic inflammatory demyelinating polyneuropathy (CIDP), as seen in human (children/adolescents), in many aspects.

Keywords: feline, neuromuscular, weakness, tetraparesis, electrodiagnostic, CIDP, GBS

INTRODUCTION

Weakness is a relatively common neurological presentation in cats and besides diseases of the muscles and the neuromuscular junction, polyneuropathies (PN) are one of the leading causes for this clinical presentation (1–3). Feline PN can be classified as inherited and acquired PN of defined or unknown cause (1–5). Examples of genetic or suspected genetic PN are sphingomyelinase-deficiency PN in Siamese cats (1, 6), primary hyperchylomicronemia in different breeds (1, 7), axonal PN in Snowshoe cats (8), or distal PN in Birman cats (1, 9). Acquired PN are described to be either of metabolic (10–12), vascular (13, 14), toxic (15–21), paraneoplastic (22–24), infectious (25–28), nutritional (4, 29), or are thought to represent immune-mediated or idiopathic PN (5, 30–34). Reports of immune-mediated/idiopathic PN in cats have been published in recent years (5, 31–33, 35). Many cats made a full recovery but relapses, a chronic disease course, or rare fatalities were also reported (5, 31–33, 35–38). In clinical neurological practice, a presumptive diagnosis is frequently based on neuroanatomical localization, age of onset, and exclusion of other diseases with further confirmation obtained by electrodiagnostic studies or muscle/nerve biopsies (1–3, 6, 31, 32, 39). Little is known about PN in young cats and detailed descriptions about clinical presentation and disease course are restricted to small patients' cohorts. Thus, uncertainties regarding outcome, recovery time, possible relapses, and efficacy of treatment modalities which are necessary for adequate counseling of cat owners, remain (5, 30, 31, 40). Therefore, the aim of this study was to describe the clinical features and disease course in a large European cohort of cats with histologically confirmed inflammatory PN.

MATERIALS AND METHODS

Archives of a single European reference laboratory for neuromuscular disorders, MASKED FOR REVIEW, were screened for biopsy diagnosis of inflammatory PN between 2011 and 2019. Only cats with histologic evidence of nerve fiber adhesive and/or invasive inflammatory infiltrates directed at the axons, nodes of Ranvier, and Schwann cells were included (**Figures 1**, **2**). Cats with PN without signs of inflammation in the intramuscular nerve branches and/or peripheral nerve biopsies were excluded from the study. In total 107 cats with inflammatory neuropathy of presumed immune-mediated origin were identified. In all cats peripheral nerve biopsies were available for review and muscle biopsies were submitted in 105/107 cats. Diagnosis was based on findings from main nerve trunk in 105/107 and from intramuscular nerve branches in 2/107. Submitting referral veterinary neurologists were asked to review the medical records of their cases and to contact the cat's owners for follow-up information, before answering the online questionnaire (https://forms.office.com/Pages/ResponsePage.aspx?id=DQSIkWdsW0yxEjajBLZtrQAAAAAAAAAAAAN__iDTpglUQVVXTzNMNlc1UlpFUlo4UEdQOTNOVzRWMy4u). The study was approved by the ethical review board of MASKED FOR REVIEW. The online survey was developed using the online application Microsoft Forms. The questionnaire included 40 single choice, five multiple choice and 48 free text questions. Briefly, five main aspects of medical records were investigated including onset of clinical signs, neurologic examination, findings related to diagnostic work-up, outcome and follow up. Results of electromyography were interpreted and graded by the examiners as follow: minimal (+), mild (++), moderate (+ + +), and severe (+ + ++) (41).The course of the disease was considered remittent when the cats recovered with none or only minor deficits, and remittent-relapsing if periods of prolonged improvement were followed by relapses. A waxing and waning clinical course implicated persistent clinical signs of variable severity. Recovery was defined as the state in which the cat could walk without assistance and could jump onto objects. Internal review of the questions by the study investigators and external review by clinicians regarding structure, phrasing, understanding, and processing of the questions was performed. All information was obtained directly from the veterinary neurologist who submitted the biopsy. A case questionnaire was considered suitable for enrolments if it was completed to the end and all mandatory questions were answered.

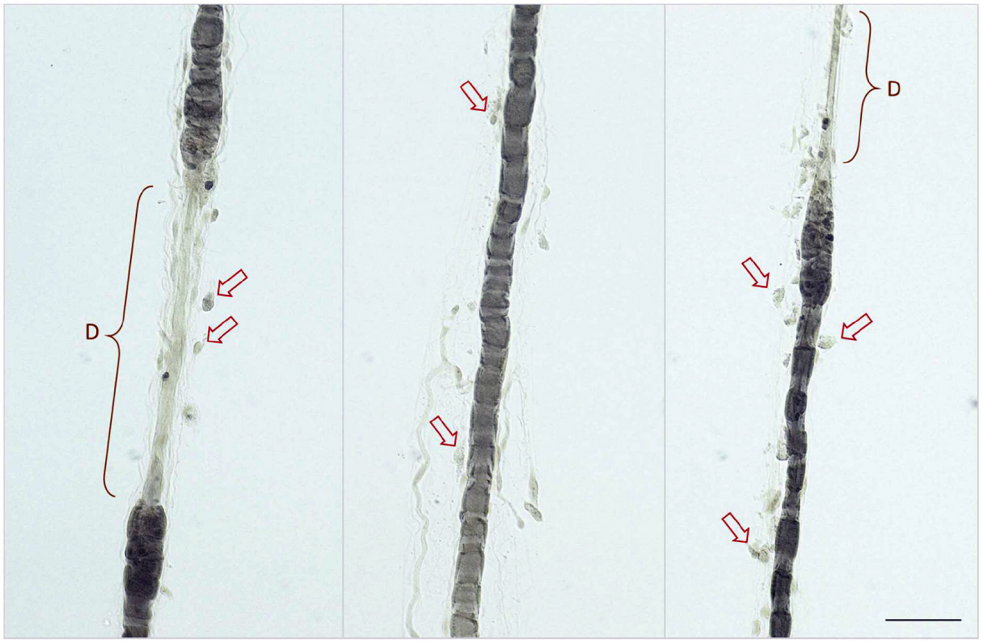

FIGURE 1 | Nerve fiber teasing preparations showing multiple fiber adhesive and invasive round mononuclear cells (lymphocytes and macrophages) indicated by red arrows at the Schmidt Lanterman clefts, paranodium and along demyelinated segments (indicated by D). Biopsy of the peroneal nerve contrasted with Osmium tetraoxide, scale bar 50 μm.

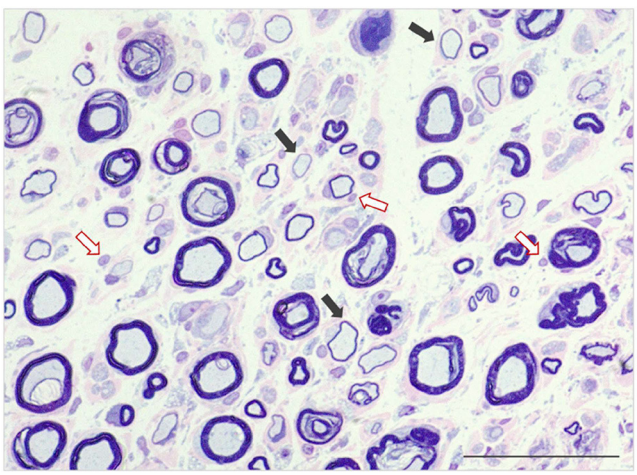

FIGURE 2 | Semithin section of peroneal nerve featuring reduced density of nerve fibers and mild expansion of the endoneurial fibrocollagenous tissue. There are numerous fibers with marked reduction of myelin sheath thickness (demyelination—black arrows) accompanied by multiple fiber adhesive mononuclear cell infiltrates and overall increased endoneurial cellularity (red arrows). Stained with Azurblue Safranin, scale bar 500 μ–m.

Statistical Evaluation

Descriptive statistic was performed and data were analyzed by Shapiro-Wilk-test for conformance with a normal distribution. Observation time was defined as the time from the submission of muscle and nerve biopsies to the last contact with the cat owner. Recovery time vs. age was studied using Kruskal-Wallis-test because age did not follow a normal distribution. Recovery time vs. all variables and groups inside of those variables between each other were studied *via* Fisher tests. Disease onset, age, clinical and electrodiagnostic parameters were analyzed for any association with outcome (recovered/not-recovered) via logistic regression. $P < 0.05$ was considered significant. Furthermore, *P*-values were adjusted for multiple comparisons by the Holm method. All analyses were done by the R Statistical Software (version 4.0.3).

RESULTS

Out of 107 identified candidates, 73 surveys were returned resulting in a response rate of 68.2%. Three were incomplete, thus 70 valid surveys were enrolled for further evaluations. Cats from this survey were geographically distributed among five European countries: Germany (42), United Kingdom (11), Italy (7), France (6), and Switzerland (4).

Demographics and History

The median age at onset of clinical signs was 10 months (range 4–120 months). Muscle/nerve biopsies were collected at a median age of 11 months (range: 4–125 months) (**Figure 3**). There were 64.3% male (45/70) and 27.1% female (19/70) cats. Sex was not specified in 6 cats. The most common breed was British short hair 25.7% (18/70). Other breeds were domestic short hair 24.3% (17/70), Bengal cat 11.4% (8/70), Maine Coon 8.6% (6/70),

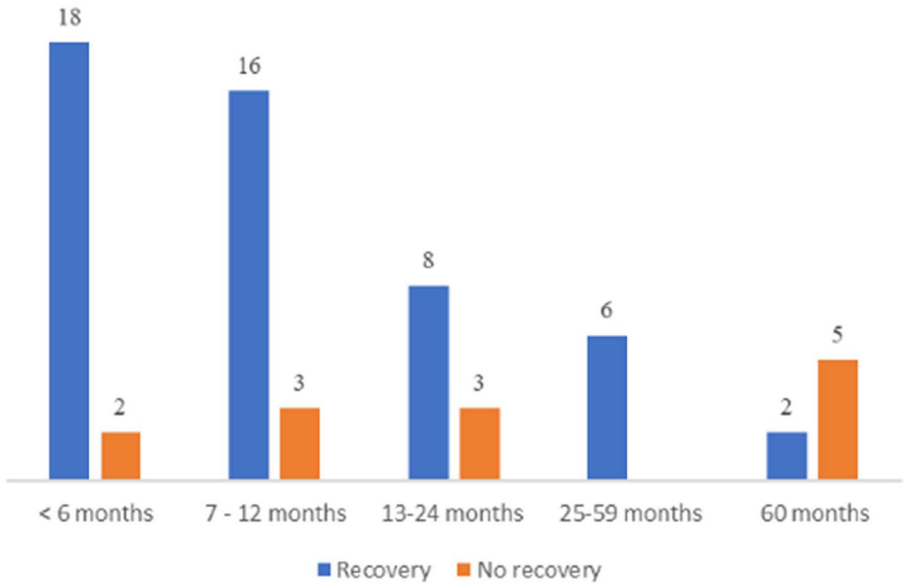

FIGURE 3 | Age at onset. Summary of the outcome in the different age groups with total number of patients for each segment.

Persian cat 5.7% (4/70), mixed breed cat 2.9% (2/70), Thai cat 2.9% (2/70), and one cat each from the following breeds: Ragdoll, Savannah, Siam cat, Siberian cat, Abyssinian cat, unknown breed, Chartreux, Devon Rex, Munchkin, Birman cat, Norwegian Forest cat, Russian Blue, and Scottish Fold. The majority of cats lived indoors (71.2%; 37/52); 7.8% (15/52) had outdoor access. Diet was indicated in 30 cats: 36.7% (11/30) received dry and wet food, 33.3% (10/30) dry food, 20% (6/30) wet food, 6.7% (2/30) biologically appropriate raw food (BARF), and 3.3% (1/30) gluten free diet. In four cats it was reported that other littermates were affected, and in 3 patients, other cats of the same household presented with similar clinical signs according to the owner.

Preceding Events

Vaccination status was known in 44 cats. Of these, 18.2% (8/44) were vaccinated 6 weeks or less before the onset of clinical signs. There were no reports on tick manifestation in temporal relationship to disease onset. Fifteen cats (21.4%) had a preexisting medical condition or an infection in the 6 weeks prior to disease onset as follows: bite wound (1), gastrointestinal infection (6), signs of feline upper respiratory disease (4), cardiac disease (2), feline leukemia virus (1), patellar luxation (1), arthritis (1), episode with transient ataxia, salivation and mydriasis 18 months before onset of clinical sign (1), and oliguric renal failure (1). In one cat, the symptoms worsened 1 month after neutering. Two cats received medications shortly before onset of signs: acyclovir (one cat) and imidacloprid/moxidectin/praziquantel (one cat).

Onset and Initial Progressive Phase

Onset was described as acute in 30.9% (21/68) and insidious in 69.1% (47/68) of the cats. No information on onset was available from two cats. An initial progressive phase was described in 74.3% of the cats (52/70). The median duration of the initial progressive phase from onset to peak/plateau of weakness was 14 days (range: 1–180 days). The median time between the onset of weakness and referral for muscle/nerve biopsy was 1 month (range: 0–28 months). The presenting complaints were generalized weakness or tetraparesis in 41 cats; other presenting complaints were pelvic limb weakness (17 cats), inability to jump (13 cats), paraparesis (nine cats), abnormal gait (seven cats), ataxia (four cats), lameness (two cats), unwillingness to play (two cats), paraplegia (two cats), tetraplegia (one cat), ventroflexion of head and neck (one cat), and/or spinal pain (one cat). Additional complaints were gastrointestinal signs in four cats. In 31 cats there was more than one presenting complaint, and in two cats the presenting problem was not specified. Weakness/paresis at the peak/plateau of the disease was characterized as lower motor neuron tetraparesis in 75.7% (53/70), tetraplegia in 2.9% (2/70), paraparesis in 18.6% (13/70) and paraplegia in 2.9% (2/70), respectively. At the peak of the weakness, 47.1% of the cats were non-ambulatory (33/70) and 51.4% (36/70) still able to walk without assistance. In one cat this information was not available.

Physical/Neurological Examination

Abnormal findings on physical examination were noted in 14 cats as follows: respiratory signs in 5.7% (4/70) (polypnea, increased vesicular lung sounds), small/thin body condition in 2.9% (2/70), heart murmur in 2.9% (2/70) and tachycardia in 1.4%, tail deformity, alopecic tail, bilateral chronic otitis externa, mild generalized lymphadenopathy, pyrexia, and mild ocular discharge in one cat each. Separate questions addressing the presence/absence of weakness, paraparesis/tetraparesis, and the presence/ absence of spinal reflexes were formulated in the survey. Here to follow are the description and summary of

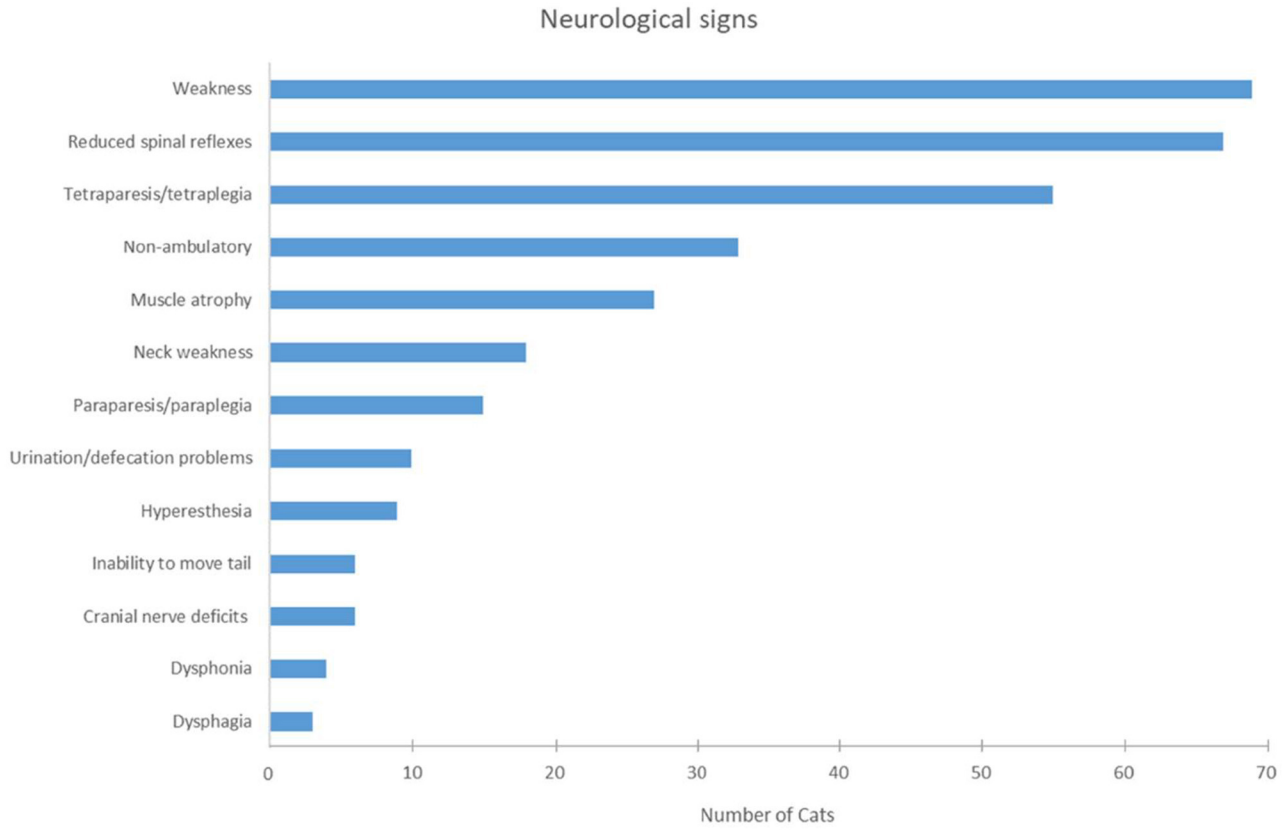

FIGURE 4 | Graphic representation of neurologic signs presented by the cats of this cohort.

the answers collected. Weakness was indicated in all cats but one (98.6%, 69/70) (**Figure 4**). Paraparesis, inability to move the tail and normal spinal reflexes were described in this cat. In summary, all cats but three had neuromuscular signs on neurological examination (95.7%, 67/70): the withdrawal reflex was reduced in all limbs in 83.6% (56/67) of the cats, in the pelvic limbs only in 13.4% (9/67), and in the thoracic limbs only in one cat, and in one cat affected limbs were not specified. Normal withdrawal reflexes were reported in two cats with paraparesis or tetraparesis, respectively, and reflexes were not described in one cat (tetraparesis). The signs showed a symmetric distribution in 91.4% (64/70) and an asymmetric distribution in 7.1% of the cases (5/70). Inability to move the tail was noted in 8.5% (6/70) of the cats. Neck weakness and flexion of the head and neck was evident in 25.7% of the patients (18/70). Signs of cranial nerve dysfunction were described in 17.1% (12/70) of the cats as follows: dysphonia (four cats), facial paresis/paralysis (four cats), dysphagia (three cats), reduced menace response (one cat), reduced palpebral reflex (one cat). Hyperesthesia was reported in nine cats (12.9%), either on spinal palpation (five cats), limb palpation (three cats), or when lifted up (one cat). Problems with urination or defecation were noted in 14.3% of the cats (10/70). Of these 10 cats, two had defecation problems, two micturition problems, three both, and three were unable to reach the litter or to stand in the litter box. Muscle atrophy was described in 38.6% (27/70) and defined as generalized with greater involvement of the hind limbs.

Electrodiagnostic Investigations

Electrodiagnostic studies were performed in 67 cats (67 Electromyography—EMG, 65 Motor Nerve Conduction Velocity—MNCV; 43 repetitive nerve stimulation—RNS; 29 F-waves; 11 Sensory Nerve Conduction Velocity—SNCV; 3 Somatosensory Evoked Potentials—SEP). Review of electrodiagnostic findings was performed considering the following normal reference ranges: MNCV 93.7 +/- 9.4 m/s in the sciatic/tibialis nerve and 82.1 +/- 11.1 m/s in the ulnaris nerve (12). EMG demonstrated spontaneous activity in 89.6% of the cats (60/67). The spontaneous electric activity (SPA) appeared generalized in 93.3% (56/60) and only in the pelvic limbs in 6.7% (4/60); one of the latter cats had only minimal EMG changes in the pelvic limbs. Information on the proximal or distal distribution of EMG changes was available in 74.6% (50/67): in 34% of the cats (17/50), the proximal and distal appendicular muscles were equally affected, in 64% (32/50) the distal muscles and in 2% (1/50) the proximal muscles were more severely affected. Only in one cat the proximal appendicular muscles were more severely affected than the distal appendicular muscles; in this cat the pelvic limbs showed SPA. The predominant abnormalities in EMG were fibrillation

potentials in 38.3% (23/60), positive sharp waves in 30% (18/60) and fibrillation potentials together with positive sharp waves in 23.3% (14/60). In five cats EMG abnormalities were not further specified. Only one cat showed additional complex repetitive discharges. Changes were mild in 38.3% (23/60), moderate in 46.7% (28/60) and severe in 11.7% (7/60). EMG changes were considered minimal and appeared only in the pelvic limbs (1/60) or not further described (one cat). Cats that only demonstrated positive sharp waves were more likely to recover than cats with fibrillation potentials ($p < 0.05$). In 65 cats, investigators reported on motor nerve conduction studies (MNCV) (**Figure 5**). Measured MNCVs (m/s) were available from 43 cats (41 pelvic limb studies, 23 thoracic limb studies), in the others, it was only indicated whether MNCV was decreased or normal. Mean MNCV was 64.6 m/s (14–127 m/s) in the pelvic limbs and 60.8 m/s (15.7–115 m/s) in the thoracic limbs. Taken together MNCV was decreased in 52.3% (34/65) of the cats; specifically, in 49.2% (30/61) of the examined pelvic limbs and in 52.9% (18/34) of the examined thoracic limbs. In cats with a decreased MNCV, MNCV was decreased to 52% in the pelvic limbs and to 65.6% in the thoracic limbs in reference to the lower limit (mean – 2SD) of normal MNCV in cats. Other findings were temporal dispersion in 26.1% (17/65) of the cats which was frequently associated with a decreased amplitude of the CMAP (15/17 cats). A decreased amplitude of the CMAP was a frequent finding (73.8%; 48/65) in general. In total, 81.5% (53/65) of the cats presented with abnormal MNCV, in 17% (11/65) MNCV was normal and in 1.5% (1/65) MNCV was not further specified. Out of the 60 patients with EMG abnormalities, 78.3% (47/60) showed concomitant MNCV changes, 15% (9/60) presented with EMG changes only, and MNCV changes were not indicated in 6.6% (4/60). Repetitive nerve stimulation was performed in 43 cats and was unremarkable in 95.3% (41/43) cats. Cord dorsum potentials were tested in three cats and described as unremarkable in all of them. Further sensory nerve conduction studies were performed in 11 cats and results were unremarkable in all of them. Details about the F-wave evaluation were indicated in 29 cases. In 72.4% (21/29) F-wave was described as abnormal: in 48.3% (14/29) the F-wave was not detected and in 24.1% (7/29) the F-wave was recorded but showed abnormalities described as: increased latency, decreased F-M-ratio, decreased amplitude, inconsistent, and irregular. Only one cat was normal on electrodiagnostic investigation.

Other Diagnostic Investigations

Advanced diagnostic imaging (Computed Tomography, Magnetic Resonance Imaging of spine/brain) was performed in 20 cats. Abnormal findings were described in four cats: One cat revealed an increased size of the sciatic nerves, one a T2-weighted hyperintensity at the level of L3/L4 (no further details were available), one a diffuse T2-weighted and STIR hyperintensity of several skeletal muscles with contrast enhancement and one a broncho-/pneumopathy and an anomaly of the descending aorta. Results of cerebrospinal fluid analysis (CSF) were available from 26 cats and were unremarkable in 73.1% (19/26). Mononuclear pleocytosis was reported in 3 cats (11.5%; 3/26) and increased protein content (>30 mg/dL) was the only abnormal finding in four other cats (15.4%; 4/26). Acetylcholine receptor antibodies were tested and were unremarkable in nine cats. Increased creatine kinase activity was reported in 51.1% (23/45) and was normal in 48.9% (22/45) of tested cats (reference range: 0–414 U/l). Negative molecular and serologic tests were reported for infectious agents: *Toxoplasma gondii* (42 cats; five cats had a positive IgG titer, but a negative IgM titer), *Feline immunodeficiency virus* (22 cats), *Feline leukemia virus* (22 cats), *Feline coronavirus* (six cats). The following infectious agents were each tested negative in one of the cats: *Bartonella spp, Borna disease virus, Neospora caninum,* and *Encephalitozoon cuniculi*; further details on diagnostic tests were not available for review. Other findings included abdominal lymphadenopathy in two cats, specified as neutrophilic cellular infiltrate by fine needle aspirate in one of these cats, and vertebral spondylosis (Th12-L1), and radiographic evidence of bilateral degenerative joint disease (coxo-femoral joints, stifles) in another cat.

Treatment

The following treatment modalities were reported: glucocorticoids (70%; 49/70), non-steroidal anti-inflammatory drugs (55.7%; 39/70), L-carnitine supplementation (48.6%; 34/70), and physical therapy/rehabilitation (20%; 14/70). Duration of these therapies was indicated in 20% of cases (14/70) and the median was 8.5 days (range 3–90 days). Other medications included antimicrobial drugs, pyridostigmine and B vitamins. In total, 62.9% of the cats (44/70) were treated before referral for biopsies and 94.3% (66/70) after muscle/nerve biopsies.

Outcome and Long-Term Clinical Course

Data on outcome and long-term clinical course were available for 63 cats. Of those 63 cats, 10 cats had a follow-up time of <1 month; the median follow-up time in the other cats was 8 months (range: 1–40 months). The overall course of the disease was characterized as remittent in 49.2% (31/63) and remittent-relapsing in 34.9% (22/63) of the cats. Thus, a remittent or remittent-relapsing course was reported in 84.1% (53/63) of the cats. In the others, clinical course was described as stagnant in 7.9% (5/63), progressive in 4.7% (3/63), or waxing-waning in 3.2% (2/63). Cats with a remittent or remittent-relapsing clinical course required a median time of 1.5 months to recover completely or to reach a clinical plateau and stable disease (range: few days to 17 months). Other endpoints were described as follows: the median time within cats could stand without assistance was 7 days, the median time until the cats could walk at least 5 steps or more without assistance was 7 days, and the median time until the cats could jump on objects was 28 days.

Relapses were reported in 35.7% (25 cats) (22 remittent-relapsing, two progressive and one waxing-waning course) after a median time of 3 months (range<1–8 months). Cats experienced only one (13/70), two (11/70), or more than three relapses (1/70). In total, 79.4% (50/63) of the cats recovered (**Figure 6**). Most patients were presented at the plateau of the weakness or during the initial progressive phase. Time from presentation until recovery was available for 37 cats: 29.7% (11/37) recovered

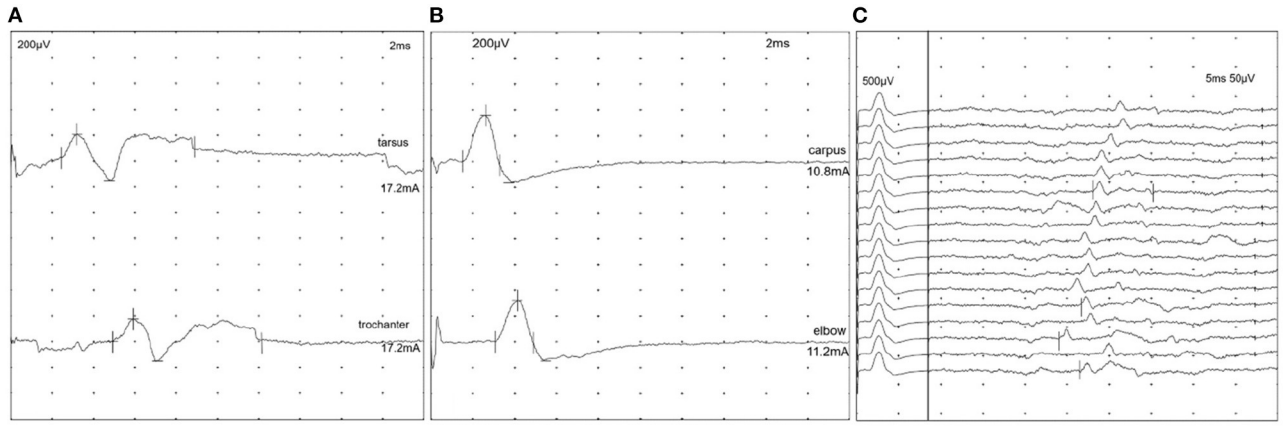

FIGURE 5 | Electrodiagnostic tests of a 14-months-old male Maine Coon cat with presumed immune-mediated polyneuropathy. **(A)** Motor nerve conduction studies of the left sciatic-tibial nerve. CMAPs show a marked reduction of amplitude/area and mild temporal dispersion. Motor nerve conduction velocity is 76 m/s [reference range 93.7 ± 9.4 m/s; Ref. (12)]. **(B)** Motor nerve conduction study of the right ulnar nerve. CMAPs show a marked reduction of amplitude/area. Motor nerve conduction velocity is 64 m/s [reference range 82.1 m/s ± 11.1 m/s; Ref. (12)]. **(C)** F-waves after supramaximal stimulation of the right ulnar nerve at the carpus. F-waves are characterized by a pronounced chronodispersion (variable minimum latency) and a severe increase in latencies. The values of minimum F-waves latencies vary between 24 and 31 ms [reference range 8.4 ± 0.9 ms; Ref. (42)].

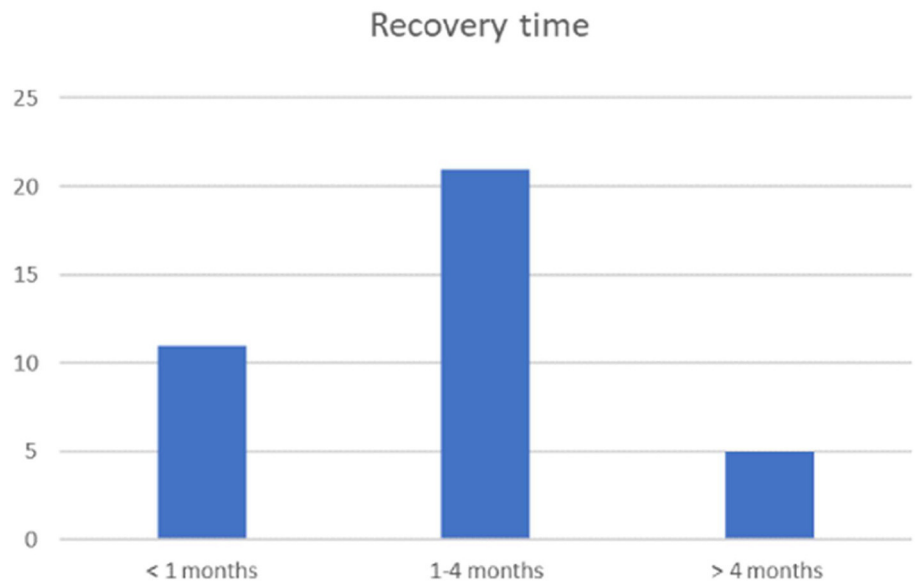

FIGURE 6 | Recovery time. Representation of recovery time in the three groups (weeks: 1–4 months, months: 1–4 months and late: >4 months), with total amount of patients indicated on the y axis.

within 4 weeks, 56.8% (21/37) within 1–4 months, and in 13.5% (5/37) recovery took longer than 4 months. A younger age at the time of the diagnosis as well as a sudden onset of clinical signs was statistically significant associated with recovery ($p < 0.05$). Only 18.6% of the cats did not recover and 7.4% died or were euthanatized due to PN.

Correlation Analysis

Statistical evaluation of clinical variables and outcome revealed that (1) young age at the time of diagnosis, (2) sudden onset of clinical signs and (3) presence of positive sharp waves alone were significantly associated with recovery ($p < 0.05$) (**Figures 7**, **8**).

DISCUSSION

This study describes the signalment, clinical sings, electrodiagnostic findings, course, outcome and predictors of outcome of a presumed immune-mediated PN in a large cohort of European cats. Characteristic features were the onset of clinical signs at an early age (median age 10 months), a variety of

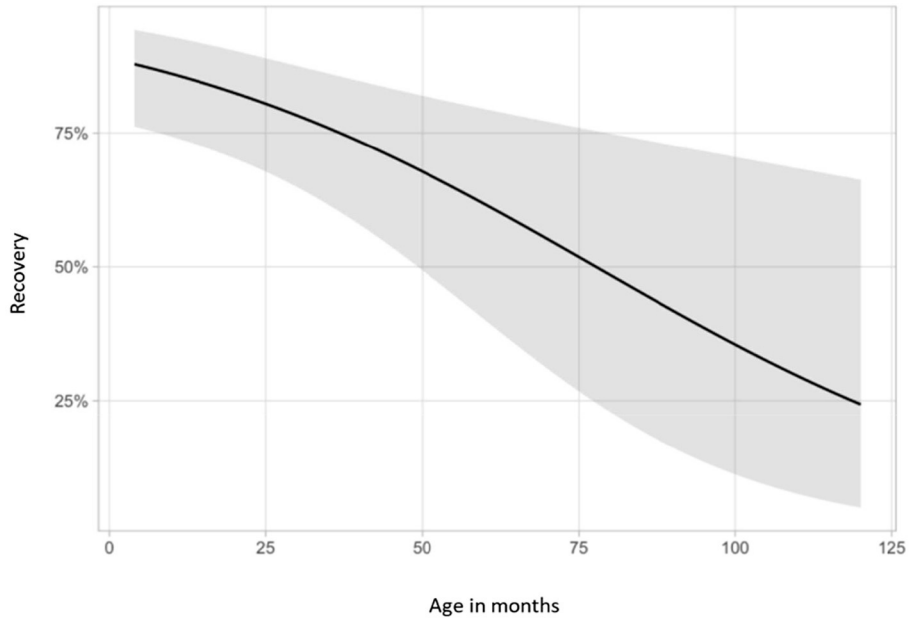

FIGURE 7 | Association between recovery and age at onset. Demonstration of the relationship between recovery and age at onset of clinical signs. The y-axis shows probabilities of recovery predicted by the logistic regression. Younger cats were more likely to recover ($p = 0.005$).

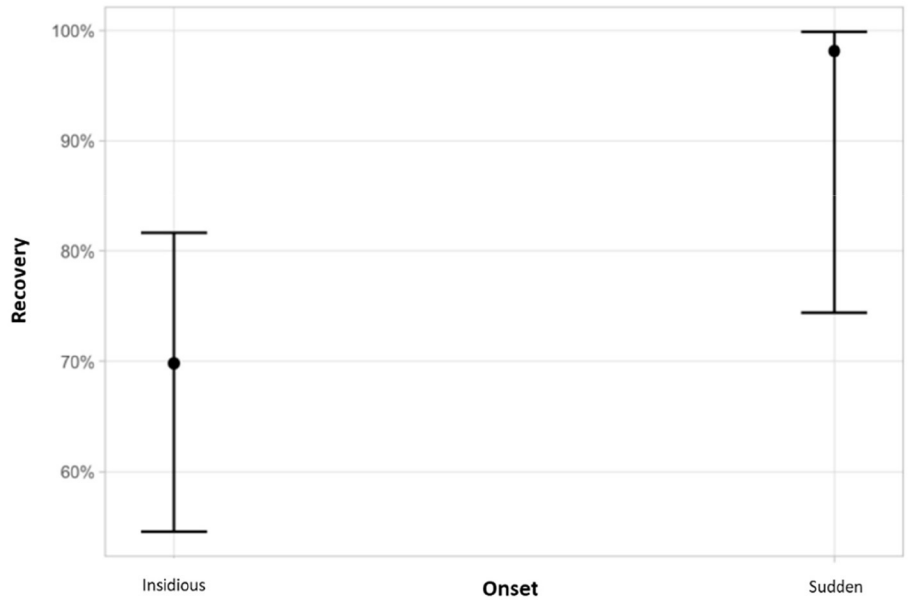

FIGURE 8 | Association between the type of onset and recovery. Cats with a sudden onset of clinical signs were more likely to recover ($p = 0.037$). The y-axis shows probabilities of recovery predicted by the logistic regression. Graph displays estimated probabilities with 95% confidence intervals.

affected breeds, male predominance, an initial progressive phase that plateaued thereafter, and a remittent or remittent-relapsing course in 84.1% of the cats with overall 79.4% of the cats recovering. Relapses occurred in 35.7% of the cats.

The clinical features of our cohort paralleled previous descriptions from US cohorts: in these, mean age of onset was either described as 10.6 +/- 7.9 months or ranged from 3 to 12 months (31, 35). A variety of breeds were affected in our cohort. The top breeds were British short hair (25.7%) and Domestic short hair cats (24.3%) followed by Bengal cat (11.4%). In previous descriptions, Bengal cats (31, 32, 35) appeared to be predisposed toward development of inflammatory

PN (31, 32, 35), but other breeds were also affected (33, 35). British shorthair cats have up to now rarely been associated with an increased risk for immune-mediated PN (43–47). The additional wide representation of domestic short hair cats in our cohort, could suggest a contribution of acquired factors to the inciting cause of inflammatory PN in cats and less of a genetic predisposition than previously assumed.

Possible triggers of immune-mediated events were investigated in the survey, but we were unable to identify unique precipitating factors. Only 18.2% (8/44) of the cats developed clinical signs in the 6 weeks following vaccination. Thus, in most of the cats, the onset had no apparent relationship with vaccination. Concomitant or preceding infection or inflammation of other organ systems was only described in a handful of cats in this study and comprised bilateral chronic otitis externa, mild lymphadenopathy, pyrexia, and ocular discharge among others. Thus, no obvious inciting triggers were identified in this cohort. Nonetheless, a potential contribution of infectious agents in inflammatory PN cannot be excluded as preceding subclinical signs might not be described or detected prior to referral for biopsies. Dietary-related sensory PN from phenylalanine and tyrosine deficiency were described in an experimental group of black cats (29). However, nutritional deficiencies appeared unlikely with regular feeding of high-quality commercial diets and inflammatory changes on peripheral nerve histology. Furthermore, in the history of our cats there was no evidence for exposure to neurotoxic substances such as organophosphates, salinomycin, thallium, mercury, acrylamide, and pyrethrins/pyrethroids or specific medications like vincristine (2, 4, 15–19, 48). Moreover, the course of the disease and widespread geographic origin of the cats throughout Europe would not suggest exposure to poisons either.

Results of the neurologic examination indicated a generalized neuromuscular disorder with a symmetric distribution. Common neurological signs were generalized weakness, tetraparesis and reduced withdrawal reflexes of all limbs, indicative of generalized PN in almost all cats. Less commonly, paraparesis was the presenting feature. Generalized muscle atrophy was a feature in 38.6%. Similarly, previous studies of feline PN reported also clinical signs indicative of generalized neuromuscular disease (1, 2, 5, 31, 32, 35, 37, 39). A proportion of the cats showed additional dysfunction of cranial nerves such as facial paresis/paralysis, swallowing deficits or, dysphonia or autonomic signs like impaired micturition and defecation problems. Other studies from North America and Europe described also decreased palpebral reflex, decreased menace response, decreased gag reflex, and difficulty in prehending food in 21.6–60% (31, 33, 35, 37). Respiratory signs, including polypnea and increased vesicular lung sounds, were reported in 5.7% of the cats but none of the cats developed dreaded consequence of peripheral nerve diseases, such as respiratory paralysis.

The onset of clinical signs was insidious in up to 70% of our cohort (69.1%; 47/68), while acute onset, characteristic of acute polyradiculoneuritis, was reported in 30.9% (21/68) of cats. Interestingly, after onset, an initial progressive phase with deterioration of weakness was described in the majority (74.3%) of the cats. The median time to reach the peak/plateau of weakness was 14 days (mean 23.9 days). An acute onset of generalized neuromuscular weakness with a progressive phase <4 weeks parallels the acute onset of immune-mediated PNs in human medicine and <2 weeks in dogs with acute polyradiculoneuritis. Both, Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP) can have acute onset in humans but may differ in the subsequent clinical course (49–51). Differences in the onset of clinical signs among different cats might reflect correct observations, but discrepancies could potentially be related to different perceptions of the cats' gait and mobility by cat owners compared to dog owners and humans. Cats do not participate in regular physical activity, as would be the case for dogs, hence precise estimation of the subtle onset of reduced fitness due to neuromuscular disorders might be more challenging to define in feline patients (3). Similar to the observations in our cats, others described the onset of PN in young cats also as either acute or insidious (31, 33, 35).

Electrodiagnostic findings confirmed the presence of generalized neuromuscular dysfunction in nearly all cats. The key finding was the widespread appearance of pathological spontaneous electric activity with a generalized symmetrical distribution together with reduced MNCV. Likewise, previous studies on PN in young cats revealed abnormal spontaneous electrical muscle activity with positive sharp waves and fibrillation potentials accompanied by reduced MNCV, increased latencies and reduced amplitudes (1, 2, 4, 5, 31, 35). Many investigators reported abnormal F-wave studies indicative of nerve root involvement, which is a common finding in acute polyradiculoneuritis in dogs or GBS in humans (49–56). In one cat normal electrodiagnostic results were reported, yet nerve pathology and denervation atrophy of the muscles was graded as moderate. Clinically, this cat showed relatively mild signs (ambulatory tetraparesis), and MNCV was only recorded from one nerve (fibular n.) In this regard it should be highlighted that in human medicine routine electrodiagnostic screening involves comprehensive testing of multiple nerves/muscles which is hardly appreciated in veterinary medicine (57, 58).

A PN with presumptive immune-mediated etiology was indicated by examination of muscle and nerve biopsies that revealed an autoreactive inflammatory neuropathy and failed to provide evidence for an infectious origin in the cats of this cohort. In particular subregion specific immune damage as in nodo-paranodopathy is a quite unequivocal feature of immune-mediated nerve disorder (36). Further support for immune-mediated PN comes from the clinical course which resembles the common PNs in people, GBS and CIDP, in many aspects, with an initial progressive phase until reaching a clinical plateaus/stable disease and subsequent remission in GBS, and chronic or acute onset with remittent-relapsing clinical course in CIDP (49, 50, 59–61). We propose that feline inflammatory PN resembles human CIDP in many aspects. Overall, unequivocal diagnosis of immune-mediated PN can be challenging. In people, diagnosis of GBS and CIDP relies on a combination of neurologic and electrodiagnostic findings (49, 50, 59, 61). Specific challenges are the diagnosis of immune-mediated disease and classification of subtypes. Guidelines in humans for the diagnosis of CIDP

are a chronic-progressive course with symmetrical weakness and motor symptoms for at least 8 weeks, non-genetic background and specific electrodiagnostic abnormalities (abnormal distal latency, abnormal MNCV and abnormal F-wave latency) (61). Guidelines for diagnosis of GBS are bilateral flaccid weakness with reduced reflexes, monophasic course with acute onset, elevated protein concentration in CSF examination, specific nerve conduction studies findings (depending on subtype) and exclusion of other causes for weakness (62). Anti-GM2 ganglioside auto-antibody may serve as biomarker and help with definition of subtypes of immune-mediated GBS and CIDP (51, 52), but at present, there are no published data available in cats and sensitivity and specificity appear yet too low for routine use.

Owners may be informed that a high proportion of patients recovers from feline inflammatory PN (79.4%; 50/63). Only 18.6% of our population did not completely recover and 7.4% died from complications or were euthanized due to their PN. Others reported that 12% of the cats were euthanized shortly after diagnosis and 36% of the cats showed only a partial recovery with residual weakness (31) or reported that 3/5 cats were lost on follow up, one cat was euthanized and one cat showed a full recovery (35). In our study cohort, cats with younger age at the time of the diagnosis were more likely to recover as well as cats with a sudden onset of clinical signs ($p < 0.05$). We hypothesize that young cats with polyneuropathy present as a homogenous group with a shared etiology, and that etiology of the polyneuropathy may be different in older cats e.g., related to different immune mechanisms. Cats without fibrillation potentials, that only showed positive sharp waves in the electrodiagnostic studies were more likely to recover ($p < 0.05$). This fact is difficult to explain considering that positive sharp waves and fibrillation potentials reflect the same underlying pathology i.e., denervation. Previous studies had pointed out that there is a discrepancy in the onset of denervation activity with positive sharp waves appearing earlier in the time course of denervation and axonal degeneration than fibrillation potentials (63). We were unable to demonstrate an influence of specific treatment protocols. In human medicine the treatment for GBS is intravenous immunoglobulin therapy or plasma exchange while corticosteroids are ineffective (48–50, 57, 58, 64, 65); on the other hand, use of corticosteroids as well as intravenous immune globulin and plasma exchange proved effective in CIDP patients (66). Most reported treatments in our cohort included glucocorticoids, non-steroidal anti-inflammatory drugs, and nutritional supplementation but clear benefits from their usage could not be identified. Likewise, Bensfield et al. failed to identify an effective treatment protocol due to the retrospective and multicentric nature of their study (31).

Inflammatory PN in young cats showed many similarities to human juvenile CIDP. A male predominance was noticed in the studied population which parallels observations in CIDP (67). The course of the disease was mostly remittent (84.1%) and relapses were a frequent feature occurring in 25 cats (35.7%). These data are in line with previous literature (31, 35). Also in CIDP the characteristic course is remittent-relapsing (59, 61). In the juvenile form of CIDP neurological motor signs predominate, whereas in the adult form sensory deficits are mostly reported (68). Further similarities with our cats are also found in the nerve conduction studies of juvenile CIDP that presents with demyelinating features on MNCV and abnormalities of the F-waves (59).

Based on these results, an inflammatory PN in young cats can be diagnosed from characteristic clinical and electrodiagnostic findings, but nerve biopsies appear still necessary for confirmation in those patients with less clear presentation. An immune-mediated cause appears most likely due to the inflammatory nature of the nerve lesions, the sudden onset or initial progressive phase followed by a plateau, electrodiagnostic findings supportive of demyelinating peripheral nerve disease, and subsequent recovery in most cases. Infectious agents were supported neither by infectious disease testing nor by examination of nerve biopsies. Retrospective multicentric studies present with some limitations and the present one is no exception. As many referral centers and many neurologists were retrospectively involved, there was no standardized way of reporting and measuring results of clinical examination comprising physical and neurological evaluations or electrodiagnostic testing. Besides, collection of data about the outcome was partially affected by missing follow up information in a second opinion referral population.

CONCLUSION

We described and characterized an underdiagnosed inflammatory PN with a likely immune-mediated origin in young cats across Europe. Clinical and electrodiagnostic features and the remittent-relapsing clinical course resembled juvenile CIDP in humans in many aspects. The disease in itself has a good prognosis and seems not to be directly influenced by conventional treatments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethikkommission der Tierärztlichen Fakultät Ludwig-Maximilians-Universität München. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MR, AF, and JR designed and coordinated the study. NK, MR, and KM provided the data. AF, FW, MR, and KM designed the questionnaire. JR, AF, and MR wrote the manuscript. All authors read and approved the final manuscript.

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Treatment With Cytarabine at Initiation of Therapy With Cyclosporine and Glucocorticoids for Dogs With Meningoencephalomyelitis of Unknown Origin Is Not Associated With Improved Outcomes

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Meningoencephalomyelitis of unknown origin (MUO) is a common disorder of dogs that results in significant morbidity and mortality. The ideal treatment regimen is not known but a second immunosuppressive agent is often utilized in combination with glucocorticoids to increase efficacy and reduce side effects. Recently, a benefit to using a cytosine arabinoside (CA) constant rate infusion (CRI) at the time of diagnosis has been demonstrated. Here, a retrospective study was performed to determine if administration of CA at the time of diagnosis would alter prognosis in dogs receiving cyclosporine and prednisone for treatment of MUO. Medical records of 51 client-owned dogs diagnosed with MUO at one institution were reviewed (2009-2019). All dogs were treated with cyclosporine and a tapering course of prednisone. Twenty-one dogs received a single initial 200 mg/m^2 treatment with CA either as a CRI or subcutaneously. Significantly more patients in the CA treatment group were obtunded on presentation but all other baseline parameters were similar between groups. No differences in success (defined as sustained improvement on neurological exam with owner perceived good quality of life), relapse, or death were identified at 1-, 3-, 6-, 9-, 12-, 18-, or 36-month time points. These results do not support treatment with CA (either as a CRI or subcutaneously) at the time of diagnosis in dogs treated with cyclosporine and prednisone.

Keywords: meningoencephalomyelitis of unknown origin (MUO), meningoencephalomyelitis of unknown etiology (MUE), meningoencephalitis, dog, cytosine arabinoside, cyclosporine

INTRODUCTION

Meningoencephalomyelitis of unknown origin (MUO) is a clinical diagnosis of non-infectious central nervous system (CNS) inflammation in dogs. It intended to represent a presumptive diagnosis of three histologically described variants of CNS inflammation that are overrepresented in young to middle-aged toy and small breed dogs: granulomatous meningoencephalomyelitis

(GME), necrotizing meningoencephalitis (NME), and necrotizing leukoencephalitis (NLE). Although the etiopathogenesis of MUO has not been fully defined, an autoimmune etiology is suspected, and, as such, immunosuppression is the mainstay of treatment. To date, there is no consensus regarding a standard treatment protocol, and the prognosis for long-term survival remains guarded (1, 2).

Glucocorticoids are the cornerstone of treatment for MUO but a second immunosuppressive agent is often added in an attempt to increase efficacy and reduce steroid side effects. Cyclosporine is a commonly prescribed second agent that may prolong survival times when used in addition to glucocorticoids (3–6). However, cases treated with cyclosporine and glucocorticoids still have up to a 48% mortality rate with 78% of cases experiencing one or more relapse events (5).

Recent studies have shown that treatment with CA at the time of diagnosis can alter MUO prognosis. Dogs that received a constant rate infusion (CRI) of CA at the time of diagnosis had improved 3-month survival times compared to those that received subcutaneous CA (7). Additionally, patients receiving a single CRI of CA at the onset of treatment had the same prognosis as those that received a CRI of CA followed by subsequent, intermittent subcutaneous injections of CA over 72 weeks (8). This led to the hypothesis that addition of a single treatment with CA at the onset of diagnosis could improve outcomes in MUO-dogs treated with cyclosporine and prednisone.

In the retrospective study presented here, all dogs were treated with long-term cyclosporine and a tapering course of prednisone. One group received a single, additional treatment with CA at the time of diagnosis. Medical records were reviewed to determine outcomes at one, three, six, nine, 12, 18, and 36 months. The primary aim was to determine if short-term (i.e., 1 and 3-month) relapse and mortality rates were improved with addition of a single administration of cytarabine either intravenously or subcutaneously at the time of diagnosis. Additionally, based on the fact that mortality most commonly occurs within the 3 months following diagnosis of MUO (9, 10), a secondary hypothesis with that long-term (i.e., up to 36-month) relapse and mortality rates would also be improved with addition of CA.

MATERIALS AND METHODS

Patient Selection

Medical records (2009–2019) were searched to identify dogs with a diagnosis of meningoencephalitis or meningoencephalomyelitis that also received cyclosporine and a tapering course of prednisone ± administration of a CA by CRI or subcutaneous injection at the time of treatment initiation.

Diagnostic inclusion criteria were based on a previous study with minor modifications (11): (i) availability of a complete medical record with neurological exams documented by either a neurology resident or board-certified neurologist; (ii) patient weight <15 kg; (iii) patient age 6 months to 12 years; (iv) focal or multifocal neuroanatomical lesion localization; (v) focal or multifocal T2-weighted hyperintensities on magnetic resonance imaging (MRI) consistent with inflammation; and (vi) cerebrospinal fluid (CSF) pleocytosis (greater than five total nucleated cells per microliter with <4,000 red blood cells per microliter) with >50% mononuclear cells. If necropsy confirmation of an MUO diagnosis was not available, dogs with a normal MRI, normal CSF, or from which CSF could not be obtained were excluded. Where possible, infectious diseases were ruled-out by negative serology for *Toxoplasma gondii*, *Neospora caninum*, and *Cryptococcus spp*. If serology for infectious diseases was not obtained, patients had to have a successful outcome (no relapse or death) for a minimum of 12 months while receiving immunosuppressive therapy or confirmation of GME, NME, or NLE by histopathology. Cases consistent with ischemic myelopathy (per-acute or acute history with clinical progression <48 h and focal T2W spinal cord hyperintensity without evidence of intracranial disease on exam or MRI) were excluded.

Additional criteria included treatment with cyclosporine (5–12 mg/kg/day) and prednisone (1.0–1.5 mg/kg/day) initiated at the time of diagnosis. Prednisone administration followed a routine tapering schedule of 25% every 4 weeks and was discontinued after every other day administration of 0.2–0.4 mg/kg/day for 4 weeks. Dogs in the CA treatment group had to receive 200 mg/m^2 CA either as a CRI over eight to 12 h or divided into four subcutaneous injections given every 12 h. Dogs could not receive additional immunomodulatory therapy unless it was added after diagnosis of a relapse event. A minimum of 36 months follow-up was required.

Data Collection

The following data were collected from each medical record: (i) signalment; (ii) time to presentation; (iii) history of seizures; (iv) presence or absence of obtundation; (v) complete neurological exams at 1, 3, 6, 9, 12, 18, and 36 months; (vi) MRI features including presence or absence of mass effect, sulci effacement, and transtentorial or foramen magnum herniation; (vii) CSF and infectious disease testing results; (viii) drug dosages, route of administration, and tapering protocol; (ix) survival times; and (x) necropsy results. Neurodisability scores (9) were calculated from recorded neurological exams.

Treatment Groups

Patients were divided into two groups based on whether or not they received CA at the time of diagnosis. The CA+ group received CA 200 mg/m^2 either as an 8 to 12-h CRI or four subcutaneous injections every 12 h. The CA- group did not receive any CA at the time of diagnosis, although it may have been subsequently administered at the time of a relapse.

Outcome Assessment

At 1, 3, 6, n9, 12, 18, and 36 months, the following outcomes were determined and recorded: success, relapse, or death. Outcome success was defined as sustained neurological improvement (as determined by repeat neurological exams and concurrent owner report of satisfaction) after initiation of the treatment protocol. Diagnosis of relapse was based on recurrence of clinical signs that necessitated a change in treatment protocol, repeat MRI and / or CSF (where available), and expected response to alterations in treatment. Death was defined by all dogs that died or were

euthanized due to clinical signs or complications related to the diagnosis of MUO. Relapse and death were only recorded once for each patient at the time of initial occurrence. Time to relapse, survival times, and change in neurodisability score were also determined as a secondary measure of outcome.

Statistical Analysis

Baseline characteristics of dogs from each group were compared. Gender, history of seizures, presence or absence of obtundation, and MRI findings were compared using the Fisher exact test. Age, time to presentation, CSF total nucleated cell count (TNCC), CSF total protein, initial prednisone dose, and cyclosporine dose were compared using the Mann-Whitney *U*-test. Outcome measures of success and relapse were compared between groups using the chi-square test where all expected cell counts were greater than five. The outcome measure of death was compared between groups using Fisher exact test. Time to relapse and survival times were conducted using Kaplan-Meier plots and were compared between groups by log-rank analysis. Cases that died of causes not related to MUO were censored in the analysis. Statistical significance for all tests was initially set at $p < 0.05$. To account for the 21 simultaneous independent analyses performed for outcomes measurements (success, relapse and death), Bonferroni correction was applied resulting in a significance level of 0.0024.

RESULTS

Patients

There were 21 dogs included in the CA+ group. Represented breeds included eight Maltese, three Chihuahuas, two Boston terriers, two French bulldogs, two miniature pinchers, two mixed breeds, and one each of rat terrier and Jack Russell terrier. There were 29 dogs included in the CA- group. Represented breeds included six Chihuahuas, five mixed breeds, three dachshunds, two French bulldogs, two Jack Russell terriers, two Maltese, two Yorkshire terriers, one affenpinscher, one American shepherd, one Boston terrier, one cairn terrier, one Pomeranian, one shi tzu, one silky terrier, and one toy poodle. There was no significant difference in gender, age at presentation, time to presentation, presence or absence of seizures, neurodisability score, presence of mass effect on MRI, evidence of increased intracranial pressure on MRI, or CSF results. There were significantly more obtunded dogs in CA+ group (43%) vs. the CA- group (13%) ($p = 0.02$) (**Table 1**). There were no significant differences in cyclosporine or prednisone doses between groups (**Table 2**). Prednisone was discontinued an average of 6 months after diagnosis in both groups. Cyclosporine was discontinued in two dogs after 12 months (both of which had successful outcomes), while all other dogs continued to receive cyclosporine for the 36-month study period. For the CA+ group, CA was administered *via* CRI in 12/21 patients (57%) and subcutaneously in 9/21 (43%). Necropsy results were available for three dogs and confirmed a diagnosis of GME in two dogs and NME in one dog.

TABLE 1 | Patient variable at initial presentation.

	CA+	CA–	*p*-value
Number of dogs	21	30	
Gender			
Male neutered	6	7	0.75
Female spayed	14	23	0.52
Female	1	0	0.41
Age at diagnosis (months)	56.5 (12–123)	67.5 (12–108)	0.33
Time to presentation (days)	19.5 (2–120)	15.2 (1–60)	0.83
History/exam findings			
Documentation of seizures	1/21 (5%)	4/30 (13%)	0.64
Patient obtunded	9/21 (43%)	4/30 (13%)	0.02
Neurodisability score	3.7 (1–7)	3.4 (1–7)	0.93
MRI findings			
Presence of mass effect	4/21 (19%)	3/30 (10%)	0.42
Presence of sulci effacement	1/21 (5%)	0/30 (0%)	0.41
Presence of transtentorial or foramen magnum herniation	1/21 (5%)	0/30 (0%)	0.41
CSF TNCC (cells/μl)	530 (6–2,271)	293 (6–1,933)	0.15
CSF total protein (mg/dl)	124 (20–348)	89 (18–371)	0.12

CA+, cytosine arabinoside treatment group; CA–, non-cytosine arabinoside treatment group; TNCC, total nucleated cell count.
Results were considered statistically significant for a p-value of < 0.05.

TABLE 2 | Treatment parameters for dogs in the cytosine arabinoside and non-cytosine arabinoside treatment groups.

	CA+	CA–	*p*-value
Cytosar 200 mg/m^2			
Constant rate infusion	12/21 (57%)		
Subcutaneous 4 doses over 48 h	9/21 (43%)		
Initial prednisone dose (mg/kg/day)	1.3 (1.0–1.5)	1.1 (1.0–1.5)	0.13
Cyclosporine dose (mg/kg/day)	7.8 (5–11)	7.4 (5–10)	0.28

CA+, cytosine arabinoside treatment group; CA–, non-cytosine arabinoside treatment group.
Results were considered statistically significant for a p-value of < 0.05.

Outcomes

After Bonferroni correction, no statistically significant differences were identified between treatment groups for success, relapse, or death at any time point (**Table 3**). The median time to first relapse was 109 days (mean 284 days, range 89–864 days) for the CA+ group and 92 days (mean 181 days, range 24–732 days) for the CA- group ($p = 0.35$). Overall median survival times could not be calculated for the treatment groups as 16/21 dogs (76%) from the CA+ group and 24/30 dogs (80%) from the CA– group were alive or lost to follow-up at the time of writing; however, the median survival of those dogs had to exceed the follow-up time of 1,095 days. A Kaplan-Meier survival curve was generated for 5/21 dogs in the CA+ and 10/30 dogs in the CA- group that were followed until their death. For these deceased dogs, the median survival for the CA+ group was 555 days (range 57–3,018 days) and the median survival for the CA- group was 344 days (range 122–2,796 days) ($p = 0.84$) (**Figure 1**). For all dogs with a successful outcome (at any time point), neurodisability scores improved to <2 with residual

TABLE 3 | Success, relapse, and death rates for dogs in the cytosine arabinoside and non-cytosine arabinoside treatment groups.

	CA+	CA–	*p*-value
1 month			
Success	19/21 (90%)	27/30 (90%)	0.95
Relapse	0/21 (0%)	3/30 (10%)	0.26
Death	2/21 (10%)	0/30 (0%)	0.16
3 months			
Success	16/21 (76%)	22/30 (73%)	0.82
Relapse	3/21 (14%)	8/30 (27%)	0.29
Death	2/21 (10%)	0/30 (0%)	0.16
6 months			
Success	15/21 (71%)	18/30 (60%)	0.40
Relapse	4/21 (19%)	10/30 (33%)	0.26
Death	2/21 (10%)	2/30 (7%)	1
9 months			
Success	15/21 (71%)	14/30 (47%)	0.08
Relapse	4/21 (19%)	11/30 (37%)	0.23
Death	2/21 (10%)	5/30 (16%)	0.68
12 months			
Success	15/21 (71%)	10/30 (33%)	0.007
Relapse	4/21 (19%)	14/30 (47%)	0.04
Death	2/21 (10%)	6/30 (20%)	0.44
18 months			
Success	13/21 (62%)	10/30 (33%)	0.04
Relapse	5/21 (24%)	14/30 (47%)	0.10
Death	3/21 (14%)	6/30 (20%)	0.72
36 months			
Success	12/21 (57%)	9/30 (30%)	0.05
Relapse	6/21 (29%)	15/30 (50%)	0.13
Death	3/21 (14%)	6/30 (20%)	0.72

CA+, cytosine arabinoside treatment group; CA–, non-cytosine arabinoside treatment group.
Bonferroni correction was applied to account for multiple independent analysis. Results were considered statistically significant for a p-value of < 0.0024.

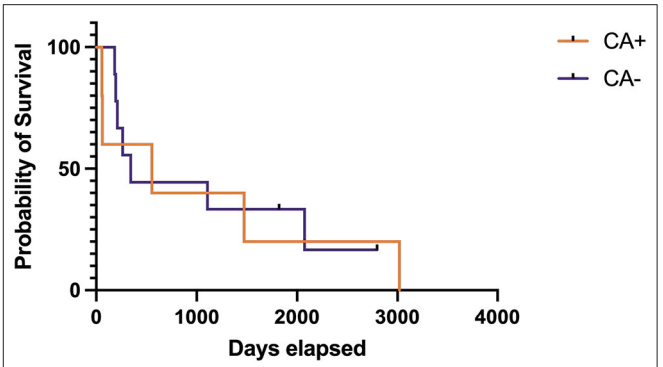

FIGURE 1 | Kaplan-Meier survival care of deceased dogs with meningoencephalomyelitis of unknown origin treated with cyclosporine and prednisone with (CA+) or without (CA–) an initial dose of cytarabine (CA). Deaths not directly related to MUO or treatment of MUO have been censored and are noted by a vertical tick mark.

deficits including one seizure less than every 2 months, head tilt, blindness, persistent lack of physiological nystagmus, and an ambulatory gait with mild weakness and / or ataxia.

DISCUSSION

We retrospectively evaluated supplemental use of CA as a single treatment at the time of diagnosis in dogs being treated for MUO with cyclosporine and prednisone. Although relapse and death rates were lower for the CA+ group at all time points, no statistically significant changes in outcome were identified. As such, the results of this study do not support addition of CA to a treatment protocol with cyclosporine and prednisone.

There are several study limitations that must be considered when interpreting these results. First, this is a retrospective study, which may have led to falsely elevated survival times. Patients were only included if they were started on a treatment regimen with cyclosporine and prednisone, which likely excluded some dogs that died prior to institution of oral medications. Additionally, although statistical significance was not reached at any time point in our study, investigation with larger case numbers might better elucidate differences in the treatment groups. Bonferroni correction was used to account for multiple simultaneous analyses. Although this conservative correction helps limit type I error, it can also lead to higher rates of false negatives. Other limitations include the fact that histological confirmation of diagnosis was only available in a minority of cases. Also, in the majority of dogs, diagnosis of relapse was based on recurrence of clinical signs and response to changes in therapy. Since numerous neurological conditions respond to treatment with prednisone, it is possible that some dogs with suspected relapse had a separate condition.

The findings in this study differ from other reports where majority of death occurs within the first 3 months after diagnosis (7, 9, 10). There were a total of nine deaths in this study but only two of these occurred before 3 months. The lower death rate early in the study might be accounted for by the previously mentioned limitation that cases were excluded if they died before administration of oral medications. But this does not account for the higher number of deaths after 3 months than previously reported. Looking more closely at the cases that died after 3 months, the majority were euthanized associated with a severe relapse, often after tapering of prednisone to a dosage of <0.4 mg/kg/day. It should also be considered that cases included here received a lower initial prednisone dosage (maximum 1.5 mg/kg/day) than many other studies that administer 2.0 mg/kg/day. This justification for this lower dosage is anecdotal; the authors have noted fewer prednisone side effects and owner complaints with no appreciable difference in outcomes. Unfortunately, it is difficult to compare outcomes between manuscripts so a dedicated study to evaluate the effects of these lower prednisone dosages is required.

Another point to consider is how CA was administered in our study. Administration of CA as a CRI instead of subcutaneously at the time of diagnosis has been shown to improve 3-month

survival times (7). Patients in our study received CA either as a CRI or subcutaneously, but statistical analysis to identify differences in these groups was not possible due to small case numbers. It is unclear if outcomes would have been improved if all dogs in this study had received a CRI instead of subcutaneous injections, but this should be considered when planning future studies.

For future studies, it is probably important to better qualify the impact of relapses on patient and owner quality of life. Multiple relapses were not addressed in this study but it must be acknowledged that not all relapses have the same impact. Many relapses are mild and respond to a minor medication adjustment like increasing the prednisone dose, while others are more severe or occur repeatedly and cause emotional and financial strain for owners.

In summary, we failed to identify improved outcomes in dogs with MUO that received a supplemental treatment of CA at the time of diagnosis in addition to treatment with cyclosporine and prednisone. Because there was a trend toward improved outcomes in the CA+ group that did not reach statistical significance, there may be benefit to a prospective, randomized, double-blinded treatment trial that could investigate similar treatment protocols in a larger number of cases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RB was responsible for study design and data collection. RB and LD were responsible for manuscript preparation. All authors approved the submitted version of the manuscript.

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Biopsy Characteristics, Subtypes, and Prognostic Features in 107 Cases of Feline Presumed Immune-Mediated Polyneuropathy

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Inflammatory polyradiculoneuropathy (IMPN) is one of the causes of sudden onset of neuromuscular signs such as para-/tetraparesis in young cats. Even though most cases have a favorable outcome, persistent deficits, relapses, and progressive courses are occasionally seen. As clinical presentation does not always appear to predict outcome and risk of recurrence, this study was initiated to screen for prognostic biopsy findings in a large cohort of histologically confirmed IMPN cases with clinical follow-up. In total, nerve and muscle specimens of 107 cats with biopsy diagnosis of presumed autoreactive inflammatory polyneuropathy and 22 control cases were reviewed by two blinded raters for a set of 36 histological parameters. To identify patterns and subtypes of IMPN, hierarchical k-means clustering of 33 histologic variables was performed. Then, the impact of histological parameters on IMPN outcome was evaluated *via* an univariate analysis to identify variables for the final multivariate model. The data on immediate outcome and follow-up were collected from submitting neurologists using a purpose-designed questionnaire. Hierarchical k-means clustering sorted the tissues into 4 main categories: cluster 1 (44/129) represents a purely inflammatory IMPN picture, whereas cluster 2 (47/129) was accompanied by demyelinating features and cluster

3 (16/129) by Wallerian degeneration. Cluster 4 (22/129) reflects normal tissues from non-neuropathic control cats. Returned questionnaires provided detailed information on outcome in 63 animals. They were categorized into recovered and non-recovered. Thereby, fiber-invasive infiltrates by mononuclear cells and mild fiber loss in intramuscular nerve branches correlated with higher probabilities of recovery. Remyelination in semithin sections, on the other hand, is correlated with a less favorable outcome. Animals grouping in cluster 1 had a tendency to a higher probability of recovery compared to other clusters. In conclusion, diagnosis of feline IMPN from nerve and muscle biopsies allowed for the identification of histologic features that were positively or negatively correlated with outcome.

Keywords: feline, neuromuscular, histology, histopathology, tetraparesis, CIDP, GBS, outcome

INTRODUCTION

Acquired polyneuropathies in cats are described as either of metabolic, toxic, paraneoplastic, immune-mediated, or idiopathic origin (1–14). Idiopathic, presumably immune-mediated, inflammatory polyneuropathy (IMPN) is sparsely reported in the current literature (1–3, 8, 15, 16), whereas in a previous study, we showed that 59% of all feline nerve biopsies submitted for diagnostic evaluation at a referral center were compatible with IMPN (6). Young animals of under 2 years of age are typically affected and present with sudden onset of neuromuscular signs, progressive course, and multiple episodes of relapses (1, 6, 15, 17, 18). Some studies suggest a breed predilection in Bengal cats (15), and a hereditary component has been proposed in Siberian cats (15, 16). Clinically, feline patients commonly present with progressive neuromuscular weakness, para- or tetraparesis, and show reduced to absent spinal reflexes (1–3, 6, 8, 16, 18). Involvement of cranial nerves is reported in a subset of patients (3, 6–8). Presumptive diagnosis of IMPN is based on the clinical presentation and electrodiagnostic evaluation, whereas histopathological evaluation of nerve and muscle specimens is required for confirmation and providing prognostic insights about the outcome (4, 18–20).

The limited literature describing the histopathologic changes in IMPN in cats reports most striking features of inflammation in nerve roots, especially in the ventral root (2), but dorsal root involvement and ganglioneuritis are also reported (7, 8) illustrating a heterogeneous motor and sensory neuropathy. Common histopathological findings in the peripheral nerves of IMPN-affected cats comprise mononuclear inflammatory infiltrates with or without signs of demyelination and remyelinating features (2, 6, 8, 15). Subsequently, muscle biopsies are affected by muscle atrophy of denervation type, and intramuscular nerve branches (IMNB) can display signs of inflammation or nerve fiber loss (2, 15). The outcome is variable ranging from full recovery, to partial recovery, and more rarely to fatal cases (1–3, 7, 8, 15, 18). The largest cohort study of Bengal cats by Bensfield et al. (15) reports a complete or partial recovery in 29 of 33 cats, and a fatal outcome in 4 cats during the first episode, followed by relapse in 12 feline patients with subsequent euthanasia in 2 further animals. Studies on large cohorts of feline patients with IMPN comparing histopathological changes and clinical outcome are currently lacking.

Immune-mediated polyneuropathies are presumed to be the veterinary correlate to the human Guillain–Barré syndrome (GBS) (3, 6, 7), the most frequent cause of acute paralytic neuropathy in people (21). Whereas the diagnosis in human medicine is entirely based on clinical, serologic, and electrodiagnostic evaluation (21), histopathological investigation of muscle and nerve biopsies is usually performed on companion animals (22). As IMPN in cats is a spontaneously occurring disease, much insight into the underlying immunobiology and prognostic indicators can be gained by the evaluation of nerve biopsies in this animal model.

The objective of this study was to perform a systematic histopathological investigation of nerve and muscle biopsies obtained from cats diagnosed with inflammatory neuropathy of presumably immune-mediated origin, and to correlate these findings with the clinical outcome. The following hypotheses were postulated: (1) IMPN can be subcategorized into histologic subtypes; (2) these subtypes correspond to diverse clinical courses and/or disease stage; (3) prognostic indicators correlating with the outcome can be identified from analysis of nerve and muscle biopsies.

MATERIALS AND METHODS

Case Selection

Nerve and muscle biopsies of cats submitted for diagnostic purposes to the Section of Clinical and Comparative Neuropathology, Institute for Veterinary Pathology, Center for Clinical Veterinary Medicine, Ludwig-Maximilians-Universitaet Muenchen, Munich, Germany from 2011 to 2019 were retrospectively reviewed. Inclusion criteria for case eligibility comprised: (1) submission of at least one nerve and/or one muscle biopsy; (2) histologic diagnosis of inflammatory, presumed immune-mediated, neuropathy, based on the changes detected on peripheral nerve biopsies and/or terminal intramuscular nerve branches on muscle biopsies. Age-matched animals submitted to the necropsy service for reasons unrelated to this study and without a history of neurologic disease represented the control group. These latter underwent the same

standard neuromuscular biopsy protocol for commonly biopsied sites including common peroneal or sciatic nerve, and cranial tibial muscle. Infectious agents were excluded by the referring neurologists according to the standard protocols for diagnostic workup of neuromuscular patients and to financial restrictions imposed by the owner.

Tissue Processing

Surgical biopsies were sent overnight either as fresh and adequately cooled or formalin-fixed samples to the neuromuscular laboratory. After submission, epineurial and mesoneurial tissue was removed from the nerve samples. In case of unfixed tissue, nerves were fixed in 2.5% glutaraldehyde for 1 to 2 h depending on the thickness of the submitted material. Thereafter, nerves were rinsed and immersed in modified Sørensen phosphate buffer (pH 7.4) until further processing. One part of the sample was divided into four segments, contrasted to 1% buffered osmium tetroxide after Caufield for 1.5 to 2 h depending on thickness, and embedded in epoxy resin (Glycidether 100, Serva®, Heidelberg, Germany) after dehydration in an ascending alcohol series. Semithin sections (0.5 μm) were cut transversally as well as longitudinally and stained with toluidine blue and safranin O. The other part of the nerve sample underwent also osmium contrast enhancement with 2% buffered osmium tetroxide after Caufield for 1.5–2 h for nerve fiber teasing preparation. At least 30 fibers per nerve were prepared on each glass slide for detailed microscopic longitudinal analysis. In case, there was no formalin-fixed muscle specimen available, a part of fresh muscle was fixed in 10% neutral-buffered formalin and subjected to paraffin-embedding in longitudinal and transverse orientation, and slides were stained with hematoxylin–eosin (H&E), as well as Giemsa stain according to the standard protocols. Remaining fresh muscle samples were snap frozen in isopentane, cooled in liquid nitrogen (−130 to −150°C), and stored at −80°C until processing. Transverse cryosections (10 μm) were stained by the standard protocols including H&E, Engel's modified Gomori trichrome, periodic acid Schiff, oil-red O, cytochrome oxidase, and nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase histochemistry, and fiber typing through immunolabeling of myosin heavy chains.

Morphological Evaluation

Histological evaluation was performed by two blinded veterinarians experienced in neuropathology using light microscopy (Zeiss Axiophot®, Zeiss Instruments, Oberkochen, Germany). Cryosections and paraffin sections of skeletal muscle and nerve fiber teasing and semithin sections of peripheral nerves were investigated.

General algorithms for diagnostic evaluation of muscle and nerve biopsies were applied and subsequently implemented by a tailored grading scheme for nerve inflammation including 36 histological variables. Briefly, the scheme focused on topographic distribution and degree of severity of inflammatory infiltrates, axonal changes, Schwann cell pathology, nerve fiber degeneration, and regenerative features. Where applicable, semiquantitative scores were applied by grading each variable as summarized in **Table 1**. The dominant feature of each case was summarized and assigned to one of the following categories: infiltrative, degenerative, or mixed. Further details on the histomorphological criteria can be found in **Table 1**. Based on the nerve fiber teasing, the IMPN subtype after Gross et al. (6) was also classified (summarized in **Table 2**).

Clinical Questionnaire

An online questionnaire was sent to submitting veterinary neurologists to gather information about patient's history, onset of clinical signs, and course of the disease, neurological examination, results of the electrophysiological examination, imaging findings including MRI and CT, laboratory workup, and outcome. The survey (https://forms.office.com/Pages/ResponsePage.aspx?id=DQSIkWdsW0yxEjajBLZtrQAAAAAAAAAAAAN__iDTpglUQVVXTzNMNlc1UlpFUlo4UEdQOTNOVzRWMy4u) was performed using Microsoft Forms (Microsoft, Redmond, Washington, USA) in accordance with the General Data Protection Regulation, and with permission of the data protection officer of the Ludwig-Maximilians-Universitaet Muenchen, Munich, Germany. Participating clinicians were specialized in the field of veterinary neurology comprising board certified neurologists [European College of Veterinary Neurology and/or American College of Veterinary Internal Medicine (Neurology)] or an equal national qualification.

Statistical Analysis

Hierarchical k-means clustering of 33 histologic variables was performed to identify similar histological patterns. Missing values were occasionally detected across the 33 variables and needed to be imputed, since cluster analysis requires complete dataset. Imputation was conducted *via* the missRanger approach, a non-parametric multivariate imputation by chained random forest algorithm with 1,000 trees (23). This method combines random forest imputation (24, 25) with predictive mean matching (26) and thus iterates multiple times until the average out-of-bag prediction error of the models stops to improve. After clustering, cluster features were analyzed to identify salient histological appearances. Individual animals were manually corrected, if the overall histological pattern was more suitable to a different cluster with confirmation of validity using Cohen's Kappa analysis. The impact of histological parameters on the outcome (recovery) and premedication was evaluated *via* univariate Bayesian generalized linear models to identify variables for the final multivariate model. Any variable having a $p < 0.2$ during the univariate test was selected as a candidate for the multivariate analysis (27). A stepwise variable selection among those variables leads to the final combination of predictors. All analyses were done by the R Statistical software (version 4.0.3).

RESULTS

Signalment and Biopsy Site

Nerve and muscle biopsies of 107 affected cats were included in the study. Investigated cases originated from various countries in Europe including: Germany ($n = 50$), United Kingdom ($n = 25$),

TABLE 1 | Morphologic criteria applied for histological examination.

	Material and histological processing		
	Nerve fiber teasing	**Semithin sections of nerve**	**Muscle (FFPE and cryosections)**
Inflammatory features	Site of inflammatory infiltrates • Nodal • Paranodal • Nodo-paranodal • Schmidt-Lanterman cleft-directed • Diffuse • Diffuse predominantly nodo-paranodal Overall extensivity of fiber-directed inflammatory infiltrates • Score 0 (not present) • Score 1 (mild) • Score 2 (moderate) • Score 3 (severe) Degree of fiber-directed inflammatory cells • Score 0 (not present) • Score 1 (mild) • Score 2 (moderate) • Score 3 (severe)	Interstitial inflammatory infiltrates Fiber-directed inflammatory infiltrates Vasculitis	Inflammatory infiltrates in intramuscular nerve branches • Score 0 (not present) • Score 1 (mild) • Score 2 (severe)
Changes of the myelin sheath	Myelin sheath pathology Site of demyelination: • Nodal • Paranodal • Internodal • Inter-/paranodal • Segmental • Nodal-segmental • Paranodal-segmental • Mixed	Percentage of nerve fiber bundle affected by de-/remyelination: • ≤12,5% of nerve fiber bundle • up to 25% of nerve fiber bundle • 50–75% of nerve fiber bundle • ≥75% of nerve fiber bundle Degree of de-/remyelination • Score 0 (not present) • Score 1 (mild) • Score 2 (moderate) • Score 3 (marked) Onion bulb formation Schwann-cell pathology and/or hypertrophy	
Axonal features	Stage of Wallerian Degeneration: • 1 • 2 • 3 • 4 Distribution of Wallerian Degeneration • Continuous • Discontinuous Temporary stadium of Wallerian Degeneration: • Synchronous • Asynchronous	Degree of nerve fiber loss • Score 0 (not present) • Score 1 (mild) • Score 2 (moderate) • Score 3 (severe) Type of fibers affected by fiber loss: • Small fibers • Large fibers • Mixed Wallerian degeneration Post-resorptive macrophages Changes in axonal diameter • Score 0 (not present) • Score 1 (mild) • Score 2 (moderate) • Score 3 (severe)	Degree of nerve fiber loss in intramuscular nerve branches • Score 0 (not present) • Score 1 (mild) • Score 2 (moderate) • Score 3 (severe)
Regenerative features		Remyelination Regenerative clusters	Degree of muscle atrophy • Score 0 (not present) • Score 1 (mild) • Score 2 (mild to moderate) • Score 3 (moderate) • Score 4 (severe) Stadium of muscle atrophy • Score 0 (not present) • Score 1 (non-reactive muscle atrophy) • Score 2 (mild reactive changes) • Score 3 (fibrosis) Muscle fiber hypertrophy • Score 0 (not present) • Score 1 (mild) • Score 2 (moderate) • Score 3 (severe)

TABLE 2 | IMPN subtypes according to Gross et al. (6).

IMPN subtype	Histological features
1	Cells are attached or enter the Schmidt-Lanterman clefts.
2A	Early invasive IMPN subtype 2 with marked demyelination, dysmorphic paranodes, paranodal retraction, and a only few cells located at the node of Ranvier.
2B	Infiltrative IMPN subtype 2 with cell-clusters in the area of the node of Ranvier.
3	Cells show an overall distribution along nerve fibers.
4	Mixed subtype with nodo-paranodal predominance.

Italy ($n = 12$), France ($n = 11$), Switzerland ($n = 8$), and Estonia ($n = 1$). Age-matched control animals were solely submitted by veterinarians or private owners in Germany ($n = 22$).

The age of affected cats ranged from 3 months to 10.4 years with a median age of 0.9 years. About 30.8% of the cats (33/107 cats) were domestic shorthair cats (DSH), whereas 64.5% (69/107 cats) were purebred cats, 2.8% (3/107 cats) were mixed breeds, and for two cats' breed information was not available. Among purebred cats, British shorthair cats were overrepresented with 16.8% (18/107 cats) together with 12.1% of Bengal cats (13/107 cats), followed by 8.4% Maine Coon cats (9/107 cats), 4.7% of Persian cats (5/107 cats), and 3.7% of Siamese cats (4/107 cats). Other affected breed with 3 or less individuals comprised Siberian cat ($n = 3$), Birman cat ($n = 3$), Ragdoll ($n = 2$), Devon Rex ($n = 2$), Abyssinian cat ($n = 2$), and one of each Russian Blue, Turkish Angora, Savannah cat, Munchkin cat, Chartreux, Scottish Fold, Norwegian Forest cat, and a Thai cat. Gender distribution of the affected cats was 43 intact males (40.2%), 30 male neutered (28.0%), 26 intact females (24.3%), and 8 female spayed (7.5%).

Control samples were collected from 22 cats at 1.3 months to 11 years of age (median 4.3 years) consisting of 13/22 DSH, 6/22 purebred cats, and 3/22 mixed breed cats with 5 males, 7 male neutered, 7 females, and 3 female spayed cats.

Nerve biopsies were available in all of the cases and were collected from the hindlimb in 99 animals, from the forelimb in 3 animals, and from both in one cat. In 4 animals, the localization of nerve biopsy was not indicated. Muscle specimens were available in 105/107 cats and gained exclusively from the hindlimbs in 64 feline patients, from the forelimb in one animal, and from both in 34 animals. In 6 animals, information about localization of muscle biopsy was not provided.

Histologic Features

Inflammatory Features Within the Nerves

The diagnosis of IMPN was based on the evidence of nerve fiber adhesive and/or invasive inflammatory infiltrates within the endoneurium, directed at the axons, nodes of Ranvier, and/or Schwann cells. Biopsies of cats without signs of inflammation in the intramuscular nerve branches and/or peripheral nerve biopsies were excluded from the study. IMPN was diagnosed in 105/107 animals based on findings in the main nerve trunk, and in 2/107 based on intramuscular nerve fiber branches. All animals showed inflammatory cells seen either in nerve fiber teasing (96/104; 92.3%), semithin preparations of the nerve (100/105 cats; 95.2%), or in the intramuscular nerve branches (69/79; 87.3%). Reduced numbers of animals result either from lacking of intramuscular nerve branches within muscle sections (26/105), and from lacking of either semithin sections or nerve fiber teasing preparations in case of limited nerve material submitted for diagnostic purposes. Only a subset of cases (38/105; 36.2%) showed fiber-invasive infiltrates in semithin sections, whereas interstitial inflammatory cells could be found in 87.3% (69/79, see above) of feline patients.

IMPN Subtypes According to Gross et al. (6)

The majority of cases with 42.1% (45/107 cats) presented with IMPN subtype 3 with diffuse inflammatory cell distribution. About 25.2% (27/107 cats) matched subtype 1 with focus of inflammation to the Schmidt-Lanterman clefts, and 16.8% (18/107 cats) were compatible with mixed subtype 4 with nodo-paranodal predominance. Only a small proportion with 5.6% (6/107 cats) fitted the criteria for the nodo-paranodal subtype 2, especially 1 animal with subtype 2a and 5 cats with subtype 2b. In 5 cats, inflammatory features presented only in semithin sections or in intramuscular nerve branches, and therefore, the subtype could not be determined. In 4 animals, IMPN type could not be defined as teasing preparations were not available due to low yield of nerve fibers from submitted material. In 2 patients, the subtype remained unclear.

Muscle Atrophy

Muscle samples were available for 105/107 cats. Of these, 86.7% (91/105) showed changes compatible with denervation atrophy. About 5.7% (6/105) of the cases had a mixed atrophy, and 7.6% (8/105) could not be further classified.

Cluster Analysis

Hierarchical k-means clustering sorted results of histologic scoring into 4 main clusters: cluster (1) (44/107) purely inflammatory IMPN; cluster (2) (47/129) IMPN accompanied by demyelinating features; cluster (3) (16/129) IMPN with signs of Wallerian degeneration; and cluster (4) (22/129) normal tissues from control animals. The cluster analysis dendrogram is shown in **Figure 1**.

Cluster 1: Purely Inflammatory IMPN

Cluster 1 comprised 41.1% (44/107) of the cats. All cases showed lesions characteristic for IMPN as shown in **Figure 2**. Semithin sections of 93.2% (41/44) of the cats showed interstitial inflammatory cells, of these 24.4% (10/41) displayed additionally also fiber-directed infiltrates. Nerve fiber teasing preparations were available for 42/44 animals and showed inflammatory cell infiltration in 88.2% (37/42) of the cases. Semiquantitative measurement of the overall extensivity of inflammatory infiltrates in teasing preparations was mild in 45.2% (19/42), moderate in 16.7% (7/42), and severe in 26.2% (11/42) of the cases. Degree of inflammatory cell infiltrates per single fiber was graded mild in 54.8% (23/42), moderate in 21.4% (9/42), and severe in 11.9%

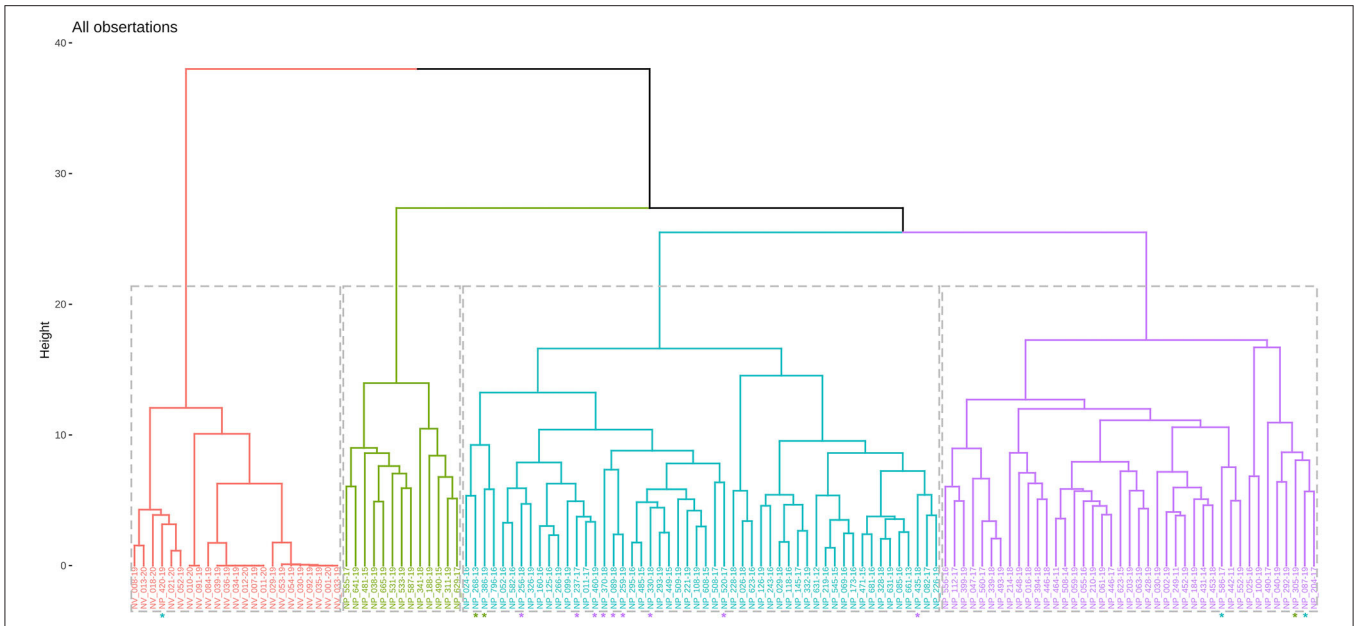

FIGURE 1 | Dendrogram of hierarchical cluster analysis. Violet tree resembles cluster 1, turquoise tree cluster 2, green cluster 3, and orange cluster 4. Manual correction of single animals are shown with an asterisk (*) in the color of the final cluster.

(5/42) of the cats. A total of 3 of 4 cases of vasculitis observed in semithin sections were present in Cluster 1.

Affected breeds comprised 34.1% (15/44) DSH, 13.6% (6/44) Bengal cats, 11.4% (5/44) BSH, 9.1% (4/44) Maine coon cats, 6.8% (3/44) Persian cats, 4.5% (2/44) Siamese cats, one of each Siberian cat, Birman cat, Ragdoll, Devon Rex, Abyssinian cat, Scottish Fold, Thai cat, and also one mixed breed cat. In 2 cats, the breed was not known. There was no significant relationship [$x^2_{Pearson}(9) = 10.34$, $p = 0.32$] between different breeds and specific clusters. Median age of the affected cats was 0.8 years (range 0.3–10.1 years). Gender distribution showed 54.5% (24/44) male or male neutered animals and 45.5% (20/44) female or female spayed cats.

Cluster 2: IMPN Accompanied by De-/remyelination

About 43.9% (47/107) of affected cats grouped in cluster 2. The hallmark of this cluster was the detection of de-/remyelination additionally to inflammatory infiltrates as shown in **Figure 2**. Demyelinating changes were seen in nerve fiber teasing preparations (33/46; 71.7%), and/or semithin sections of the nerve (32/45; 71.1%). The reduced number of animals in both preparation types results from lacking of teasing preparations in one cat or semithin preparations in 2 cats due to limited size of submitted nerve material. Demyelination could be localized in 33 cats *via* teasing preparation as paranodal (8/33; 24.2%), internodal (2/33; 6.1%), paranodal and internodal (1/33; 3.0%), segmental (17/33; 51.5%), nodal-segmental (1/33; 3.0%), paranodal-segmental (2/33; 6.1%), and mixed (2/33; 6.1%). The overall degree of de-/remyelination of individual nerve fibers could be semiquantitatively measured as mild in 62.5% (20/32) and moderate in 37.5% (12/32) of the cases with de-/remyelination present in semithin sections of the nerve. Remyelination was identified on semithin sections in 62.5% (20/32) of the cases. Extensiveness of de-/remyelinated fibers could be determined *via* semithin sections in 95.6% (29/32) of which 69.0% (20/29) with $\leq$ 12,5% of nerve fiber bundle affected, 31.0% (9/29) up to 25% of de-/remyelinated nerve fiber bundle, and 3.4% (1/29) of the cats with $\geq$75% of affected fibers. Onion bulb formation observed in semithin sections was present in 22.2% (10/45) of the cats. The remaining forth case of vasculitis was present in this cluster 2.

Breeds in this group comprised 27.7% (13/47) DSH, 21.3% (10/47) BSH, 12.8% (6/47) Bengal cats, 6.4% (3/47) Maine coon cats, 6.4% (3/47), Birman cat, 4.3% (2/47) Persian cats, 4.3% (2/47) mixed breed cats, one of each Siamese cat, Abyssinian cat, Russian blue, Turkish Angora, Savannah cat, Munchkin cat, Chartreux, and Norwegian Forest cat. Median age of cats in cluster 2 was 1 year (range 0.3–10.3 years). About 74.5% (35/47) of cats in cluster 2 were male or male neutered animals and 25.5% (12/47) female or female spayed.

Cluster 3: IMPN Accompanied by Wallerian Degeneration

About 15.0% (16/107) of the patients grouped in cluster 3. Wallerian degeneration was the unique feature in this cluster besides inflammatory infiltration shown in **Figure 2**. Wallerian degeneration was identified in nerve fiber teasing preparations in all but one animal (15/16; 93.8%) and in 37.5% (6/16) of the cases within semithin nerve sections. Stage of Wallerian degeneration could be determined based on nerve fiber teasing as follows: 1 in 6.7% (1/15), 2 in 13.3% (2/15), 3 in 26.7% (4/15), and 4 in 53.3% (8/15) of the affected cats. Along single teased nerve fibers, changes were considered as continuous in 80.0% (12/15) and discontinuous in 40.0% (6/15) of the cases. Temporal relationship of Wallerian degeneration among affected nerve fibers could be

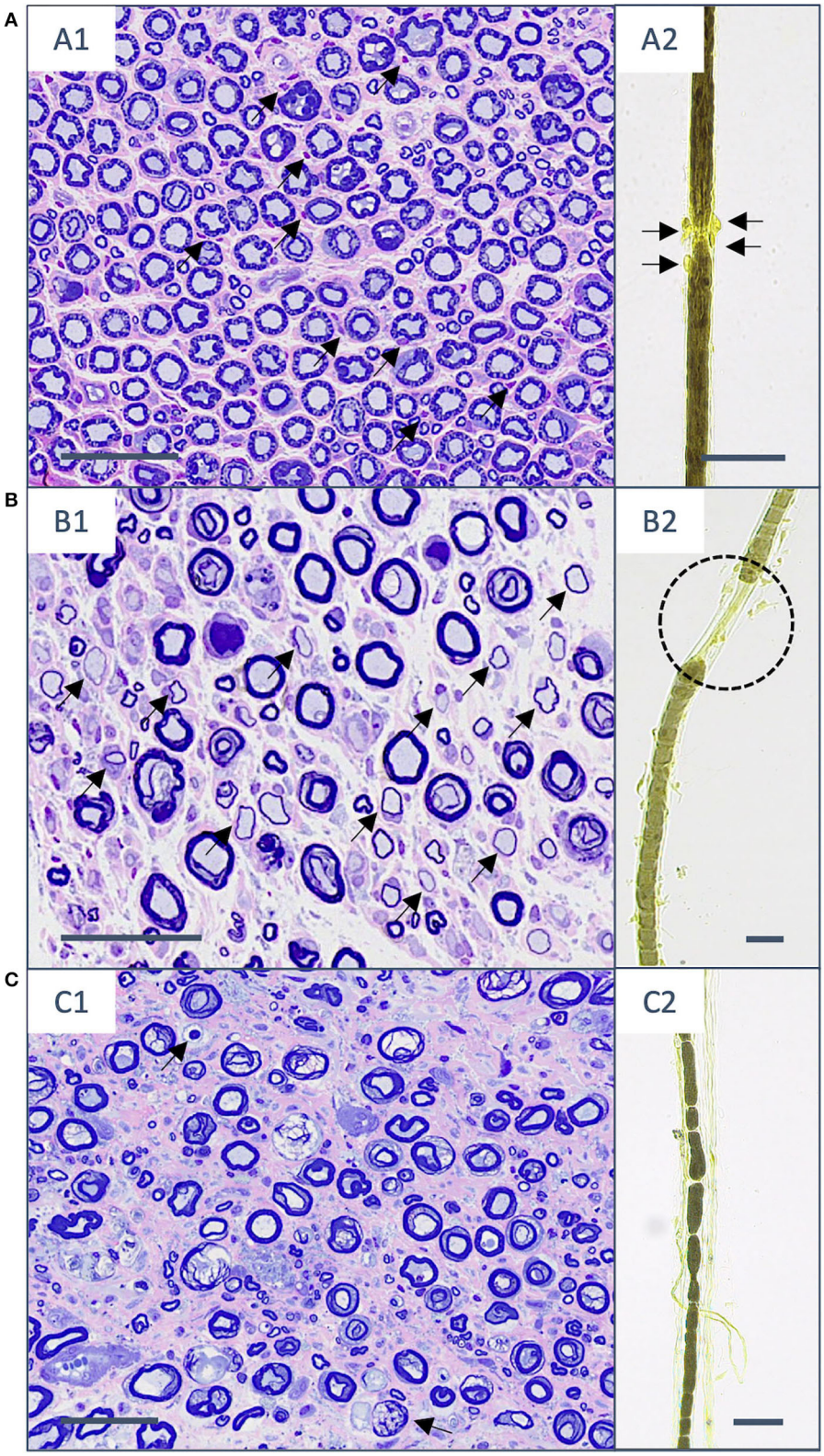

FIGURE 2 | Histopathological changes in semithin sections (stained with toluidine blue and safranin O) and nerve fiber teasing preparations (contrasted with Osmium tetroxide) in the three different clusters. **(A)** Cluster 1. **(A1)** Transverse section of fibular nerve with interstitial and fiber-adhesive mononuclear infiltrates (arrows). **(A2)**

(Continued)

FIGURE 2 | The same cat shows mononuclear cell infiltrates directed to the nodo-paranodal area (arrows). **(B)** Cluster 2. **(B1)** The sciatic nerve with a moderately reduced number of myelinated fibers and multiple hypomyelinated fibers (arrows) on top of mononuclear interstitial and fiber-invasive infiltrates. **(B2)** Teasing preparations of the same animal shows paranodal retraction as a sign of demyelination (black dotted line). **(C)** Cluster 3. **(C1)** The peroneal nerve with markedly reduced number of myelinated fibers, endoneurial fibrosis, and signs of Wallerian degeneration (arrows). **(C2)** The fibular nerve of another cat with sings of stage II Wallerian degeneration. Scale bar: 50 μm.

determined in 12 animals and defined as synchronous in (11.7%; 11/12) and asynchronous (8.3%; 1/12).

In cluster 3, 31.3% (5/16) were DSH, 18.8% (3/16) BSH, 12.5% (2/16) Maine coon cats, 12.5% (2/16) Siberian cats, and one of each Bengal cat, Siamese cat, Ragdoll, and Devon Rex. The median age of cluster 3 animals was 0.7 years (range 0.3–10.4). Male or male neutered cats in cluster 3 comprised 87.5% (14/16) and 12.5% (2/16) female cats.

Online Questionnaire

A total of 73 survey answers were obtained, 3 were considered incomplete for further analysis, and 70 were valid surveys. Data on outcome were available in 63 animals, and information about therapies prior to biopsies collection was indicated in 70 survey responses. Outcome was classified into 2 categories, namely, recovered and not recovered. Recovery was defined as the state in which the cat could walk without assistance and could jump onto objects. Results of patient history, onset of clinical signs, course of the disease, neurological examination, results of the electrophysiological examination, imaging findings including MRI and CT, laboratory workup, and outcome are described by van Renen et al. (28).

Prognostic Histologic Parameters

A univariate model with 31 of the histological parameters revealed 9 variables as significantly related to recovery of the animal (**Table 3**), which were then used for the final multivariate model. In the final model, remyelination in semithin sections was most significant related to recovery ($p = 0.006$), followed by fiber-invasive infiltrates in semithin sections ($p = 0.022$), and intramuscular nerve fiber loss ($p = 0.045$). The presence of remyelination was correlated with a lower probability of recovery compared to the absence of remyelinating features. When animals presented with fiber-invasive infiltrates, the probability of recovery was higher compared to cats where these infiltrates were mainly endoneurial. Fiber loss in intramuscular nerve branches observed in muscle sections was inhomogeneously correlated with probability of recovery. When fiber loss was moderate or absent, the probability of a favorable outcome was lower compared to mild fiber loss. Probabilities of recovery are shown in **Figure 3**.

TABLE 3 | Significance of histological parameters in the univariate model.

Histological phenomenon	*P*-value
Paraffin sections of the muscle	
Nerve fiber loss in intramuscular nerve branches	0.035
Semithin sections of the nerve	
Remyelination	0.044
Wallerian degeneration	0.076
Fiber-invasive infiltrates	0.105
Degree of de-/remyelination	0.130
Regenerative clusters	0.166
Nerve fiber teasing preparations	
Extensivity of inflammatory infiltrates	0.068
Degree of inflammatory infiltrates observed on single teased nerve fibers	0.103
Demyelination	0.198

Histopathology Compared to Clinical Data

Therapies Prior to Biopsy Collection

About 62.9% of the cats (44/70) received medication prior to biopsy collection. Evaluation of the impact of medications on distribution and magnitude of inflammatory infiltrates on nerve biopsies did not reveal significant differences between pretreated and untreated patients. Medications included corticosteroids (all $p > 0.395$), non-steroidal anti-inflammatory drugs (NSAIDs) (all $p > 0.601$), and L-carnitine (all $p > 0.123$).

Outcome

The probability to recover among the three clusters did not differ significantly, but showed a tendency toward a higher rate of recovery in cluster 1, compared to cluster 2 ($p = 0.188$), and 3 ($p = 0.201$) as shown in **Figure 4**. A correlation between the course of the disease (chronic: clinical signs lasting longer than 1 month; acute: clinical signs lasting less than 1 month) to the three different clusters could not be identified.

DISCUSSION

To date, this is the largest retrospective study on histopathological findings and clinical features of presumed IMPN in cats. Hierarchical cluster analysis defined three groups of IMPN with different histologic phenotypes: cluster 1 purely inflammatory, cluster 2 IMPN with additional demyelination, and cluster 3 where IMPN was accompanied by Wallerian degeneration. Salient histologic features with impact on patients' outcome were also identified through a multivariate analysis combining clinical and histologic data. Premedication with corticosteroids, NSAIDs, and L-carnitine had no significant impact on the magnitude of inflammatory features observed by neuropathologists on peripheral nerve biopsies.

In this cohort, 30.8% of cats were DSH and 64.5% purebreds with BSH being the most common breed accompanied by various purebreds not reported to be affected by IMPN. Previous studies described inflammatory/demyelinating/idiopathic polyneuropathy mainly in purebred cats (1, 3, 6, 15–17, 19),

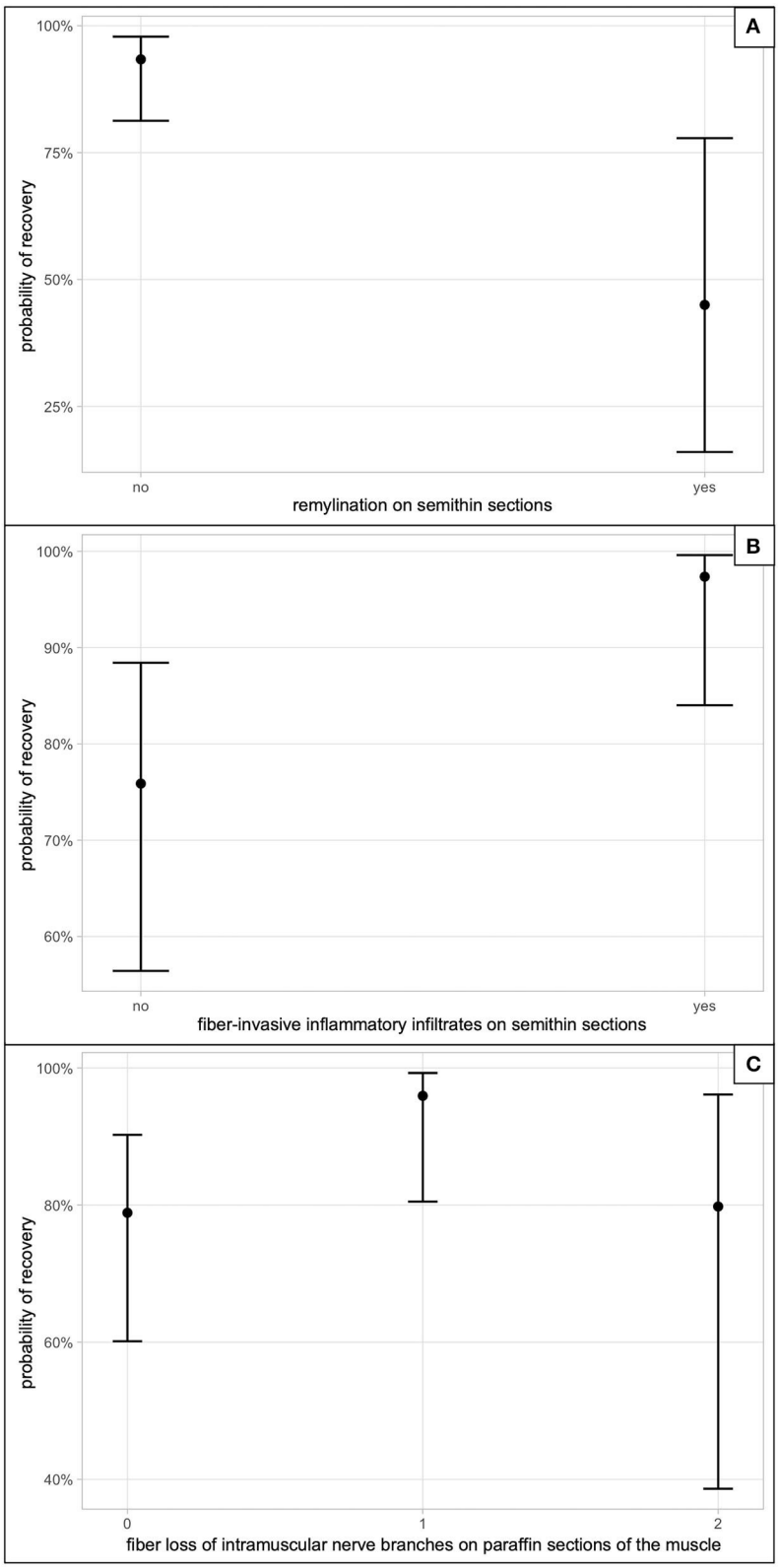

FIGURE 3 | Probability of recovery in the multivariate model. **(A)** The impact on recovery, if remyelination on semithin sections was observed. **(B)** The correlation between presence of fiber-invasive inflammatory infiltrates on semithin sections and recovery. **(C)** Correlates the semiquantitative fiber loss score of intramuscular nerve branches seen on paraffin sections of the muscle in degree; 0, absent; 1, mild; 2, moderate; 3, severe ($n = 0$).

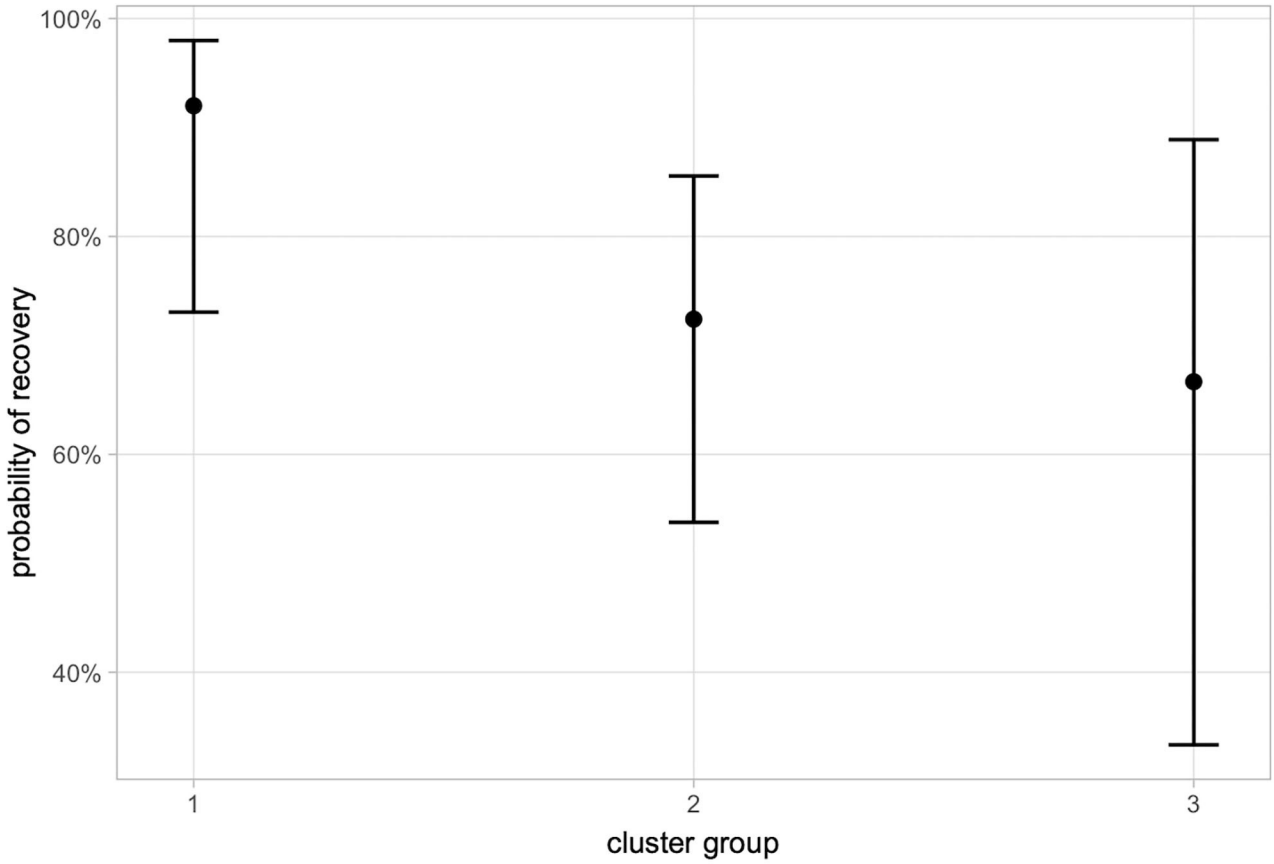

FIGURE 4 | Probability of recovery in different cluster groups. 1, Purely inflammatory IMPN; 2, IMPN accompanied by de-/remyelination; 3, IMPN accompanied by Wallerian degeneration.

leading to the hypothesis of a hereditary neuropathy and/or a genetic predisposition in Siberian and Bengal cats (15, 16). Based on our findings, an inherited breed predisposition appears less likely as the number of breeds increased considerably with the present observations compared to previous descriptions, and biopsies were submitted from several referral centers distributed in 6 European countries. Breeds were distributed among all three different clusters with DSH as the most common. In a case series of Gerritsen et al. (3), DSH was also the most reported breed with 5/9 cases. Histopathology in DSH in other studies comprised also inflammatory infiltration, demyelination, and axonal degeneration with fragmentation in peripheral nerves (3, 8). Also, Bengal cats were found in all clusters, which fits to the previous study by Bensfield et al. where inflammation, demyelination, and axonal degeneration were described. In a Siberian cat, histologic evaluation of Crawford et al. (16) revealed a mononuclear cell infiltration within IMNB and the peripheral nerve. In our study, Siberian cats were present in clusters 1 and 3, but not in the de-/remyelinating cluster 2. In a case report of Jeandel et al., an Abyssinian cat showed loss of myelinated fibers and inflammatory infiltrates in the peripheral nerve. The two Abyssinian cats in our study were found in clusters 1 and 2, but not in cluster 3. Statistical analysis of a possible association of specific breed dispositions to the three different clusters revealed no correlation. Due to low numbers of animals in many breeds, further studies are needed to further exclude breed dispositions for specific histological subtypes.

As observed in previous investigations (1–3, 6, 8, 15, 17), IMPN is a disease mainly manifesting in young patients with 0.9 years as median age of onset in this study. However, 15/107 cats were older than 4 years in addition to what was described in the literature (6, 7, 18), we suggest to consider IMPN among differential diagnosis of neuromuscular signs even in adult cats. Gender distribution showed an overall predominance of male or male neutered animals (68.2%). This is in parallel with the biggest cohort study of Bensfield et al. (15) in Bengal cats, where 65% were male, and also Gross et al. (6) found 11/15 cats to be male or male neutered. Gender distribution in case series or case reports varies (1–3, 7, 8, 16, 17). Interestingly, gender distribution was close to equal in cluster 1, whereas in clusters 2 and 3, the gender gap was markedly skewed toward male sex. Similar findings are also recorded for human immune-mediated polyneuropathies such as Guillain–Barré syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP), where men are about 1.5 times more frequently

affected compared to women (21). The reason for this gender predisposition remains unclear.

Diagnosis of IMPN was achieved from the evaluation of biopsies of the main peripheral nerve trunk and/or terminal nerve branches from muscle biopsies. Distribution of the lesions in inflammatory neuropathies can vary and show proximal (radiculitis), main nerve trunk (neuritis), and distal (terminal neuritis) changes (2, 6–8, 15–17). Multifocal lesions can severely affect one nerve fascicle and spare those neighboring as seen in a study of Aleman et al. (19) and further complicate diagnostic interpretation of peripheral nerve biopsies. Diagnosis of IMPN was obtained from main nerve trunk in 105/107, terminal nerve branches in 2/107, and from both sites in 67/79 animals. As in 87.3% of the investigated muscle biopsies intramuscular nerve branches were affected, muscle biopsy can help in diagnostic settings in case of multifocal distribution of the lesions. We conclude that a combination of nerve and muscle biopsies provides the highest yield of diagnostic samples in case of IMPN. Influence of medical treatments prior to biopsies collection and the yield of diagnostic features on submitted neuromuscular samples was also evaluated. Patients are often referred to veterinary neurologists with some delays from the onset of clinical signs, and empirical treatments are already established by the time of referral. In our population, corticosteroids, NSAIDs, and L-carnitine were administered prior to biopsy in 62.9% of the cases, but there was no significant impact on the diagnostic yield of inflammatory features, and a histologic diagnosis of IMPN could still be made. However, due to the retrospective nature of this study, more sound conclusions on the effects of prior therapies and their dosages (anti-inflammatory vs. immunosuppressive) could not be drawn.

Systematic evaluation of nerve biopsies identified changes comparable to those previously described including endoneurial mononuclear cell infiltration, demyelination, and axonal damage as indicated by Wallerian degeneration of nerve fibers (2, 3, 8, 15–17). Apart from unanimous identification of inflammatory infiltrates in peripheral nerves, in this cohort, as well as in past descriptions, there are some variabilities regarding distribution of inflammation along nerve fibers and damage of the Schwann cells and axons. According to Gross et al. (6), IMPN in cats and dogs can be subclassified based on nerve fiber teasing analysis of inflammatory cell distribution into 4 subtypes. In that study, the most common subtype in a cohort of 15 cats was subtype 4 mixed predominantly nodo-paranodal, followed by subtype 3 mixed cell distribution, subtype 1 Schmidt-Lanterman clefts distribution, whereas subtype 2 purely nodo-paranodal types were only present in one cat (6). Application of that scheme in this investigation revealed subtype 3 as the most common, followed by 1, 4, and 2 possibly reflecting a homogeneous density of antigenic triggering proteins along nerve fiber.

Hierarchical cluster analysis, applied in our study, sorted cases into three clusters based on histopathologic similarities. About 43.9% of affected cats grouped in cluster 2 making demyelination and Schwann cell pathology one of the main features of this condition besides inflammation. Demyelination is frequently mentioned also in previous reports (3, 6, 8, 17), and Bensfield et al. (15) report this feature in 11/17 Bengal cats, where peripheral nerves were available and pathologically affected. Wallerian degeneration characterizing cluster 3 was detected in 15% of our cases, and similarly, it has been rarely described in previous studies (3, 6, 7, 15). Cluster 1 with 41.1% of cats showed inflammatory changes in peripheral nerves only.

Histologic changes in our study are comparable to the human diseases GBS and CIDP, where inflammatory infiltrates in peripheral nerves and nerve roots, demyelination, and signs indicative of Wallerian degeneration can be found (29). Perivascular inflammatory infiltration can also be seen as additional features in few cases (29). Diagnosis of CIDP and GBS is based on clinical findings including muscle weakness, paresthesia, and reduced or absent tendon reflexes, electrophysiological examination, and CSF showing elevated protein content (30–32). In contrast to GBS, which has an acute onset, CIDP evolves over a course of more than 8 weeks (30).

At present, there is no clear reason and/or rational for patients grouping into one cluster instead of the other. We hypothesize that the pathomechanisms involved might be different, and that epitopes differ in topography along the axon-Schwann cell unit. As seen in human CIDP and GBS, multiple forms are described and characterized by clinical, serologic, and electrodiagnostic findings (30–33). Further studies are needed to elucidate and further characterize our observations.

Association of these clusters with signalment and course of the disease could not be identified. Though not statistically significant, recovery showed minor differences among clusters as cluster 1 had a tendency to a better outcome (**Figure 4**). Demyelination, nerve fiber loss, and Wallerian degeneration represent structural damage of the axon-Schwann cell unit requiring prolonged timespan to regenerate, and if regeneration is unsuccessful, residual deficits persist. In this vein, the absence of remyelination in semithin sections of the nerves was significantly correlated with a higher probability of recovery ($p = 0.006$). Remyelination [histologically seen as hypomyelinated nerve fibers (34)] follows segmental loss of Schwann cells and requires different signaling pathways, transcriptional regulators, and activation of epigenetic mechanisms (35, 36).

Mild fiber loss in intramuscular nerve branches was also associated with recovery ($p = 0.045$). Distally accentuated IMPN might have their target epitopes on the terminal portion of peripheral nerves close to the end organ. In this scenario, re-establishment of connectivity with the skeletal muscle benefit from (1) integrity of the perineurial sheaths, (2) less time required to fill a short gap, and (3) stimulation by trophic factors produced by the effector organs (37, 38). In line with these findings, fiber-invasive infiltrates in semithin sections were positively correlated with recovery ($p = 0.022$), and this might partly reflect the tendency to higher recovery seen in cluster 1.

In summary, our study shows three different clusters of IMPN based on histologic evaluation of nerve and muscle biopsies, characterized by inflammatory, demyelinating, and axonal changes. Age, gender distribution, and predominance of demyelinating features parallel findings of juvenile forms of CIDP.

Even when medication was already started before biopsies collection, diagnostic features of IMPN were still retained. Purely

inflammatory changes are associated with a good prognosis, whereas chronic remodeling of the myelin sheath, as seen with remyelination, was negatively correlated with recovery.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethikkommission der Tierärztlichen Fakultät Ludwig-Maximilians-Universität München. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MR and NK designed, coordinated the study, and wrote the manuscript. NK, MR, and KM provided the data. AF, FW, MR, and KM designed the questionnaire. All authors read and approved the final manuscript.

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Differential T-cell responses in dogs with meningoencephalomyelitis of unknown origin compared to healthy controls

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Meningoencephalomyelitis of unknown origin (MUO) is a common disorder in dogs that results in mononuclear inflammation in the brain and/or spinal cord. MUO is presumed to be autoimmune but specific immunological aberrations have not been identified. This exploratory study aimed to evaluate T cell production of two cytokines commonly implicated in autoimmune disease, interferon-gamma (IFNg) and interleukin-17 (IL17). Peripheral blood mononuclear cells were obtained from 12 dogs with MUO and 10 healthy controls, stimulated to activate intracellular signaling pathways, and stained with a cluster of differentiation 4 (CD4), cluster of differentiation eight (CD8), IFNg, and IL17 antibodies prior to analysis by flow cytometry. Mean differences in absolute cell numbers are represented as MUO cases minus healthy controls, and 95% Cis are reported. Overall IFNg-producing lymphocytes (mean difference = 241.8 cells/ul, 95% CI = 65.6 to 418.1) and CD4+ IFNg-producing T-cells (mean difference = 188.4, 95% CI = 77.3 to 299.5) were fewer in MUO cases. Additionally, CD4+ IL17-producing T-cells were greater in MUO cases (mean difference −34.9, 95% CI = −50.54 to −19.17) and CD8+ IL17-producing T-cells were fewer in MUO cases (mean difference = 73.5, 95% CI = 6.8 to 140.1). These results support that immunological changes can be identified in peripheral blood cells of dogs with MUO and suggest that T-helper type 17 (Th17) cells may play a role in pathogenesis.

KEYWORDS

meningoencephalomyelitis of unknown origin (MUO), T-helper cell, interferon-gamma, interleukin 17, flow cytometry

Introduction

Meningoencephalomyelitis of unknown origin (MUO) is a presumptive autoimmune disease affecting the central nervous system (CNS) of dogs. It is overrepresented in young to middle-aged toy and small breed dogs and after histological evaluation of tissue can be divided into three variants: granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME), and

necrotizing leukoencephalitis (NLE). Affected dogs have progressive neurological deficits and unpredictable, often temporary responses to standard immunosuppressive therapy. The etiopathogenesis of MUO is poorly understood, which has precluded the development of accurate antemortem diagnostic tests and consistently effective treatments.

Evaluation of brain tissue from MUO-affected dogs revealed elevated interferon-gamma (IFNg) and interleukin-17 (IL17) mRNA levels and protein (1), suggesting Th1 and Th17 responses may contribute to inflammation in cases of MUO. Th1 and Th17 responses are known to be important in many autoimmune diseases. In people, Th1 and Th17 have been shown to play a key role in the pathogenesis of numerous autoimmune diseases, including ankylosing spondylitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and inflammatory bowel disease (2–5). Th17 responses have also been implicated in several canine diseases, including immune-mediated hemolytic anemia and steroid-responsive meningitis arteritis (6, 7).

This exploratory study was designed to evaluate T-cell cytokine production of IFNg and IL17 in the peripheral blood of dogs with MUO compared to healthy controls. Specifically, the authors expected to identify more clusters of differentiation 4 (CD4)+ IFNg+ and CD4+ IL17+ T-cells in MUO cases than controls.

Materials and methods

Case identification

This prospective study was approved by the University of Georgia Clinical Research Committee and performed with the informed consent of the owners. From 2018 to 2022, 12 dogs with MUO and 10 healthy dogs were enrolled in the study. Diagnostic inclusion criteria for MUO were based on a previous study with minor modifications (8): (i) evaluation by a neurology resident or board-certified neurologist; (ii) patient age 6 months to 12 years; (iii) focal or multifocal neuroanatomical lesion localization; (iv) focal or multifocal T2-weighted hyperintensities on magnetic resonance imaging consistent with inflammation; (v) cerebrospinal fluid (CSF) pleocytosis (> five total nucleated cells per microliter with <4,000 red blood cells per microliter) with >50% mononuclear cells; and (vi) where possible, exclusion of infectious diseases by negative serology for *Toxoplasma gondii*, *Neospora caninum*, and *Cryptococcus spp*. If serology for infectious diseases was not obtained, patients had to have a successful outcome (no relapse or death) for a minimum of 12 months while receiving immunosuppressive therapy or confirmation of GME, NME, or NLE by histopathology. Patients were excluded if they had received immunomodulatory therapy within the 12 weeks preceding sample acquisition. Where possible, necropsy results were recorded, but were infrequently available because the majority of cases were alive at the time of writing this manuscript.

Flow cytometry

Cell isolation and flow cytometry were performed as previously described (9–13). Specifically, peripheral blood mononuclear cells (PBMCs) were isolated from sodium-heparinized peripheral blood using density gradient centrifugation with Ficoll-Hypaque (Histopaque®-1077, Sigma-Aldrich). PBMCs at the interface were collected and washed two times with phosphate-buffered saline (PBS). Cells were adjusted to a concentration of 1 x 10^6 cells/ml and suspended in RPMI 1,640 with GlutaMAX™ and HEPES (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific). Cells were stimulated *ex vivo* for 4 h at 37° with and without 25 ng/ml Phorbol 12-Myristate 13-Acetate (PMA, Sigma Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of a 1:1,000 dilution of GolgiPlug™ Protein Transport Inhibitor (containing Brefeldin A) (Thermo Fischer Scientific) (14). Cells were then washed in serum-free PBS medium containing GolgiPlug and stained with the LIVE DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen). Cells were washed with Flow Staining Buffer (eBioscience) containing GolgiPlug and stained with anti-canine CD4-Pac Blue (clone YKIX302.9, Bio-Rad) and anti-canine CD8-AF700 (clone YCATE55.9, Bio-Rad) antibodies prior to being fixed and permeabilized (Foxp3 Transcription Factor Staining Buffer Set, eBiosience). Anti-bovine IFNg-RPE (clone CC302, Bio-Rad), and anti-human IL17/IL-17A (R&D Systems) were used for intracellular staining. The anti-IL17 antibody was conjugated using the Zenon™ Alexa Fluor™ 647 Goat IgG Labeling Kit (Thermo Fisher Scientific) per the manufacturer's instructions. Samples were analyzed on an LSR-II (BD Bioscience) or a Quanteon (Agilent) flow cytometer analyzer. Analysis gates were set on lymphocytes according to forward and side scatter properties with a stopping gate of 30,000 single, viable CD4+, and/or CD8+ cells. Flow cytometry (FC) data were analyzed using FACSDiva (BD Biosciences) or NovoExpress (Agilent) software. Minimum thresholds of positivity were determined by employing fluorescence-minus-one controls and, for the IFNg and IL17, verified with unstimulated (PMA and ionomycin negative) biologic controls. Relative percent FC data were translated into absolute cell counts as a product of percent IFNg or IL17 of Th or Tc, percent T-cell subset of lymphocyte gate, and absolute lymphocyte cell count data from a current clinical CBC test (<24 h prior).

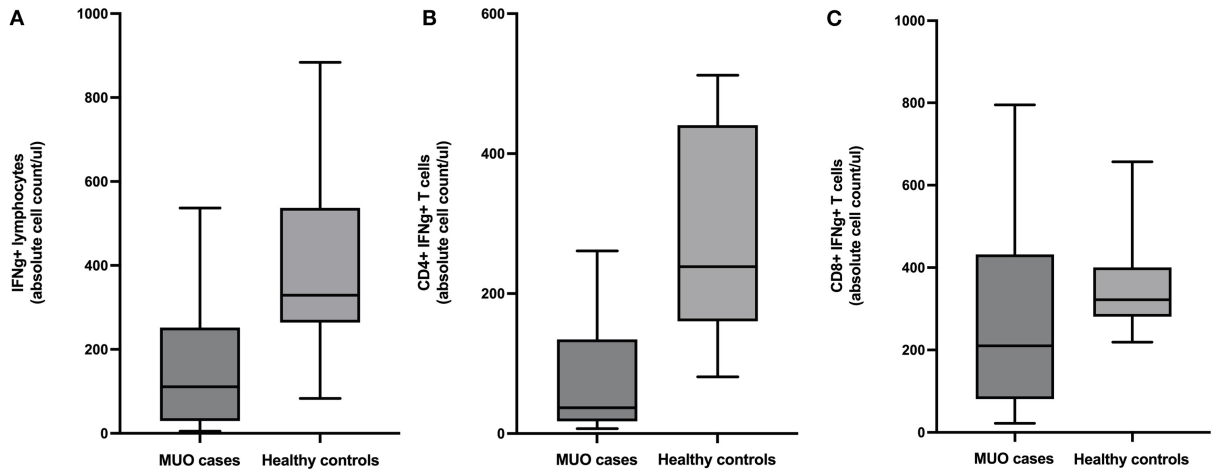

FIGURE 1
Box and whiskers plots showing absolute cell counts of **(A)** IFNg+ lymphocytes, **(B)** CD4+ IFNg+ T-cells, and **(C)** CD8+ IFNg+ T-cells in cases of meningoencephalomyelitis of unknown origin (MUO) versus healthy controls.

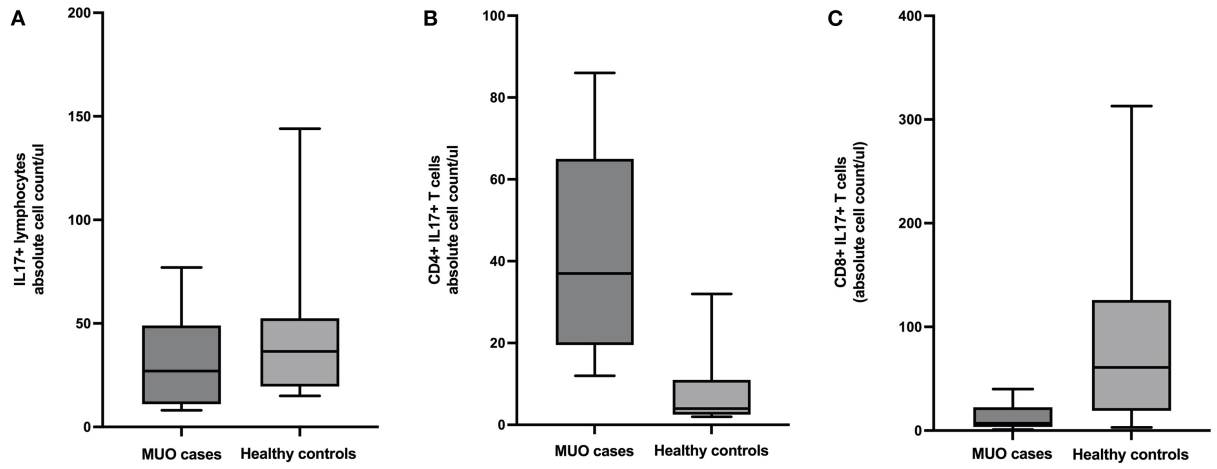

FIGURE 2
Box and whiskers plots showing absolute cell counts of **(A)** IL17+ lymphocytes, **(B)** CD4+ IL17+ T-cells, and **(C)** CD8+ IL17+ T-cells in cases of meningoencephalomyelitis of unknown origin (MUO) versus healthy controls.

Statistical analysis

Absolute cell numbers for the following categories were analyzed between MUO cases and healthy controls by the unpaired *t*-test with the Welch's correction using the GraphPad Prism version 9: IFN-g+ lymphocytes, CD4+ IFNg+ T-cells, CD8+ IFNg+ T-cells, IL17+ lymphocytes, CD4+ IL17+ T-cells, and CD8+ IL17+ T-cells. Q–Q plots of the residuals were examined to confirm the assumption of approximate normality. There was non-homogeneous variance with the diseased group having higher variability than the healthy control group generally, so the Welch's *t*-tests were used. Mean differences in absolute cell numbers are represented as MUO cases minus healthy controls. Ninety-five percentage of CIs are reported.

Results

Case information

A total of 12 MUO cases were evaluated, including three Maltese, three mixed breeds, and one each of Pomeranian, Boston terrier, Jack Russell terrier, Shih Tzu, Chihuahua, and Yorkshire terrier.

The mean age was 6.9 years (range 2–12 years). Among the animals, nine were spayed females and four were neutered males. Necropsy results were available for one case with a diagnosis of GME.

A total of 10 healthy controls were evaluated, including three mixed breeds, two Maltese, two poodles, two Yorkshire terriers, and one Chihuahua. The mean age was 6.6 years (range 2–12 years). Among the animals, six were spayed females and four were neutered males.

Flow cytometry

To identify CD4+ and CD8+ T-cells producing IFNg and IL17, we performed a flow cytometric analysis of PBMCs stimulated with PMA and ionomycin (Supplementary Figure S1).

There were fewer lymphocytes producing IFNg in MUO cases than healthy controls (mean difference = 241.8, 95% CI = 65.6 to 418.1, $p = 0.01$). Additional analysis of lymphocyte subpopulations revealed there were fewer CD4+ IFNg+ T-cells in MUO cases compared to healthy controls (mean difference =188.4, 95% CI = 77.3 to 299.5, $p = 0.003$) and fewer CD8+ IFNg+ T-cells in MUO cases compared to healthy controls (mean difference = 91.9, 95% CI = −73.8 to 257.6, $p = 0.26$) (Figure 1).

There were fewer lymphocytes producing IL17 in MUO cases compared to controls (mean difference =13.78, 95% CI = −15.3 to 42.9, $p = 0.33$). Additional analysis of lymphocyte subpopulations revealed there were more CD4+ IL17+ T-cells in MUO cases compared to healthy controls (mean difference = −34.9, 95% CI = −50.54 to −19.17, $p = 0.0002$) and fewer CD8+ IL17+ T-cells in MUO cases vs. healthy controls (mean difference =73.5, 95% CI = 6.8 to 140.1, $p = 0.03$) (Figure 2).

Discussion

Analysis of lymphocyte populations in MUO cases vs. healthy controls identified differences in IFNg- and IL17-producing cells. There were fewer IFNg-producing lymphocytes, specifically characterized by fewer CD4+ IFNg+ T-cells, in MUO compared to controls. Additionally, there were more CD4+ IL17-producing T-cells but fewer CD8+ IL17-producing T-cells in MUO compared to controls. The results of this exploratory study support that immunological differences can be identified in the peripheral blood of dogs with MUO and yield some insight into pathogenesis, suggesting IL17 and Th17 responses may play a role in disease pathogenesis.

There are only a small number of studies looking at specific components of the immune system in dogs with MUO. Several groups have used immunohistochemistry to look at B and T-cells in GME lesions with differing results as to the predominant cell type (15–18). Other groups have looked at various cytokines and chemokines in brain tissue and CSF (19). Specifically, evaluation of mRNA and protein expression levels in brain tissue from a small number of cases revealed elevated IFNg in NME and elevated IL17 in GME (1). To the author's knowledge, no studies looking at peripheral components of the immune system in MUO have been published. Although it is hard to compare the evaluation of brain tissue with peripheral blood, the findings presented here are consistent with the identification of elevated IL17 in brain tissue of GME. However, in that study, IL17 was more commonly associated with macrophages than T-cells (1) and in the study presented here, histological evaluation to determine the MUO subtype could not be performed for all cases.

There are several limitations to this study. This was an exploratory study so only small numbers of cases and controls were utilized, which can increase the margin for error and, and additionally, precluded correlation of results with clinical findings. Where possible, controls were age and breed matched but this was not always possible. Also, the diagnosis was not confirmed by histopathology in the majority of dogs.

Ultimately, this exploratory study suggests that immunological differences can be identified in peripheral blood from dogs with MUO and supports that IL17-producing T-cells may play a role in disease pathogenesis. These results should be validated with a larger sample size prior to additional research to determine the biological and clinical significance of these changes. Ultimately, components of Th17 responses may provide useful biomarkers and could inform the development of targeted immunotherapies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by Clinical Research Committee at the University of Georgia College of Veterinary Medicine. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

RB contributed to study design, case identification, sample acquisition and processing, and manuscript preparation. JB contributed to study design, sample processing, data analysis,

and manuscript preparation. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor AT, declared a past co-authorship with the author, RB.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.925770/full#supplementary-material

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Case report: Lympho-histiocytic meningoencephalitis with central nervous system vasculitis of unknown origin in three dogs

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Meningoencephalitis of unknown origin (MUO) is an umbrella term for a variety of subtypes of meningoencephalitis of dogs and cats with no identifiable infectious agent. In dogs, granulomatous meningoencephalitis (GME), necrotizing meningoencephalitis (NME), and necrotizing leukoencephalitis (NLE) are the most commonly reported subtypes. However, sporadically there are reports about other subtypes such as greyhound encephalitis or eosinophilic meningoencephalitis. The following case series presents three dogs with peracute to acute progressive signs of encephalopathy. The magnetic resonance imaging (MRI) of two dogs (*post mortem* $n = 1/2$) showed severe, diffuse swelling of the cortical gray matter with increased signal intensity in T2weighted (w) and fluid-attenuated inversion recovery (FLAIR) and decreased signal intensity in T1w. Additionally, focal to multifocal areas with signal void in both dogs and caudal transforaminal herniation of the cerebellum in one dog was observed. *Post mortem* histopathological examination revealed lympho-histiocytic encephalitis and central nervous system (CNS) vasculitis in all dogs. No infectious agents were detectable by histopathology (hematoxylin and eosin stain), periodic acid-Schiff reaction (PAS), Ziehl-Neelsen stain and immunohistochemistry for Canine adenovirus-1, Parvovirus, *Listeria monocytogenes*, Parainfluenzavirus, *Toxoplasma gondii*, Herpes-suis virus, Pan-Morbillivirus, Tick born encephalitis virus, Severe acute respiratory syndrome coronavirus (SARS-CoV) 2. Furthermore, two dogs were tested negative for rabies virus. To the best of the authors' knowledge, this is the first report of a lympho-histiocytic encephalitis with CNS vasculitis with no identifiable infectious agent. It is suggested to consider this as an additional subtype of MUO with severe clinical signs.

KEYWORDS

meningoencephalitis of unknown origin (MUO), central nervous system (CNS), sterile, canine (dog), inflammation, brain

Introduction

Meningoencephalitis of unknown origin (MUO) is an umbrella term for a variety of subtypes of meningoencephalitis of dogs where no infectious agent can be identified (1–6). Granulomatous meningoencephalitis (GME), necrotizing meningoencephalitis (NME), and necrotizing leukoencephalitis (NLE) of dogs are the most commonly reported histopathological subtypes (3, 7–10). Other less commonly reported subtypes of MUO include eosinophilic meningoencephalitis, and greyhound encephalitis (11–13). The specific etiopathology of MUO is unknown so far, but a multifactorial pathology is suspected, involving an underlying - suspected mostly genetic - immunological defect (8, 14) and possible environmental triggers, for example an infectious or toxic agent (1–6). MUO is typically treated with anti-inflammatory or immunosuppressive drugs with varying prognosis depending on the subtype of MUO (4). A diagnosis is usually based on clinical signs, diagnostic imaging findings, cerebrospinal fluid examination, and exclusion of possible infectious agents. (1–4, 6, 8). However, a definitive diagnosis and especially the determination of the MUO subtype requires histopathologic examination (15). The known MUO subtypes present with distinct histopathological features. GME is characterized by an angiocentric, lymphocytic and granulomatous inflammation of the CNS (16). Here, inflammatory cell infiltrates are most often found within the white matter (17). In NME and NLE, lesions compromise CNS necrosis as well as lympho-histiocytic, often perivascularly located inflammation (17). In NME, the predilection site is the cerebral cortex, while in NLE, the white matter is primarily affected (17). Idiopathic eosinophilic meningoencephalitis shows necrosis and inflammatory infiltrates consisting of eosinophils and macrophages within the cerebral cortex (12). Greyhound encephalitis is considered a breed-associated MUO, which presents with non-suppurative inflammatory changes mainly found in the frontal lobe and olfactory bulb bilaterally (13). Furthermore, a large number of cases of MUO have not been specified as a distinct subtype (18, 19). None of the mentioned subtypes of MUO is typically accompanied by CNS vasculitis (12, 13, 16, 18, 19).

Vasculitis is defined as an inflammation of blood vessels with inflammatory cells infiltrating the damaged vascular wall as well as the perivascular space (20). Aside from primary vasculitis with no evident cause, secondary vasculitis due to different triggers represents the more common type reported (20, 21). Typical causes of secondary vasculitis include environmental noxae, reaction to different kinds of medication as well as hypersensitivity reactions (18). Vasculitis confined to the CNS is rarely reported (20, 22).

The present case series describes the macroscopic and histopathological findings of dogs that suffered from a so far undescribed meningoencephalitis with vasculitis restricted to the CNS. A causative infectious agent was not detectable.

Materials and methods

All examinations were performed with written informed owner's consent according to ethical guidelines of the University of Veterinary Medicine Hannover, Germany, between 2017 and 2021.

Blood examinations were performed immediately after blood sampling and included blood cell count (ADVIA 120 Hematology System, Siemens Healthcare GmbH, Erlangen, Germany), biochemistry (cobas c 311 analyzer, Roche Deutschland Holding GmbH, Mannheim, Germany), and electrolytes (RAPIDLab 1260, Siemens Healthcare GmbH).

Radiography (Philips Bucky Diagnost, 2001, Hamburg, Germany, and AGFA CR85-X Digitalizer, 2007, Mortsel, Belgium) of the thorax in three planes was performed in case 1.

Magnetic resonance imaging (MRI; 3.0 T MRI scanner Achieva, Philips Medical Systems, Best, The Netherlands) of the brain was obtained under general anesthesia in one dog, and 20 min after euthanasia in another. MRI was not available in the third dog. After premedication with diazepam [0.5 mg/kg intravenously (i.v.)] and levomethadone with fenpipramide [0.2 mg/kg i.v. (L-Polamivet ®, MSD Tiergesundheit, Unterschleißheim, Germany)], anesthesia was inducted with propofol (dose to effect 1-3 mg/kg i.v.) followed by orotracheal intubation and connection to a semiclosed circle absorber system (Anesthesia ventilator, Cato®; Dräger, Germany). Anesthesia was maintained with isoflurane in an oxygen/air mixture (1:1, flow 50 ml/kg/min) in one dog (case 3). MRI was obtained in transversal, sagittal, and dorsal view in T2weighted (w) and T1w sequences pre and post contrast administration (gadoterate meglumine, 0.2mmol/kg i.v.). No contrast medium was applied in case 1. Fluid-attenuated inversion recovery (FLAIR) and gradient echo (GE) images were obtained in transversal plane. Cerebrospinal fluid (CSF) was sampled suboccipitally from the *cisterna magna post mortem* only in case 1 and was immediately examined for cell content *via* Fuchs-Rosenthal-chamber and microscopical cell differentiation. Case 1 and 3 were euthanized by i.v. administration of pentobarbital (100–357 mg/kg, Euthadorm® CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany). A *post mortem* examination of all dogs was performed at the Department of Pathology, University of Veterinary Medicine Hannover, Germany. Following necropsy, specimens of various organs including brain, spinal cord, peripheral nerves, tonsil, lung, spleen, liver, heart, eye, thyroid gland, diaphragm, skeletal musculature,

pituitary gland, adrenal glands, kidneys, urinary bladder, tongue, trachea, stomach, and small and large intestine were collected for further histological, histochemical, and immunohistochemical investigation. Samples were fixed in 10% neutrally buffered formalin for at least 24 h, paraffin wax-embedded, and cut into approximately 2 µm thick sections. Sections were stained with hematoxylin and eosin (HE) and central nervous system (CNS) regions with lesions were evaluated semi-quantitatively by using mild (single inflammatory cells in the perivascular space), moderate (1–3 layers of perivascular inflammatory cells), and severe (>3 layers of inflammatory cells in the perivascular space). Selected sections of CNS with inflammatory changes were further assessed by immunohistochemistry (IHC) and additional special stains including a periodic acid-Schiff reaction (PAS) and Ziehl-Neelsen stain. IHC for the detection of potential causative infectious agents was performed as described previously (18). This included using an anti-canine adenovirus-1 (DV4-1A; Custom Monoclonals International, Chris K. Grant, CA, USA, mouse monoclonal), anti-parvovirus (CPV1-2A1; Custom Monoclonals International, Chris K. Grant, CA, USA, mouse monoclonal), anti-listeria monocytogenes (DIFCO Laboratories, 2,469–563, rabbit polyclonal), anti-parainfluenzavirus (SV5-NP-C, Dr Randall, Department of Biochemistry and Microbiology, University of St Andrews, UK, mouse monoclonal), anti-toxoplasma gondii (Quartett, Cat. No. 201500102, rabbit polyclonal), anti-neospora caninum (Dr. Schares, Institute of Epidemiology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany, monoclonal mouse), anti-herpes-suis (Dr. Eskens, Veterinär-Untersuchungsamt Mittelhessen, Germany, polyclonal mouse), anti-pan-Morbillivirus (D110; kind gift from Prof. Dr. A. Zurbriggen, University of Bern, Switzerland, mouse monoclonal), anti-tick born encephalitis virus (K-D-3.BA; Prof. Holzmann, Department of Virology, University of Vienna, Austria, rabbit polyclonal) antibody. Additionally, IHC for the detection of severe acute respiratory syndrome coronavirus (SARS-CoV) 2 antigen using an anti-SARS CoV 2 nucleoprotein (NP) antibody (Sino Biological, 40143-MM05, mouse monoclonal) was performed on sections of brain and lung of all dogs using the Dako EnVision+ polymer system (Dako Agilent Pathology Solutions) and 3, 3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St.Louis, MO, United States) as described previously (23, 24). Investigation of rabies-virus infection was performed in two dogs (case 2 and 3) at the Lower Saxony State Office for Consumer Protection and Food Safety. Furthermore, a set of immunological markers including anti-CD3 (Agilent Dako, Cat.No. A0452, rabbit polyclonal) for the detection of T-lymphocytes, anti-CD20 (Thermo Fisher Scientific, Cat. No. RB-9013-P, rabbit polyclonal) for the detection of B-lymphocytes as well as anti-CD204 (Abnova Corporation, Cat. No. MAB1710, mouse monoclonal) for the detection of macrophages was applied in IHC as described previously (25). In addition, Luxol Fast Blue (LFB) – Cresyl-Echt-Violet was performed to investigate myelin loss in all dogs.

Cases

Case 1

A 1.25 year old, male-neutered Chihuahua was presented with a four-day history of lethargy, decreased appetite, and tachypnea. He was regularly dewormed and vaccinated. In the general examination, the dog showed mild apathy and generalized high-frequency, low-amplitude tremor. Rectal body temperature was 38.7°C. Breathing pattern was normal, frequency was 32/min with physiological effort. Auscultation was inconclusive due to whole body tremor. Coughing and retching was provoked when palpating the larynx. On cardiac auscultation the heartbeat was regular with 120 beats per minute (bpm), no heart murmur was audible. Femoral pulses bilaterally were strong, regular, and synchronous with heart beat. Capillary refill time was under 2 s, the mucous membranes were pale-pink and moist. Peripheral lymph nodes were non-painful, soft and under 1cm in diameter on palpation. Abdominal palpation revealed no abnormal intraabdominal structures and no signs of pain. Mild serous ocular discharge in both medial canthus was noted without any other ocular abnormalities. Macroscopical evaluation of external ears, nose, and skin were unremarkable. Gait and posture were normal. At this timepoint, a neurological examination was not performed due to the lack of obvious involvement of the nervous system on general examination. Complete blood count, clinical chemistry, serum electrolytes, and abdominal ultrasonography were without clinically relevant abnormalities. The owner declined thoracic radiographs at this time point and decided for further outpatient therapy with non-steroidal anti-inflammatory and antibiotic medication. At home, 12 h after first presentation in the clinic, the dog developed generalized tonic-clonic seizure. The seizure had lasted for more than 2 h before the dog was presented to the emergency service again. The dog showed tonic-clonic movements, impaired consciousness, salivation, and a gurgling laryngeal stridor during in- and expiration. Body temperature was measured at 42°C, the heart rate was 162 bpm, no heart murmur was audible. Femoral pulses were strong bilaterally, regular, and synchronous with the heartbeat. Breathing frequency was 52/min. Capillary refill time was under 2 s, the mucous membranes were pale-pink and moist. The dog had brown-reddish diarrhea. Neurologic examination revealed generalized tonic-clonic seizure while the dog was in lateral recumbency. Menace response was absent in both eyes. The dog showed no facial paralysis and an increased tone of the masticatory muscles. Pupillary light reflex, gagging, strabismus, vestibulooccular reflexes, facial sensation, pain sensation and spinal reflexes were not evaluable due

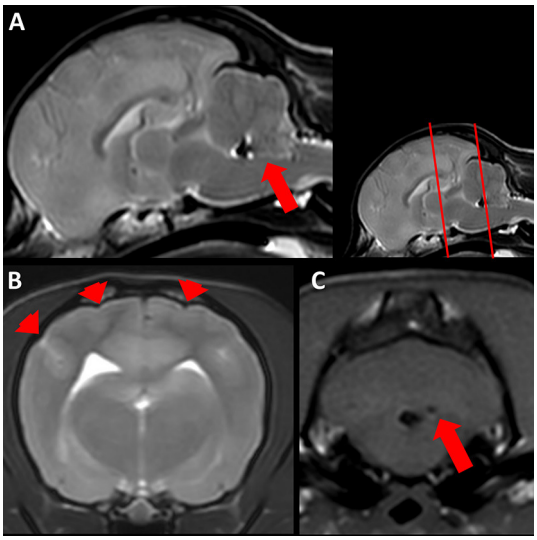

FIGURE 1
Magnetic resonance imaging (MRI) of a 1.25 year old Chihuahua (case 1). **(A)** Sagittal T2 weighted (w) *post mortem* MRI of the brain. Note the mild indentation of the rostral cerebellum due to increased volume of the cerebrum. Hypointense material fills the fourth ventricle (arrow), intraventricular hemorrhage is suspected. **(B)** Transversal T2w MRI of the cerebrum at the level of the caudal part of the hippocampus (level is indicated in the small inlay as the first red line). Note the generalized swelling of gray matter with flattened gyri and sulci (arrowheads). **(C)** Transversal T1w MRI of the cerebellum and brainstem (level is indicated in the small inlay as the second red line). Round, well demarcated intraaxial lesion with signal void (arrow), hemorrhage is suspected.

to ongoing seizure. The dog was stabilized with diazepam (4 × 2 mg/kg i.v., Diazepam Lipuro, B.Braun, Melsung, Germany) and phenobarbital (2 × 2 mg/kg i.v., Luminal, Desitin, Hamburg, Germany) and resuscitative fluid therapy (20 ml/kg/h for 3 × 20 min as bolus infusion, Sterofundin ISO Vetcare, B.Braun, Melsung, Germany) followed by continuous rate infusion (3 ml/kg/h, Sterofundin BG-5, B.Braun, Melsung, Germany). Thoracic radiographs were unremarkable. After cardiovascular stabilization and treatment of seizures leading to their interruption, neurological examination showed coma, bilateral non-responsive miotic pupils, and generalized absent cranial reflexes, while the breathing pattern remained normal, why a severe brainstem lesion with primary forebrain disease was suspected. Due to a grave prognosis, the owners elected for euthanasia.

Post mortem MRI (Figure 1) showed generalized swelling of the gray matter in the cerebrum and cerebellum with secondary flattening of gyri and sulci. Cortical gray matter displayed increased signal in T2w and FLAIR and decreased signal in T1w. The boundary between subcortical white matter and cortical gray matter was mostly blurry in all sequences. Caudal brainstem and cerebellum showed multifocal, intraaxial, small, round lesions with signal void in T2w and T1w. The fourth ventricle and cisterna magna were filled with material causing signal void in T2w and T1w without significant mass effect.

Examination of CSF sampled *post mortem* atlanto-occipitally revealed severely elevated number of erythrocytes without signs of erythrophagocytosis. Leukocyte value was elevated, the exact number was not countable. Protein content measured 188 mg/dl (reference values <25 mg/dl). Cell differentiation showed 91% lymphocytes, 7% monocytes and 2% neutrophils.

At necropsy, a moderate flattening of the gyri and narrowing of sulci of the brain (Figure 2A) was observed in conformity with the MRI results and interpreted as edema. In addition, mild cerebellar vermal herniation into the *foramen occipitale magnum* was visible. The meningeal vessels were moderately and diffusely congested. The dog had hemorrhagic intestinal content and a single nematode was found in the small intestine. Histopathologicallly, the gray matter of the cerebrum as well as the cerebellum showed moderate, multifocal, non-symmetrical, lympho-plasma-histiocytic, necrotizing inflammation. Inflammatory cell infiltrates were mainly found in the perivascular space. Furthermore, primarily small to medium sized blood vessels in the gray matter and especially prominent in the leptomeninx displayed loss of integrity of the vascular wall with moderate to severe infiltration with partially degenerated lymphocytes and macrophages resembling vasculitis of the leukocytoclastic type (Figure 2B). The spinal cord did not show any morphological changes.

IHC of selected sections of the CNS revealed infiltration of a moderate to high number of CD3-positive T-lymphocytes, low to moderate number of CD204-positive macrophages and few CD20-positive B-lymphocytes, respectively, in the partially destructed vascular wall and the perivascular space (Figures 2C–E). Using IHC and histochemistry, no infectious agent or signs of demyelination were detected.

In addition, mild, mucosal hemorrhages and few dilated crypts and mild flattening of villi were observed in the small intestine. Due to the macroscopic and histological lesion in the small intestine, parvovirus infection was ruled out by IHC. The spleen showed moderate lymphoid depletion. In the lung only moderate, diffuse, acute alveolar edema and hyperemia were found. These changes were interpreted as having developed during agony. No signs of parasites, e.g. *Angiostrongylus vasorum*, were found in the lungs, heart, gastrointestinal tract, or CNS via gross and microscopic examination. The remaining investigated tissues lacked significant microscopic lesions.

Case 2

A 10 year old, male-neutered, medium sized, mixed breed dog was presented with a one-week history of progressive neurological signs. Initially, the dog was dull and showed a low

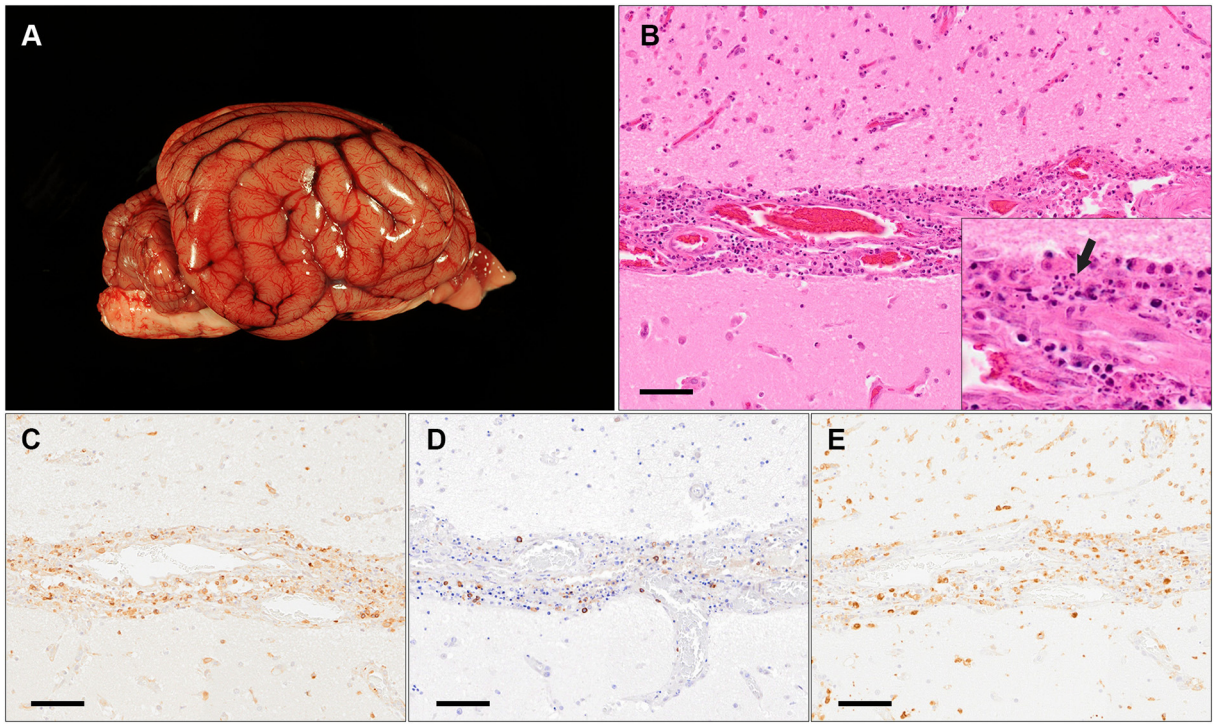

FIGURE 2
Macroscopic and histopathological findings of a 1.25 year old Chihuahua (case 1). **(A)** Macroscopic image of the brain with mild narrowing of sulci and flattening of gyri interpreted as edema. **(B)** Histopathology of the cerebral cortex at the level of the temporal lobe with moderate, leukocytoclastic vasculitis of the leptomeningeal blood vessels with fragments of degenerated inflammatory cells (arrow in insert) visible within the destructed vascular wall (HE stain; scale bar: 50μm). **(C–E)** Immunohistochemistry for CD3 **(C)**, CD20 **(D)** and CD204 **(E)** shows that the majority of infiltrating cells are comprised of T-lymphocytes [**(C)**; CD3-positive] and macrophages [**(E)**; CD204-positive] (Scale bars: 50 μm).

head carriage. Clinical signs progressed to pacing in circles to the right, dysphoria, and suspected right sided visual deficits. There was no travel history. The dog was regularly vaccinated and dewormed.

The dog was presented in lateral recumbency. The dog was stuporous. Rectal body temperature was 38.2°C. Breathing pattern was normal, frequency was 34/min with physiological effort. Auscultation revealed mildly increased vesicular sounds, which were interpreted as still within physiological limits. On cardiac auscultation the heartbeat was regular with 90 bpm, no heart murmur was audible. Femoral pulses were strong, regular, and synchronous with heartbeat. Capillary refill time was under 2 s, the mucous membranes were pale-pink and mildly sticky on palpation. Macroscopical evaluation of eyes, external ears, nose, and skin were unremarkable.

The owner declined any further diagnostic attempt or therapy. After 3 h in the clinic, the dog suffered spontaneous cardio-respiratory arrest and died.

Macroscopically, an approximately 4 cm in diameter sized accumulation of coagulated blood was found within the lateral ventricle of the left hemisphere of the brain (Figure 3A). The adjacent brain parenchyma showed multifocal hemorrhages. Other findings comprised mild endocardiosis of the atrioventricular valve as well as multiple nodular masses within the spleen. Histopathologically, focal, severe hemorrhage corresponding to the macroscopic finding was visible in the left lateral ventricle and the neighboring brain parenchyma. Furthermore, moderate to severe, lympho-histiocytic infiltrates were detected within the meninges and brain parenchyma, and associated with non-leukocytoclastic vasculitis, most frequently in the cerebral cortex, hippocampus, brain stem, and cerebellum (Figure 3B). Furthermore, similar, but mild inflammatory changes of leptomeningeal blood vessels were found in the cervical, thoracic, and lumbar spinal cord. Additionally, within the perineural tissue of the optic nerve of the left eye mild, lympho-histiocytic vasculitis was detected as well as a mild cataract. In between nerve fibers of the trigeminal nerve, moderate, focal, acute hemorrhage was found. Using IHC, perivascular infiltrates in the CNS comprised equal numbers of CD3- and CD20-positive lymphocytes and multifocal, irregular infiltration with few CD204-positive macrophages. Using IHC and histochemistry, no infectious agent or signs of demyelination were detected.

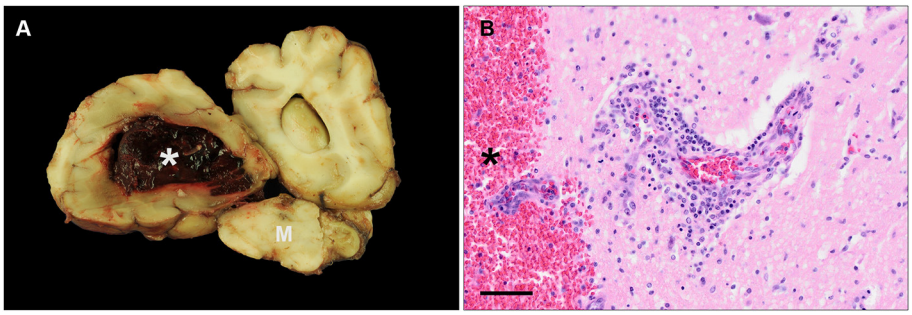

FIGURE 3
Macroscopic and histopathological findings of a 10 year old, mixed breed dog (case 2). **(A)** Macroscopic image of a transverse section of the cerebrum including the cerebral hemispheres and parts of the mesencephalon (M). The lateral ventricle of the left hemisphere displays severe, focal hemorrhage (asterisk). **(B)** Histopathology of the same area displayed in **(A)**. A blood vessel shows moderate vasculitis and perivascular infiltrates consisting of lymphocytes and macrophages. The lateral ventricle and adjacent parenchyma reveal hemorrhage (asterisk) (HE stain; scale bar: 50 μm).

Further alterations of minor significance comprised mild, follicular hyperplasia in the tonsils and mild, multifocal anthracosis in the lung as well as mild, acute, diffuse, alveolar edema. The splenic nodules were diagnosed as nodular hyperplasia. No signs of parasites, e.g. *Angiostrongylus vasorum*, were found in the lungs, heart, gastrointestinal tract, or CNS via gross and macroscopic examination.

Case 3

A 10.75 years old, male-neutered Australian Shepherd with a 2-day history of progressive gait abnormality and two self-limiting generalized tonic-clonic seizures was presented. Two months before presentation, the dog showed a left sided facial paralysis, which completely resolved after 15 days of prednisolone treatment by the primary veterinarian. The vaccination status was not reported.

At the time of presentation, the dog showed orofacial seizures, which developed into generalized tonic-clonic seizures and could be controlled with diazepam (2 mg/kg i.v.). Rectal body temperature was 39.4 °C. Heartbeat was 90 bpm without murmur on auscultation. Breathing pattern was normal, frequency was 40/min with physiological effort. Auscultation revealed mild vesicular lung sounds. Capillary refill time was under 2 s, the mucous membranes were pink and moist. Abdominal palpation was within normal limits. Macroscopical evaluation of eyes, external ears, nose, and skin were unremarkable. Blood examination revealed mildly elevated alanine aminotransferase (102 U/l; reference <50 U/l) and alkaline phosphatase (193 U/l; reference >150 U/l) activity. Clinical signs progressed within 24 hours, and the dog showed severe bradypnea and cyanosis. At neurological examination the dog was in lateral recumbency with generalized increased muscle tone and high-frequency, low-amplitude generalized tremor. He was comatose, showed bilateral absent palpebral reflexes and menace response and decreased pupillary light response on both eyes. Vestibuloocular and spinal reflexes were not tested. Consequently, the dog was intubated and mechanically ventilated.

Subsequent MRI (Figure 4) showed generalized flattened sulci and reduced volume of internal and external CSF space due to swelling of the CNS parenchyma. The FLAIR sequence revealed a subtle, diffuse, increased signal intensity of the internal capsule. There was transtentorial forebrain herniation as well as caudal foraminal herniation of the cerebellum with severe compression of the brainstem. At the site of compression, there was an intraaxial, T2w hyperintense lesion with multifocal signal void in the brainstem. There was no physiological contrast enhancement in the choroid plexus, which was suspected to be secondary to the compressed basilar artery preventing contrast agent to reach the CNS parenchyma.

Due to an infaust prognosis, no further examinations were performed and the dog was euthanized on owner's request.

Necropsy revealed a generalized swelling of the brain with mild herniation of the cerebellar vermis into the *foramen occipitale magnum* as well as herniation of the occipital lobe underneath the *tentorium cerebelli osseum* as indicated in the MRI. Furthermore, there was severe, acute hemorrhage and softening of the neuroparenchyma within the brain stem (Figure 5A).

Histologically, the cerebral cortex, cerebellum, brain stem as well as the spinal cord showed severe, lympho-histiocytic and plasmacytic panencephalomyelitis and meningitis with perivascular edema (Figure 5B). Additionally, severe vasculitis with inflammatory infiltrates in the damaged vascular wall (leukocytoclastic vasculitis) and in the perivascular space was found within the brain and the spinal cord, accompanied by moderate to severe hemorrhage. Spinal ganglia displayed mild to moderate infiltration of lymphocytes and macrophages,

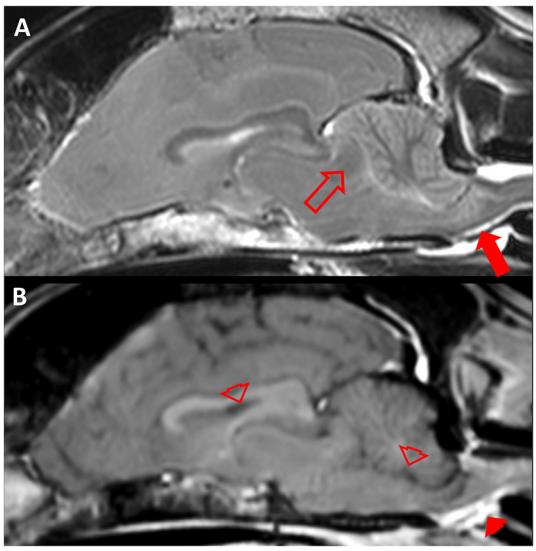

FIGURE 4
Magnetic resonance imaging (MRI) of a 10.75 year old Australian Shepherd (case 3). **(A)** Sagittal T2 weighted (w) MRI of the brain. Note the caudal cerebral herniation (empty arrow) which causes concave distortion of the rostral cerebellum and secondary transforaminal cerebellar herniation with compression of the brainstem and an intramedullary hyperintense lesion (filled arrow). **(B)** Sagittal T1w MRI approximately 3 min after intravenous contrast medium application. Note that there is physiological intravenous contrast enhancement extracranially (e.g., filled arrowhead) but no contrast medium is visible in structures which physiologically take up contrast medium (empty arrowheads).

too. Additionally, the right eye displayed a mild to moderate, lympho-histiocytic to granulomatous neuritis and perineuritis of the optic nerve.

IHC of the CNS (Figures 5C–E) revealed that the majority of inflammatory cells infiltrating the vascular wall as well as the perivascular space were comprised of B- and T-lymphocytes. B-lymphocytes outnumbered T-lymphocytes in most of the investigated areas. Few infiltrating cells represented CD204-positive macrophages in these regions. However, the number of infiltrating macrophages was variably increased in other areas, where less lymphocytes were visible.

Using IHC and histochemistry, no infectious agent or signs of demyelination were detected.

Additionally, the tracheobronchial lymph node showed severe anthracosis. The bone marrow revealed a dominating myeloid cell population. In lungs, heart, gastrointestinal tract, and CNS no parasites were found.

Summary of the cases

This case series reports about three dogs with signs of severe forebrain disease, which rapidly progressed, additionally involved the brainstem, and subsequently led to death. Extracranial clinical signs were mild and involved respiratory signs in one dog. MRI examination in two dogs showed generalized swelling of cerebral gray matter and subsequent features of increased intracranial pressure as well as signs of cerebellar and brainstem hemorrhage or herniation. Pathological contrast uptake could not be evaluated, because it was not administered in *post mortem* MRI in case 1, because no MRI was performed due to peracute death in case 2, and because of suspected insufficient intracranial circulation of contrast medium in case 3. Cerebrospinal fluid examination was only performed in case 1 and revealed hemorrhage, and lymphocytic dominance in cell differentiation analysis.

Macroscopically, the brains of two dogs displayed edema of varying degree and cerebellar herniation, and the brains of all dogs displayed hemorrhages occasionally. Microscopically, the main findings comprised lympho-histiocytic inflammation in the brain and/or spinal cord with associated leukocytoclastic and non-leukocytoclastic vasculitis.

An infectious causative agent could not be determined in any of the cases.

Discussion

Meningoencephalitis of unknown origin (MUO) in dogs is defined as a primary inflammatory brain disease with a so far unknown trigger (2, 3). Clinical signs of focal to multifocal encephalopathy, advanced diagnostic imaging (preferably MRI) revealing focal or multifocal, intraaxial lesions (frequently with increased contrast enhancement), CSF pleocytosis as well as the exclusion of potential causative infectious agents (3), suggest MUO in a clinical setting. However, definitive diagnosis requires histopathological confirmation (3, 15, 26).

MUO subtypes, like GME, NME, NLE, and Greyhound encephalitis, present with distinct histopathological features (12, 13, 16–19). In none of them, CNS vasculitis is a predominant finding (12, 13, 16–19). Vasculitis confined to the CNS is rarely reported (20, 22). Vasculitis is typically generalized or most pronounced in other organs than CNS such as the skin (20, 21) of which approximately 50% of the cases have an idiopathic pathogenesis (27). A focal dermal vasculitis with alopecia is described after subcutaneous rabies vaccination at the side of injection (28, 29). In dogs, the most common form of CNS vasculitis is found in Steroid-responsive meningitis-arteritis (SRMA) (20, 30–32). Acute hemorrhage and/or focal ischemic events secondary to vascular stenosis due to chronic changes of the vascular wall can cause signs of encephalopathy or myelopathy occasionally (20, 33). Histopathologically, SRMA is characterized by fibrinoid necrotizing polyarteritis of small to medium sized predominantly leptomeningeal arteries as well as perivascular and transmural infiltration with lymphocytes, plasma cells, macrophages, and neutrophils (34). Involvement

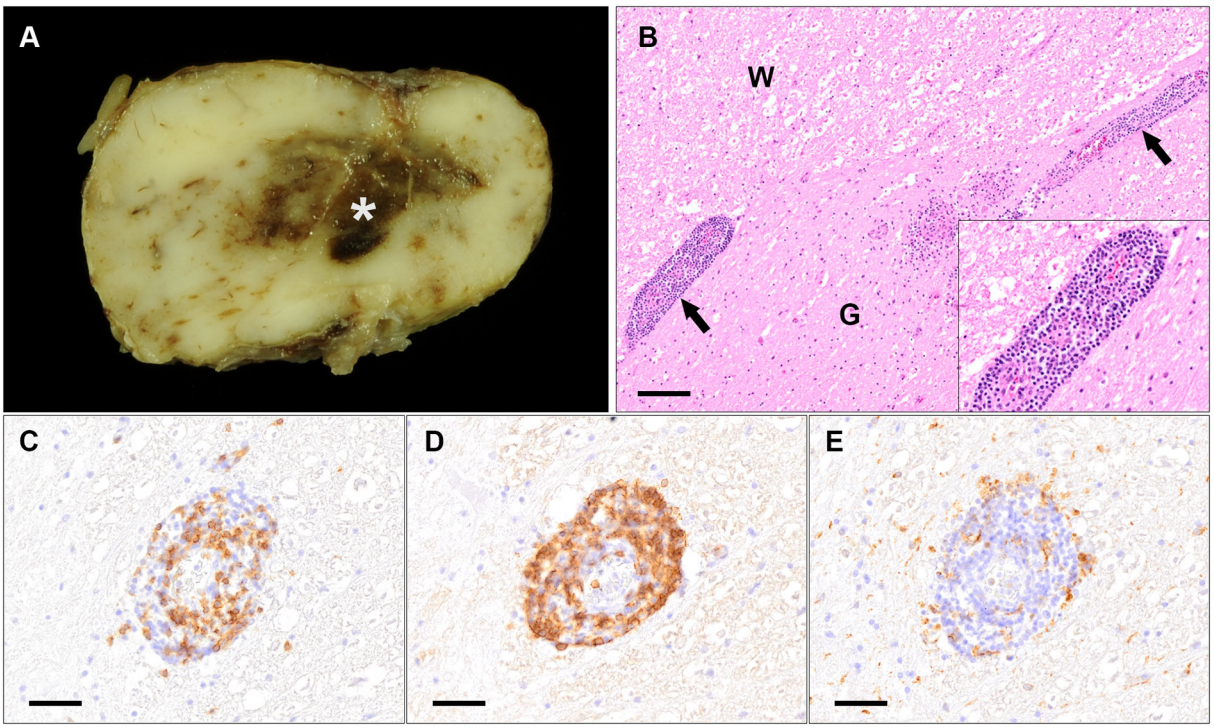

FIGURE 5
Macroscopic and histopathological findings of a 10.75 year old Australian Shepherd (case 3). **(A)** Macroscopic image of the brain stem displaying focal, severe hemorrhage within the neuroparenchyma (asterisk). **(B)** Histopathology of the spinal cord shows severe, multifocal vasculitis characterized by infiltrating inflammatory cells in the damaged vascular wall and the perivascular space (arrows; insert displays higher magnification of the perivascular infiltrates) at the border between white matter (W) and gray matter (G) (HE stain; scale bar: 100 μm). **(C–E)** Immunohistochemistry for CD3 **(C)**, CD20 **(D)** and CD204 **(E)** shows that the majority of inflammatory cells infiltrating and surrounding damaged vessels are comprised of many **(B–D)** and less T-lymphocytes **(C)**. Only very few infiltrating cells represent CD204-positive macrophages **(E)** (Scale bar: 50 μm).

of the arteries in other organs including heart, thyroid, and mediastinum are observed (29). Though, a substantial encephalitis is uncommon and rarely reported (30, 35).

Anecdotal reports about other sterile CNS vasculitis include localized or generalized, fibrinoid necrotizing vasculitis with and without associated ischemic lesions (20, 36), fibrinoid necrotizing changes of leptomeningeal blood vessels with purulent inflammation (20), segmental mononuclear vasculitis of the ventral spinal artery branches in a Miniature Schnauzer (20), and chronic demyelinating vasculitis in a middle-aged Weimaraner (31). The pathological findings of the cases presented in this report are characterized by meningoencephalitis and CNS vasculitis and do not resemble any of these described cases. Here, vasculitis was restricted to the CNS. Mostly small to medium sized blood vessels of the parenchyma as well as the leptomeninx were affected. The changes were asymmetrical, affected both the gray and white matter or only the gray matter and severity ranged from mild to severe depending on the area. The clinical signs of the dogs in this case series were acute, rapidly progressive, and severe. Most likely discontinuity of the inflamed vascular walls led to break down of the blood-brain barrier and generalized edema and hemorrhage (37) as observed macroscopically and histologically. This led to a quick increase of intracranial pressure, partly with caudal cerebellar herniation; as a consequence, centrally controlled vital functions most likely got compromised (38). If the acute and fatal outcome of these cases is representative for the here presented lympho-histiocytic meningoencephalitis with CNS vasculitis is unclear, as necropsy was one of the inclusion criteria in this case series, which might cause a clinical bias. Therefore, no statement can be made about the clinical prognosis or about specific therapy recommendations.

Clinical signs in MUO are mostly restricted to neurological abnormalities; signs of systemic disease are rare (1). In this case series only case 1 presented with coughing while no other extracranial sings were evident *ante mortem*. Respiratory signs could have been indicative for a triggering respiratory infection or might have been unrelated to the encephalitis. However, necropsy and histopathology did not reveal signs

of respiratory or systemic disease. In case 3, hyperthermia, tachycardia, and hemorrhagic diarrhea were suspected to be secondary to prolonged seizure activity not primarily due to the disease itself.

Interpretation of CSF examinations were restricted. Severe contamination with blood prevented adequate leukocyte cell count. It might be caused by iatrogenic vessel damage during CSF tap or might reflect intracranial hemorrhage (39). The latter seems more likely, as MRI was indicative of multifocal caudal fossa hemorrhage (40).

For the here presented meningoencephalitis with CNS vasculitis no infectious agents could be detected. However, this does not necessarily exclude an infectious etiology. This could be due to the fact that a potentially causative infectious agent was no longer detectable at the time of investigation ["hit and run theory" (41)]. Furthermore, the risk of false-negative results in the performed investigations needs to be taken into account as well. Pathogenetically, similar lesions can be triggered directly by an infectious agent or could be the result of immune-mediated pathogen-triggered mechanisms such as molecular mimicry or epitope-spreading [reviewed by Pederson (42)]. Moreover, so far unknown pathogens not discovered by immunohistochemistry need to be considered, since several recent investigations using next generation sequencing have revealed new, so far unknown etiologies (43, 44).

To the best of the author's knowledge, this is the first case series describing clinical signs, diagnostic imaging and histopathological findings of an acute, progressive, fatal non-suppurative meningoencephalitis with vasculitis restricted to the CNS. It is proposed to consider the described changes of the three dogs as a new subtype of MUO with CNS vasculitis as the histopathological distinguishing feature.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the animal study because retrospective case report. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

IZ performed and interpreted results of necropsy and histopathology and drafted manuscript. JR performed and interpreted findings of diagnostic imaging and finalized manuscript. FS performed and supervised anesthesia, took care for patients, and finalized manuscript. WB interpreted results of necropsy and histopathology and finalized manuscript. AT interpreted findings of clinical and diagnostic imaging examination and finalized manuscript. JN performed and interpreted findings of clinical and diagnostic imaging examinations, drafted, and finalized manuscript. All authors agree on authorship and publication of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Concomitant necrotizing encephalitis and granulomatous meningoencephalitis in four toy breed dogs

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The term "meningoencephalitis of unknown origin" (MUO) describes a group of different encephalitides in dogs in which no infectious agent can be identified and a multifactorial etiology is suspected. Among others, genetic factors and unknown triggers seem to be involved. Included are necrotizing leukoencephalitis (NLE), necrotizing meningoencephalitis (NME), and granulomatous meningoencephalitis (GME). In this case series, we describe the histopathological findings of four toy breed dogs with focal or multifocal necrotizing encephalitis and mainly lymphocytic perivascular infiltrates on histopathological examination. At the same time, however, in all dogs, focal or multifocal high-grade angiocentric granulomatous inflammatory lesions were evident with focal histiocytic perivascular infiltrates in the brain. The former changes are typical for NLE and NME. In contrast, the latter changes are indicative of GME. This case series shows that the boundaries between the necrotizing and granulomatous variants of MUO might be smooth and suggests that NLE, NME, and GME are not as distinct as previously described. This finding could be a crucial piece of the puzzle in the study of the pathogenesis of MUO as individual susceptibility and specific triggers could be responsible for the manifestation of the different MUO subtypes.

KEYWORDS

meningoencephalitis of unknown origin (MUO), necrotizing meningoencephalitis, necrotizing leukoencephalitis, canine (dog), inflammatory brain disease, histopathology (HPE), granulomatous meningoencephalitis (GME)

Introduction

Granulomatous meningoencephalitis (GME), necrotizing meningoencephalitis (NME), and necrotizing leukoencephalitis (NLE) are supposed to be subtypes of canine meningoencephalitis of unknown origin (MUO) (1, 2). MUO seems to be multifactorial: a trigger is suspected to cause exaggerated immune response in susceptible individuals, but both the trigger and the underlying individual predisposition remain mostly elusive (1), though a genetic defect is suspected to predispose to NME in some toy breeds (3). Typically, patients display progressive clinical signs of focal or multifocal central nervous system (CNS) dysfunction, with focal to multifocal CNS lesions in advanced imaging and often non-suppurative cerebrospinal fluid (CSF) pleocytosis, indicating CNS inflammation. An infectious agent can be found neither in the CSF nor in the neuronal parenchyma (4–9), and immunomodulatory therapy often improves clinical signs (2, 10, 11).

Although signalment, history, clinical signs, cross-sectional imaging modalities of the brain and CSF examination, and exclusion of infectious agents may allow a tentative diagnosis of MUO *ante mortem* (9, 10), different subtypes can often not be separated (2). Thus, the gold standard for diagnosing different types of MUO still remains histopathology (1).

Findings in histopathology are reported to be relatively specific for each subtype: GME is characterized by angiocentric or nodular granulomatous lesions due to focal eccentric nodular proliferation of macrophages within histiocytic perivascular cuffs in the Virchow-Robin space (12) in the cerebellum, medulla oblongata, and spinal cord. In contrast, non-suppurative perivascular inflammation and necrotic lesions of NLE occur mostly in the white matter of the cerebrum and brain stem, while in NME, they affect predominantly the gray matter and meninges of the telencephalon (12, 13).

Due to frequent overlap of histopathological and clinical features as well as MRI findings, NME and NLE are often taken together as necrotizing encephalitis (NE), and the authors of several reviews have speculated that NME and NLE are even just different manifestations of the same disease entity with breed specific characteristics (1, 14).

While NLE is mostly found in young Yorkshire Terriers and French Bulldogs (14–16), NME appears predominantly in young Maltese, Chihuahua, and Pug dogs (3, 17). GME, on the contrary, might occur in any breed at any age, although preferably in young to middle-aged toy breed dogs (2).

Till present, it is still under debate whether all subtypes of MUO belong to an array of different manifestations within the same disease entity or if they display completely different diseases (1).

This multicentric case study presents histopathological findings of four toy breed dogs, each with overlapping phenotypes of NME/NLE and GME.

Materials and methods

All clinical examinations were performed with the informed written owner's consent by a resident or diplomate of the European College of Veterinary Neurologist (ECVN). Anesthesia was induced with levomethadone, diazepam, and propofol and maintained by the administration of isoflurane in oxygen-nitrous oxide or air. The magnetic resonance imaging (MRI) examination of the brain was performed under general anesthesia with the dog in sternal or dorsal recumbency using 0.3 or 3.0 Tesla (Philips, Achieva 3.0T TX MRI, Phillips Healthcare, Hamburg, Germany, or Hitachi Airis II, Hitachi Medical Systems, Düsseldorf, Germany) and consisted of turbo spin echo sequences with T2-weighted (T2W), fluid attenuation inversion recovery T2 (T2W FLAIR), and T1-weighted images (T1W) before and after intravenous administration of MRI contrast (Gadolinium-Dotarem, 0.2 mmol/kg, or Gadopentetate Dimeglumineb, 0.1 mmol/kg). Computed tomography (CT) was performed using Siemens Somatom AR.T (Siemens Switzerland AG, Fahrweid, Switzerland) pre- and postintravenous administration of iodinated contrast medium.

Necropsies and histopathology of formalin-fixed and paraffin-embedded tissues were performed at the Department of Pathology of the University of Veterinary Medicine Hannover, Foundation, and the Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern. All cases were investigated by a diplomate of the European College of Veterinary Pathologists (ECVP). Brain and spinal cord were fixed in 10% neutral buffered formalin, processed, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E) in all dogs. Immunohistochemistry was performed to exclude an infection with rabies virus, canine distemper virus, *Neospora caninum*, and *Toxoplasma gondii* using the avidin-biotin-complex (ABC)-method with 3,3′-diaminobenzidine (DAB) as chromogen (6, 18–20). Selected sections of the brain were stained with Grocott's methenamine silver, Gram, Periodic acid–Schiff (PAS), or Ziehl-Neelsen (20).

Dogs were included if, on histopathological examination, concomitant signs of NME or NLE and GME were evident,

Abbreviations: CBC, complete blood count; CSF, cerebrospinal fluid; CT, computer tomography; CNS, central nervous system; EAE, experimental autoimmune encephalitis; ECVN, European College of Veterinary Neurology; GME, Granulomatous meningoencephalitis; IFAT, immunofluorescence antibody test; Ig, immunoglobulin; IHC, immunohistochemistry; MRI, magnetic resonance imaging; NE, necrotizing encephalitis; NLE, necrotizing leukoencephalitis; NME, necrotizing meningoencephalitis; PAS, periodic acid–Schiff.

TABLE 1 All cases of dogs with meningoencephalitis of unknown origin (MUO) examined in both pathological institutes from 2010 to 2021.

Histopathological diagnosis	Number of dogs
GME	22
NLE	9
NME	14
Concomitant findings of NME and NLE	1
Lymphohistiocytic MUO (findings not consistent with GME, NME or NLE)[a]	12
MUO with CNS vasculitis[b]	3
Concomitant findings of GME and NLE/NME	4
Total	**65**

GME, granulomatous meningoencephalitis; NLE, necrotizing leukoencephalitis; NME, necrotizing meningoencephalitis; MUO, meningoencephalitis of unknown origin; CNS, central nervous system; [a]as described in (21); [b]dogs described in (22).

which are multifocal asymmetric necrosis with non-purulent inflammation predominantly in the gray or in the white matter of the cerebrum and thalamus for NME or NLE, respectively, and additional angiocentric lymphoplasmacellular or granulomatous inflammatory infiltration predominantly in the white matter of the brain stem, cerebellum, and spinal cord for GME (12, 13), and no infectious agent could be found. Data banks from 2012 to 2021 of both institutes were searched. In total, 65 dogs with histopathological signs of (meningo-)encephalitis without a known infectious agent were identified (Table 1), of which 4 dogs matched the inclusion criteria of concomitant signs of NME or NLE and GME.

Case descriptions

Case 1

A 6.5-year-old male neutered Yorkshire Terrier was presented with a 3-week history of progressive gait abnormality in the pelvic limbs, two suspected seizures with tremor, restlessness, and increased water intake, according to the owner.

On presentation, the general physical examination was normal with respiratory sinus arrhythmia. In the neurological examination, the dog had mild kyphotic posture, paraparesis accentuated on the left side, and a reduced to absent paw placement of the left pelvic limb. The dog also had a reduced to absent menace response bilaterally and a ventromedial strabismus of the left eye. Based on these findings, a multifocal meningoencephalopathy was suspected.

On complete blood count (CBC), mild leukopenia ($5.32 \times 10^3/\mu l$, reference range $6–12 \times 10^3/\mu l$) was evident, the serum biochemistry profile was within reference ranges.

On MRI of the brain (Figure 1), multifocal bilateral asymmetric forebrain lesions were seen, mainly affecting the subcortical white matter but also the gray matter of the left parietal lobe, the right occipital lobe, the left hippocampus, and thalamus with no to mild mass effect. The lesions were hyperintense in T2W and FLAIR and hypointense in T1W and were mostly well demarcated. In the lesions, heterogeneous strong contrast enhancement was present.

The suboccipital CSF sample contained mildly increased total protein of 34 mg/dl (reference range <25 mg/dl), lymphocytic pleocytosis (270 cells/μl, reference range <5 cells/μl; 84% lymphocytes, 12% monocytes, and 4% macrophages), and mild suspected iatrogenic blood contamination (190 cells/μl, reference range 0 cells/μl) was evident. MUO was suspected, but the owners denied any further examinations and elected euthanasia.

On pathological examination of the brain, multifocal marked necrotizing leukoencephalitis was evident with many gitter cells (malacia) and severe lymphohistiocytic perivascular cuffing in the subcortical white matter of the left parietal lobe (Figure 2). A moderate lymphohistiocytic inflammation with severe gliosis, gemistocytes, dilated myelin sheaths, and spheroids was found in the periventricular white matter at the level of the hippocampus. Additionally, there was marked granulomatous inflammation with severe lymphocytic perivascular cuffing in the right striatal body and caudate nucleus (Figure 2). Moreover, mild lymphoplasmacytic meningitis of the cerebral hemispheres as well as a mild lymphoplasmacytic inflammation of the right optic nerve were detected. No infectious agents were observed using immunohistochemistry (IHC) against rabies virus, canine distemper virus, *Neospora caninum*, and *Toxoplasma gondii*.

Case 2

A 1-year-old male intact Maltese was presented due to 3-day history of vestibular ataxia. The blood examination of the referring veterinarian was unremarkable. The dog was mildly apathic, but otherwise the general examination was unremarkable. In the neurological examination, mild head tilt and head turn to the left were present. The dog was non-ambulatory and ataxic. Increased extensor-muscle tone was evident in both right limbs. Proprioception (paw placement and hopping) was reduced on both left limbs. The menace response was mildly and inconsistently reduced in both eyes, and occasionally the dog displayed a head tremor. No other cranial nerve deficits were visible at the time of examination. Multifocal intracranial lesions pronounced in the left brain stem and cerebellum were suspected.

On MRI of the brain in general anesthesia, an intraaxial lesion was evident in the white matter of the cerebellum, extending bilaterally into the brain stem over the cerebellar peduncles. In the brain stem, the left side was more affected and involved the nerve roots of the facial, vestibulocochlear,

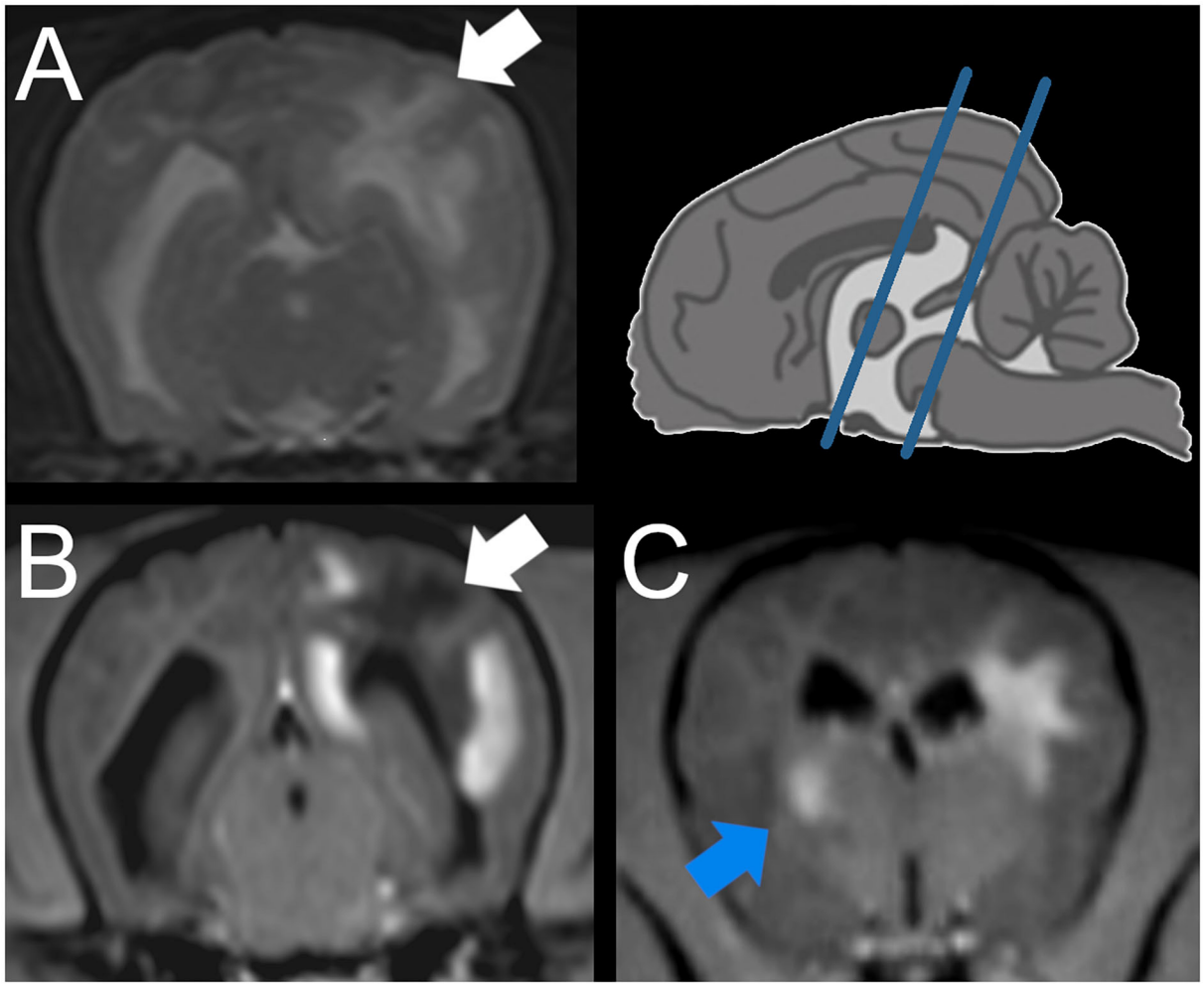

FIGURE 1
Magnetic resonance imaging (MRI) of a Yorkshire Terrier with meningoencephalitis of unknown origin (case 1). Transverse plane at the level of the mesencephalon **(A,B)** and interthalamic adhesion **(C)** (see blue lines in the schematic overview upper right), A: T2 weighted (w), B, C: T1w post contrast injection. Intraaxial lesion of the subcortical white matter of the cerebral hemisphere, identified on histopathology as predominantly necrotic (**A,B**: white arrow). Intraaxial lesion of the striated body identified as granulomatous lesion (**C**: blue arrow).

and trigeminal nerves. The lesions were mildly heterogeneous, moderately hyperintense in T2w, and mildly hypointense in T1w. They were ill demarcated, causing a mild mass effect and increased volume of the brain stem. Marked contrast enhancement was evident in the brain stem meninges and ependymal layer of the fourth ventricle, and there was mild to moderate meningeal enhancement in the left temporal lobe. Additionally, there was mild secondary enlargement of the cervical central canal.

Suboccipital CSF contained increased protein content of 135 mg/dl (reference range <25 mg/dl) and a mononuclear pleocytosis (221 cells/µl, reference range <5 cells/µl; 69% monocytes, 30% lymphocytes, and 1% neutrophilic granulocytes) was evident. *Toxoplasma gondii* antibody titer of immunoglobulin (Ig)M and IgG and antibody titer for *Neospora canis* in serum were below 1:32 (immunofluorescence antibody test (IFAT); Laboklin, Bad Kissingen, Germany).

MUO was suspected. Clindamycin 12 mg/kg body weight and prednisolone 0.5 mg/kg body weight *per os* twice daily were given until negative infectious agent titers were confirmed, then clindamycin was discontinued and prednisolone increased to 1 mg/kg bodyweight twice daily.

Initially, clinical signs improved mildly but relapsed and deteriorated after 5 days. Therefore, owners elected euthanasia 2 weeks after the onset of clinical signs.

On pathological examination, a marked focal-extensive malacia was evident in the area of the cerebellar lingula, brain stem, and midbrain ventral to the aqueduct. On histopathology, extensive necrosis and edema were associated with marked nodular and perivascular infiltration with

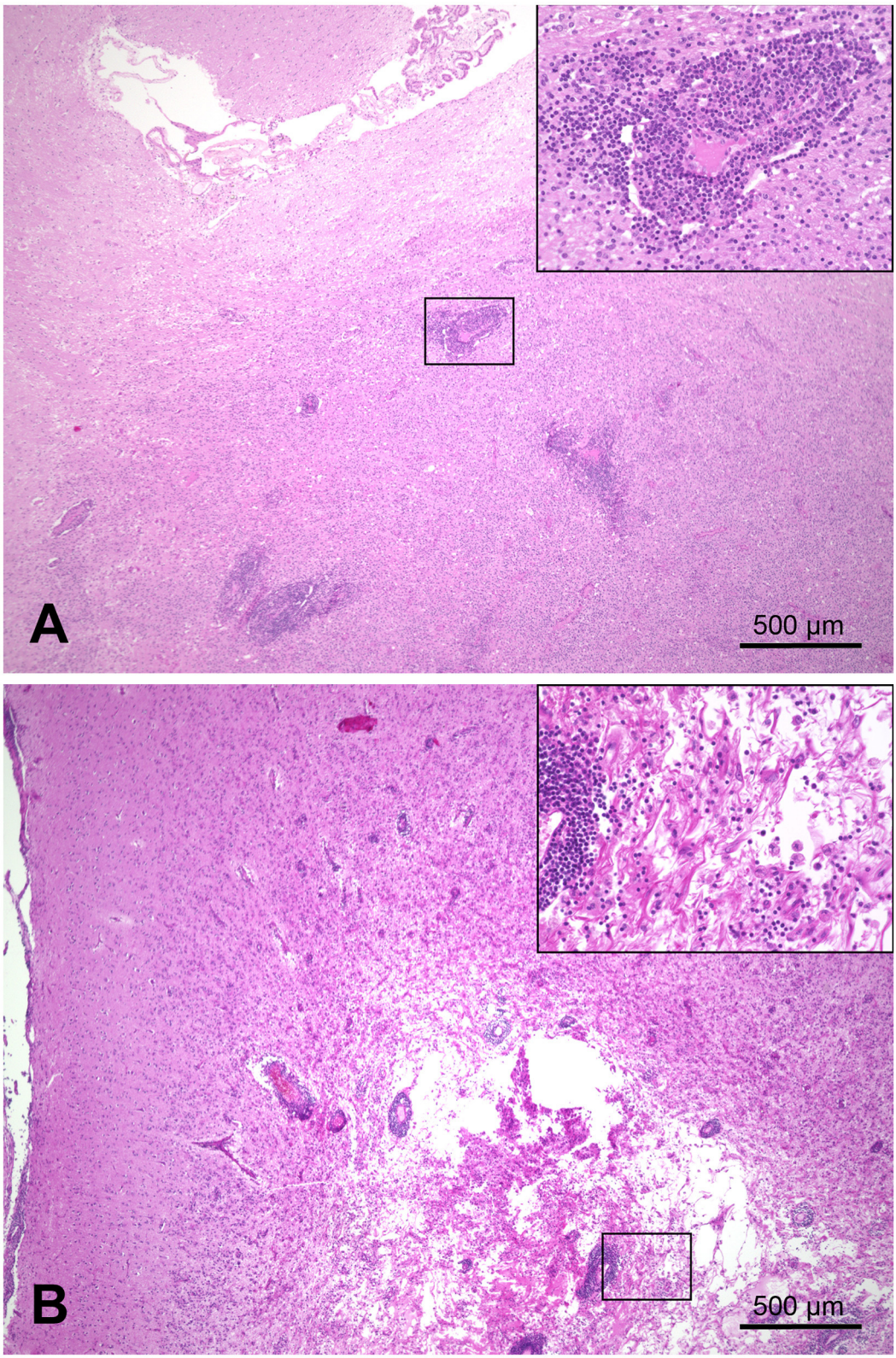

FIGURE 2
Combined necrotizing leukoencephalitis and granulomatous meningoencephalitis in the brain of a Yorkshire Terrier (case 1). **(A)** right striatal body; marked granulomatous inflammation with severe lymphocytic perivascular cuffing. **(B)** cerebrum, left parietal lobe; marked necrotizing leukoencephalitis in subcortical white matter with gitter cells (malacia) and severe lymphohistiocytic perivascular cuffing; bars = 500 µm.

epithelioid macrophages and lymphocytes in the midbrain, cerebellum, and brain stem (Figure 3). The mesencephalic meninges adjacent to the lesion were also infiltrated by lymphocytes and macrophages. Periodic acid–Schiff (PAS) reaction, Grocott, Gram, and Ziehl-Neelsen staining as well as IHC for *Toxoplasma gondii* and canine distemper virus did not detect any infectious agent.

Case 3

A 7.5-year-old female Yorkshire Terrier was presented due to status epilepticus. She gave birth to five puppies 4 weeks prior to the presentation, which all died within 3 days *post partum*. She was progressively anorectic for 2 weeks and obtunded. The dog experienced several generalized tonic-clonic cluster seizures for 2 days.

The dog was presented with a generalized tonic-clonic seizure, not responding to external stimuli. The body temperature was normal. Her mucous membranes were pale; general examination was otherwise unremarkable. In the blood examination, mildly increased white blood cell count (18.7 $\times$ 10^3/μl, reference range 4.7–11.3 $\times$ 10^3/μl) with mild lymphopenia was evident with decreased urea 1.6 mmol/L (reference range 3.8–9.4 mmol/L), creatinine 37 μmol/L (reference range 50–119 μmol/L), without any further pathological changes.

On computed tomographic examination of the brain, multifocal, hypoattenuating, and intraaxial lesions were evident in the right piriform and frontal lobes, with moderate mass effect causing a midline shift to the left. Mild heterogeneous contrast enhancement was evident in the lesions. Additionally, there was asymmetrical moderate dilatation of both lateral ventricles.

In suboccipital CSF samples, lymphocytic pleocytosis (88% lymphocytes, 9% monocytes, and 3% neutrophils) was evident. Due to the small sample size, no further examinations could be performed, and the exact cell count was not evaluated.

Seizures could only be poorly controlled with midazolam continuous rate infusion, propofol and phenobarbital bolus injections, and prednisolone therapy, hence her owners elected for euthanasia.

On pathological examination, multifocal malacic changes were evident in the right piriform lobe, extending to the basal nuclei and meninges, and additionally in the brain stem. On histology, severe inflammation was scattered over the entire brain with multifocal marked distinct perivascular cuffs containing many lymphocytes and macrophages, frequent groups of large epithelioid macrophages, and few plasma cells. Lesions were most evident in the gray and white matter of the frontal lobes and basal nuclei, but they also affected the brain stem, midbrain, thalamus, and hippocampus, and extended into the subarachnoid space (Figure 4). Frontal lobes and basal nuclei were affected by marked multifocal areas of necrosis infiltrated with numerous gitter cells. These lesions were surrounded by numerous gemistocytic astrocytes. The PAS reaction and Grocott staining did not reveal any infectious pathogens.

Case 4

The formalin-fixed brain of a 7-year-old Chihuahua was sent to the Institute of Pathology in Bern. Before death, the dog had a 4-day history of progressive pacing and circling to the right. No further information was given.

On neuropathology, focal extensive white matter necrosis was evident in the occipital lobes of both hemispheres. Ventral and left of the midline in the midbrain and pons, there was a focal, circular, white-beige, and space-occupying lesion. On histopathology, the space-occupying lesion resembled a granuloma consisting of a central cavitary necrosis, which was intersected with astrocytes and hyperplastic capillaries, surrounded by a thick band of numerous epithelioid macrophages, lymphocytes, and astrocytes. In the cerebral hemispheres, the inflammatory process was more extensive and mainly focused on the white matter. In some areas, the lesions consisted of chronic cavity-like necroses interspersed with gliotic tissue, while in other areas, lesions consisted of active inflammation with perivascular cuffs of numerous epithelioid macrophages, lymphocytes, and fewer plasma cells, intermixed with prominent gemistocytic astrocytes. The inflammatory infiltrates extended multifocally to the gray matter and occasionally to the meninges. The PAS reaction and Ziehl-Neelsen staining did not detect any infectious pathogens.

Summary

This multicentric case series describes the clinical and pathological findings of two Yorkshire Terriers, one Maltese, and one Chihuahua, with meningoencephalitis of unknown origin. Microscopically, in all dogs, areas of marked necrosis were evident in the cerebral hemispheres (n = 3/4; mainly cortical white matter n = 2/4, gray and white matter of the frontal lobe and basal nuclei n = 1/4), cerebellar white matter (n = 1/4), or brain stem (n = 1/4) with mainly lymphocytic perivascular infiltrates. Gemistocytes and gitter cells were found in two and three dogs, respectively. At the same time, all four dogs also had focal or multifocal high-grade angiocentric granulomatous inflammatory lesions in the cerebrum (n = 2/4; thalamus n = 1/2, corpus striatum including basal nuclei n = 2/2, cortex n = 1/2), and rhombencephalon (n = 4/4; brain stem n = 3/4, midbrain 2/4, cerebellum n =1 /4). Meningitis was found in all the dogs. Infectious agents were excluded using H&E, special staining techniques or IHC.

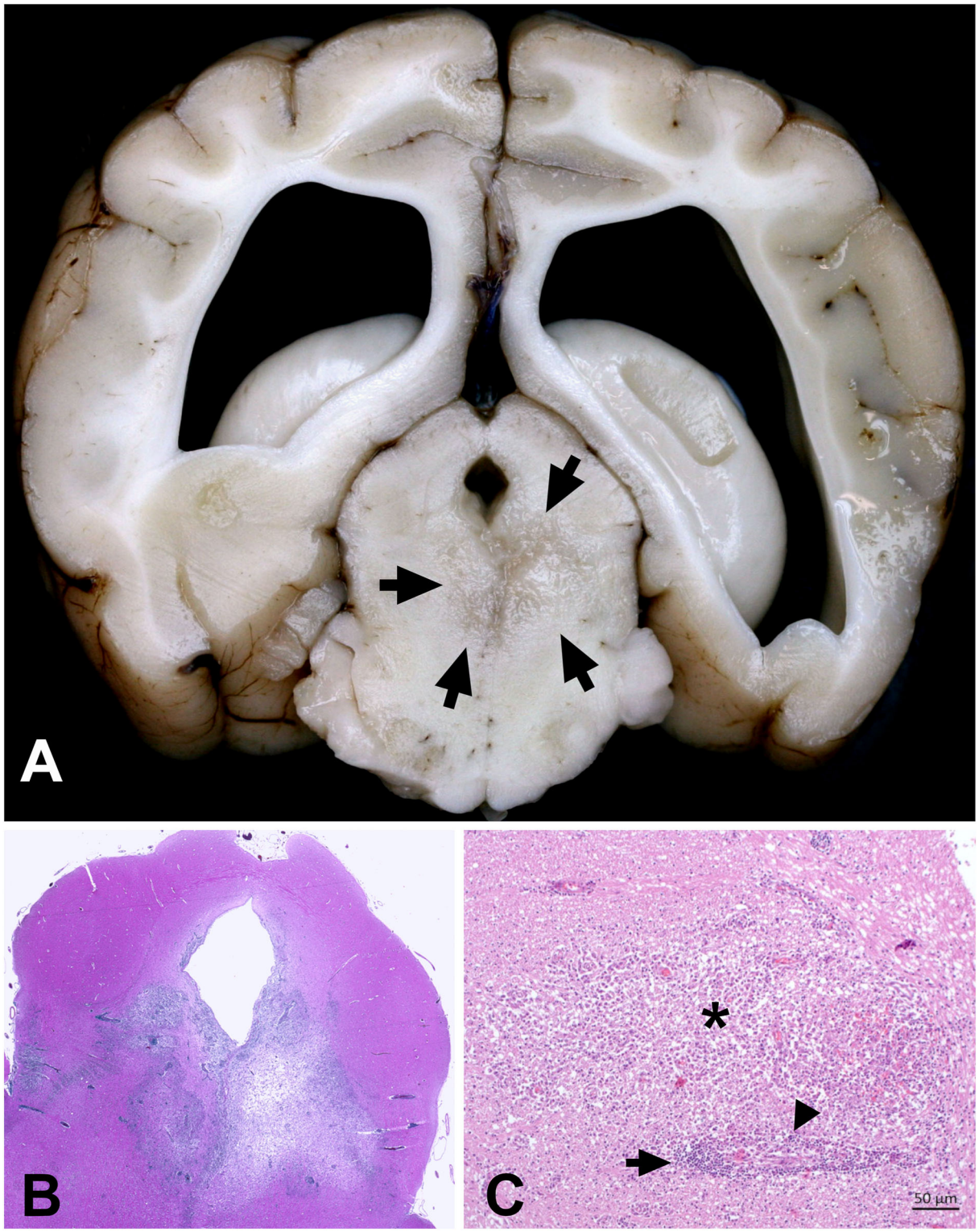

FIGURE 3
Combined necrotizing and granulomatous meningoencephalitis in the brain of a Maltese (case 2). **(A)** Focal-extensive malacia in the midbrain (arrows). **(B)** Subgross picture of a H&E stained section of the midbrain shown in A. Focal-extensive pallor indicates necrosis and edema, which is associated with a prominent inflammatory process (dark blue color due to nuclei of infiltrating inflammatory cells). **(C)** Area of granulomatous inflammation with lymphocytes (arrows) and activated macrophages (arrowheads) around a vessel. The activated macrophages markedly extend into the neuroparenchyma (asterisk).

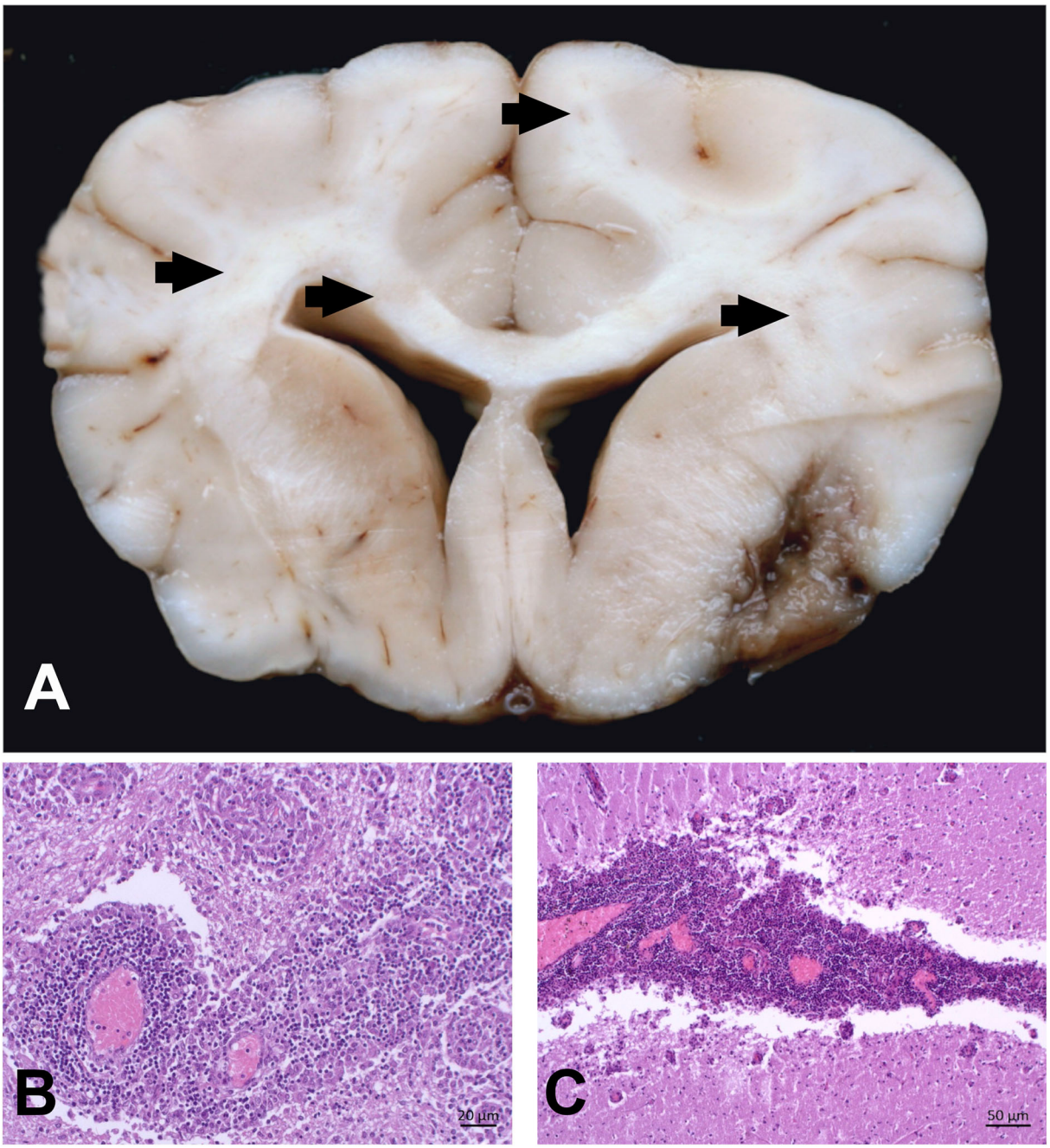

FIGURE 4
Combined necrotizing and granulomatous meningoencephalitis in the brain of a Yorkshire Terrier (case 3). **(A)** Severe, focal-extensive and delineated malacia in the right rhinal cortex and extending to the surface. This lesion mimicks NME. Additionally, there are multifocal areas of beige discoloration in the corona radiata (arrows). **(B)** Severe and multifocal-coalescing perivascular cuffs with lymphocytes and large activated macrophages. Macrophages tend to compartmentalize within these cuffs. **(C)** The same dog is affected by a marked cerebellar meningitis that extends into the molecular layer.

Discussion

This case series describes four dogs with histopathological signs of NLE/NME and concomitant GME.

NME, NLE, and GME are often summarized under the umbrella term "MUO" where an unknown multifactorial etiopathogenesis is suspected (1, 2). The different MUO subtypes cannot be reliably differentiated in a clinical setting without

histopathological confirmation (2, 11), but in all of them, clinical sings respond to a certain degree to immune-suppressive therapy (2, 10, 11).

Typical histopathological features of GME allow relatively clear differentiation from NME and NLE (13). In GME, angiocentric or nodular granulomatous lesions in the white matter of the cerebrum, cerebellum, medulla oblongata, and spinal cord contain mainly macrophages, epithelioid cells, lymphocytes, plasma cells, and neutrophils (13). In NLE, multifocal lymphoplasmacellular encephalitis and necrotic foci are typical in the cerebral white matter and sometimes in the brain stem (13). For NME, a predominant involvement of the cerebral gray matter and meninges is typical (13). Due to the frequently seen overlap of histopathological and clinical features, as well as MRI findings, NME and NLE are often taken together as NE, and the authors of several reviews have speculated that NME and NLE are rather different manifestations of the same disease entity with breed specific characteristics (1, 14). This study suggests that there might be additionally significant overlaps between GME and NE based on the presented histopathological findings in 4 dogs with concomitant features of GME and NLE/NME. It is already reported that in the absence of necrosis in very acute NME, NME, GME, and NLE might be less easy to distinguish due to overlap in infiltrating inflammatory cell population (13). On the contrary, mild necrotic foci are reported in GME but were not as extensive as in NME or NLE (23).

All dogs were presented with clinical signs of progressive intracranial disease. Signalment, clinical signs, diagnostic imaging, and CSF findings were most consistent with NLE in case 1 and NME in case 2 (14). Although Yorkshire Terriers are predominantly affected by NLE (14), MRI findings of case 3 included an intraaxial lesion with increased contrast enhancement and mass effect, which is more likely to occur in GME than in NLE (14, 24, 25). Due to the incomplete availability of clinical data in case 4, the clinical diagnosis was uncertain. Advanced diagnostic imaging might be rather unspecific for the diagnosis of inflammatory brain lesions (26), and biopsy might be preferred to diagnose MUO subtypes (1, 9).

On histopathology, necrosis and lymphocytic perivascular inflammation in subcortical white matter, in the cerebral cortex, deep gray matter of the telencephalon, or in the brain stem and cerebellum were indicative of NE. Additionally, in all dogs, concomitant granulomatous meningoencephalitis was evident with epithelioid macrophages mostly in the brain stem and also in the telencephalon or spinal cord. In none of the dogs, infectious agents were found with basic histopathological examinations.

Attempts by several working groups to identify an infectious agent in any subtype of MUO failed (4–9, 27). As in this case series, no further in-depth search for infectious agents was performed, e.g., next generation sequencing (4), it cannot be completely ruled out that these dogs might have suffered from a so far unidentified infection, which causes this specific mixed pattern of granulomatous and necrotizing meningoencephalitis. However, immunohistochemistry and special staining for known infectious agents were negative, supporting the classification of MUO.

It is also unknown why some dogs develop GME instead of NME and NLE or vice versa (1, 13). A certain trigger (infectious or environmental) might lead to the development of MUO, and the clinical and histopathological phenotype depends on individual genetic susceptibility. Moon (28) induced experimental autoimmune encephalitis (EAE) in dogs by injecting whole cerebral homogenate subcutaneously together with an inflammatory stimulation agent. While 7 out of 12 dogs developed necrotizing encephalitis, others did not develop any obvious clinical signs (28). The design of the study does not allow any further conclusion on the exact pathogenesis of the encephalitis as the study population was too heterogeneous regarding breed, gender, and even injected brain homogenate derived from different canine donors with various underlying brain disease. Nevertheless, not all dogs, which received brain homogenate from the same donor, did develop EAE (28) pointing to individual predisposing factors.

Additionally, breed-specific disease patterns of MUO are reported (1, 14). The dogs with mixed inflammation patterns of NME/NLE and GME described here were all toy breed dogs, mainly with the necrotizing variants of MUO (14): Maltese dogs and Chihuahuas seem to develop preferably NME (3, 17), while Yorkshire Terriers and French Bulldogs seem to be prone to develop NLE (15, 16, 29). In Maltese dogs and Chihuahua, a genetic defect in dog leukocyte antigen (DLA) class II is suspected to predispose to NME (3). It seems that, among other factors, genetic susceptibility might determine the subtype of MUO. Unfortunately, no statement can be made about the underlying DLA-II genotype of the dogs with mixed GME and NME/NLE in this case series, as no genetic examination was performed.

On the contrary, it seems that different triggers can cause different inflammatory patterns in EAE (30). In the study of Moon, all dogs with EAE, very similar histopathological signs of NLE-like or NME-like lesions were evident after injection of the similar trigger (homogenized cerebral tissue) despite the heterogeneous genetic background in the study population (28). In EAE in rats, injection of homogenized cerebral tissue induced lesions similar to NME in the forebrain, while injection of homogenized cerebellar tissue induced completely different lesions with demyelination and inflammation in the rhombencephalon and spinal cord (30). It seems that both the individual susceptibility and the underlying trigger might have an influence on the expression of a specific EAE subtype (28, 30). The same might also be true for naturally occurring MUO in dogs, and the pattern of concomitant GME and NME/NLE described here might be the result of a particular combination of individual

susceptibility and triggers, which differs from classical GME or NME/NLE.

The described pattern of neuroinflammation with concurrent signs of NME/NLE and GME might be either a so far undescribed new subtype of MUO or an atypical variant of either NME, NLE, or GME. Furthermore, it could be hypothesized that NME/NLE and GME are the same disease entity with an individual histopathological expression. Another theory could imply that the presented dogs might have suffered from two different diseases—GME and NE—at the same time, coincidentally.

Although all of these theories are speculative in nature and it cannot be told, what caused this atypical inflammation pattern in the presented dogs, this case series is an additional hint that the subtypes of MUO might not be as distinct as it is generally portrayed, which was already stated by other authors before (1, 14).

Additionally, for *ante mortem* diagnosis of MUO, it is very important to keep in mind that *in vivo* diagnosis of NME/NLE or GME based on biopsy sampling could be misleading: Biopsies only give a focal impression and might not necessarily be representative of the entire inflammatory process in the whole brain. Depending on the side of tissue sampling, a biopsy could lead to an incomplete or even false impression of the overall nature of the inflammation, and either a necrotic or a granulomatous component of the encephalitis could be overlooked in cases of concomitant GME and NME/NLE. The consequence could be an incomplete or false diagnosis, which might have an influence on the results and interpretation of future studies or on the development of clinical therapy strategies.

Conclusion

Although rarely reported, concomitant signs of GME and NME/NLE can be presented in the diagnostic imaging and histopathological examinations of small toy breed dogs, which should be considered for future studies. Especially in a clinical setting without extensive histopathological examination, the diagnosis MUO should be preferred over the more specific diagnoses of GME, NME, or NLE.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JN contributed to conception and design of the study, collected and organized the data, and drafted and finalized the report. AO performed and interpreted results of necropsy, histopathology, and drafted and finalized the report. MS performed neurological examination, interpreted diagnostic imaging findings, and drafted and finalized the report. IS performed and interpreted results of necropsy and histopathology, and drafted and finalized the report. IG performed and interpreted results of necropsy, histopathology, and finalized the report. SB performed neurological examination, interpreted diagnostic imaging findings, and finalized the report. FS performed neurological examination, interpreted diagnostic imaging findings, and finalized the report. MJS performed neurological examination, interpreted diagnostic imaging findings, and finalized the report. AT supervised neurological examination and interpretation of diagnostic imaging findings, contributed to conception and design of the study, and finalized the report. All authors agree to the authorship and the publication of the report. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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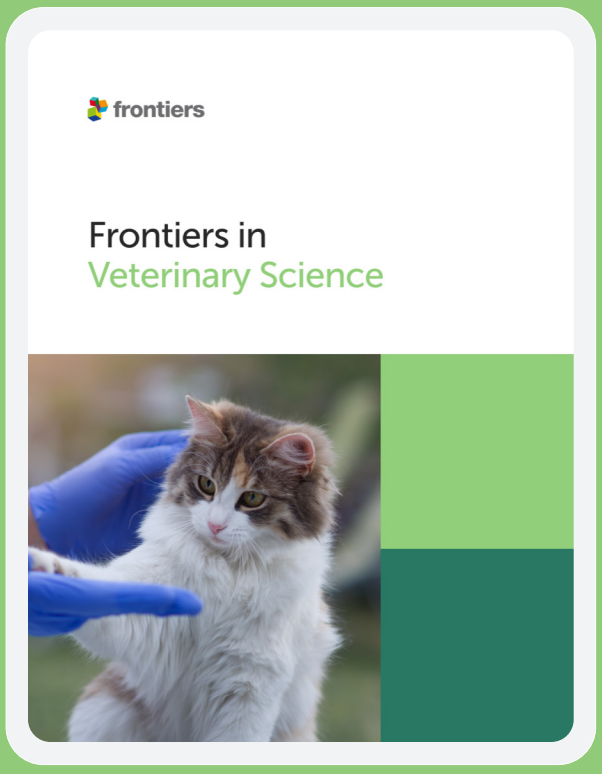

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