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Immunohistochemical evaluation of fibrin/fibrinogen, D-dimers, and intravascular thrombosis in brains of dogs with meningoencephalitis of unknown origin

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ABSTRACT

Granulomatous meningoencephalitis (GME) and necrotizing encephalitides (NE) are the most common immune-mediated inflammatory diseases of the central nervous system in dogs. Activation of the fibrinolytic system in multiple sclerosis, a similar immune-mediated disease affecting the central nervous system in humans, seems to be related to disease progression. The aim of this study was to identify fibrin/fibrinogen and D-dimer deposition, as well as presence of intravascular thrombosis (IVT) in brains of dogs with a diagnosis of GME or NE. Immunohistochemical studies using antibodies against fibrin/fibrinogen and D-dimers were performed. Statistical analyses were performed to determine whether there were differences in the presence and location of fibrin/fibrinogen, D-dimers deposits, and IVT between GME and NE. Samples from sixty-four dogs were included in the study: 32 with a diagnosis of GME and 32 with a diagnosis of NE. Fibrin/fibrinogen depositions were detected in all samples and D-dimers were detected in 43/64 samples. IVT was present in 29/64 samples, with a significantly higher score in samples from dogs with NE than in samples from dogs with GME ($P = 0.001$). These data support hemostatic system activation in both diseases, especially NE. This finding might be related to the origin of the necrotic lesions seen in NE, which could represent chronic ischemic lesions. Further studies are needed to investigate the association between vascular lesions and the histopathological differences between GME and NE and the hemostatic system as a potential therapeutic target.

Introduction

Inflammatory diseases are among the most common causes of central nervous system (CNS) dysfunction in dogs (Fluehmann et al., 2006). Granulomatous meningoencephalitis (GME) and necrotizing encephalitides (NE) are different subtypes of meningoencephalitis of unknown origin (MUO) and histopathological analysis is essential for a definitive diagnosis (Tipold, 1995; Granger et al., 2010; Talarico and Schatzberg, 2010; Coates and Jeffery, 2014). GME is characterized by concentric perivascular accumulations of mononuclear inflammatory cells, mainly macrophages with smaller numbers of lymphocytes and plasma cells, predominantly affecting the CNS white matter. NE include necrotizing meningoencephalitis (NME) and necrotizing leukoencephalitis (NLE), which are differentiated by the distribution and appearance of the microscopic lesions (Summers et al., 1995; Vandevelde et al., 2012). The asymmetric necrotic lesions affecting the cerebral white or gray matter in the NE are the main histopathological difference between these and GME.

Inflammation and coagulation are two systems that cannot be considered separately, since extensive cross-talk exists between them (Cicala and Cirino, 1998; Esmon, 2008; Levi and Van Der Poll, 2005, 2010; O'Brien, 2012). The inflammatory response causes release of tissue factor and a downregulation of the physiological anticoagulant pathways. At the same time, coagulation proteases and anticoagulant proteins interact with cell receptors inducing the synthesis of pro-inflammatory cytokines (Levi and Van Der Poll, 2005, 2010).

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Thereby, inflammation influences coagulation while coagulation activates the inflammatory response.

Similarities have been noted between MUOs in dogs and multiple sclerosis (MS) in people such as their idiopathic origin, the female predilection, young age of the patients, and the same MHC class II genetic association between NME in Pugs and human MS (Greer et al., 2010; Church et al., 2021). MS is one of the most common causes of neurologic disability in young adults that results from an autoimmune disease of the CNS (World Health Organization. and Multiple Sclerosis International Federation., 2008). An association between the dysregulation of the hemostatic system and the development and progression of has been described in both humans with MS (Wakefield et al., 1994; Gverić et al., 2003; Vos et al., 2005; Aksungar et al., 2008; Göbel et al., 2016; Yates et al., 2017; Petersen et al., 2018) and in the most commonly used experimental model for MS, experimental autoimmune encephalomyelitis (EAE) in rodents (Han et al., 2008; Davalos et al., 2014). Blood-brain barrier (BBB) disruption in MS causes extravasation and deposition of serum fibrin/fibrinogen in the brain parenchyma. In addition to their role in the coagulation cascade, fibrin/fibrinogen induce activation of the microglia, axonal damage, and demyelination, and hinders axonal regeneration (Yates et al., 2017; Petersen et al., 2018). Deposition of fibrin/fibrinogen and D-dimers, and presence of intravascular thrombosis (IVT) in canine gliomas (de la Fuente et al., 2014) and meningiomas (Font et al., 2015) has been described, but information is lacking about their occurrence in canine MUOs.

Coagulation factor dysregulation and impaired fibrinolysis is also important in the development of MS (Gverić et al., 2003; Aksungar et al., 2008; Han et al., 2008; Davalos et al., 2014; Göbel et al., 2016). The upregulation of some factors in the coagulation cascade, such as factor X or thrombin, is linked to neuroinflammation and progression in MS and EAE (Davalos et al., 2014; Göbel et al., 2016). D-dimers, a specific degradation product of the turnover of cross-linked fibrin, are a sensitive marker of activation of coagulation and fibrinolysis (Weitz et al., 2017). Serum D-dimer concentrations are increased in MS patients (Aksungar et al., 2008). While in dogs, 54% (n = 20/37) of dogs with inflammatory/infectious CNS disease had detectable D-dimer concentrations in their CSF, whereas D-dimers were not detectable in the CSF of control dogs (de la Fuente et al., 2012).

The aim of this study was to evaluate the presence and pattern of fibrin/fibrinogen and D-dimer deposition in brain samples of dogs with a diagnosis of GME and NE using immunohistochemical techniques, to evaluate the presence of IVT in these samples, and to compare the presence and location of these features between the two conditions.

Materials and methods

Case selection

Our veterinary neuropathology database was searched to identify dogs with a histopathological diagnosis of GME and NE, excluding dogs with focal inflammation involving the optic nerve or spinal cord, made by an ECVP board-certified pathologist (MP), for which there was archival tissue suitable for immunohistochemical studies between 2001 and 2019.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (3 μm thick) were used. The immunohistochemical procedures, using antibodies against fibrin/fibrinogen (A0080, Dako) and D-dimers (ABS 015–22–02, Thermo Fisher Scientific) were the same as those described previously for canine gliomas and meningiomas (de la Fuente et al., 2014; Font et al., 2015). Sections from a canine brain with intra-parenchymal hemorrhage were used as a positive control. For a negative control, an isotype-specific immunoglobulin was used as a substitute for the primary antibody. Healthy canine brain tissue was used to determine normal distribution of fibrin/fibrinogen and D-dimers in the CNS.

Fibrin/fibrinogen deposition was graded using a semiquantitative score for intensity of immunostaining and location of deposits. All samples were evaluated under 20x objective lens. Fibrin/fibrinogen deposition was graded as follows: score 0, no staining; score 1, mild (pale) staining; score 2, moderate (evident) staining; and score 3, severe (strong) staining. Location of deposits was classified as intracellular (inside glial cells), extracellular (extracellular space), or mixed (both intra- and extracellular in the same sample).

D-dimer immunoreactivity was classified as positive if D-dimer deposits were observed in the sample, or negative if there were no D-dimer deposits. IVT was considered to be present when a vessel was occluded by a deposit of D-dimers. Twenty high power fields (HPF; 40x objective lens) within the main lesion were evaluated and were graded as follows: score 0: no vessels occluded; score 1: at least 1 but < 33% of total vessels occluded; score 2: 33–66% of total vessels occluded; and score 3: > 66% of total vessels occluded.

Statistical analysis

Variables were tabulated according to sample GME / NE classification. Statistical analyses were performed using a Fisher's Exact test to compare the location of fibrin/fibrinogen deposits and the presence of D-dimer deposits between GME and NE samples. For ordinal variables, a Mann-Whitney U test was used to compare the scores of fibrin/fibrinogen immunostaining and the grade of IVT between GME and NE samples. All statistical analyses were performed using a statistical software package (SPSS version 26, IBM Corp). Statistical significance was set at $P < 0.05$.

Results

Case selection

Samples from 64 dogs were included in the study. Dogs were aged between 6 months and 11 years (median 4 years). Breeds included Yorkshire terrier (n = 13), Crossbreed (n = 9), Chihuahua (n = 6), Maltese (n = 6), French bulldog (n = 5), Alaskan Husky (n = 2), Belgian shepherd (n = 2), Cocker spaniel (n = 2), Shih tzu (n = 2), Pug (n = 2), West Highland white terrier (n = 2), Miniature poodle (n = 2), and one of each of German shepherd, Pomeranian, Greyhound, Labrador retriever, English Springer spaniel, Rottweiler, Miniature Pinscher, Parson Russell terrier, Dachshund, Samoyed, and Old English sheepdog. Thirty-two dogs had a diagnosis of GME, and 32 dogs a diagnosis of NE. Within the NE group, 15 had a diagnosis of NME and 17 a diagnosis of NLE.

Fibrin/fibrinogen immunostaining

Fibrin/fibrinogen deposits were detected in all samples, with a score of 3 in 35/64 (55%) samples, a score of 2 in 22/64 (34%) samples, and a score of 1 in 7/64 (11%) samples (Table 1). No fibrin/fibrinogen deposits were detected in the control tissue. There was no significant difference in fibrin/fibrinogen scores between the GME and the NE groups (P = 0.801).

Two different patterns of fibrin/fibrinogen depositions were detected. In one, fibrin/fibrinogen deposits were primarily located inside glial cells (reactive astrocytes/gemistocytes and microglia/macrophages) combined with a more diffuse staining usually associated with areas of loss of brain parenchyma (Fig. 1A, B). The other was a reticular pattern of fibrin/fibrinogen deposits in the extracellular space, mainly surrounding blood vessels and forming perivascular cuffs (Fig. 1C). When both patterns were present, deposits were classified as mixed (Fig. 1D). There was a significant difference in the location of fibrin/fibrinogen deposits between GME and NE groups (P < 0.001; Table 1), with extracellular deposits being present in 18/32 (56%) of GME samples as

Table 1
Comparison of fibrin/fibrinogen deposition, fibrin/fibrinogen pattern, presence of extracellular D-dimers, and intravascular thrombosis (IVT) between granulomatous meningoencephalitis (GME) and necrotizing encephalitides (NE).

	GME ($n = 32$)	NE ($n = 32$)	P
Fibrin/fibrinogen deposition[a]	Score 1: 4 (13%) Score 2: 11 (34%) Score 3: 17 (53%)	Score 1: 3 (9%) Score 2: 11 (34%) Score 3: 18 (56%)	0.801
Fibrin/fibrinogen pattern	Extracellular: 18 (56%) Intracellular: 5 (16%) Mixed: 9 (28%)	Extracellular: 6 (19%) Intracellular: 20 (63%) Mixed: 6 (19%)	< 0.001
Presence of extracellular D-dimers	Yes: 10 (31%) No: 22 (69%)	Yes: 8 (25%) No: 24 (75%)	0.782
IVT[b]	Score 0: 24 (75%) Score 1: 4 (13%) Score 2: 3 (9%) Score 3: 1 (3%)	Score 0: 11 (34%) Score 1: 9 (28%) Score 2: 8 (25%) Score 3: 4 (13%)	0.001

GME, Granulomatous meningoencephalitis; NE, Necrotizing encephalitides; IVT, Intravascular thrombosis

[a] Samples with an absence of staining received a score of 0; samples with mild staining received a score of 1; samples with moderate staining received a score of 2; and samples with strong staining received a score of 3.

[b] Samples with no vessels occluded received a score of 0; samples with at least one but < 33% of total vessels occluded received a score of 1; samples with 33–66% of total vessels occluded received a score of 2; and samples with > 66% of total vessels occluded received a score of 3.

compared to 6/32 (19%) of NE samples and glial cell deposits in 20/32 (63%) of NE samples as compared to 5/32 (28%) of GME samples.

D-dimers immunostaining and IVT

D-dimer deposition was detected in 43/64 (67%) samples (Table 1). Extravascular D-dimer deposition was observed in 14 samples, intravascular deposition in 25 samples, and a further four samples with both intra- and extravascular D-dimer deposition. Extravascular D-dimer immunostaining showed a reticular pattern in the extracellular space and co-localized with the fibrin/fibrinogen deposition (Fig. 2A-C). Intracellular D-dimer immunoreactivity was not observed in any sample. D-dimers were not detected in control tissue. There was no significant difference in extravascular D-dimer presence between GME and NE groups ($P = 0.782$).

IVT was detected in the microvasculature within the lesions of 29/64 (45%) samples, comprising 8 with GME and 21 with NE (Fig. 2D, F). Only five (17%) of the positive samples had a score of 3, of which four had NE (Table 1). The IVT score was significantly higher in the NE group than the GME group ($P = 0.001$).

Discussion

Fibrin/fibrinogen deposits were found in all samples of the study, as was found in previous studies on MS and EAE (Vos et al., 2005; Marik et al., 2007; Yates et al., 2017). The presence of fibrin/fibrinogen in the extracellular compartment in GME, mostly perivascular, could be related to the acute damage of blood vessels and the breakdown of the BBB. This is similar to what happens in the early stages of MS, preceding the demyelinating stage (Marik et al., 2007). In NE, the fibrin/fibrinogen deposits were located mostly within glial cells, particularly reactive astrocytes and gitter cells. The presence of fibrin/fibrinogen deposits within astrocytes is suspected to be a result of phagocytosis (Hsiao et al., 2013) and is consistent with the findings of a study on progressive MS, in which fibrin/fibrinogen were found in glial cell processes and within cell bodies in the more chronic stages of the disease (Yates et al., 2017). The results of the study described herein support GME and NE as naturally-occurring disease models of MS and evidenced the more chronic nature of the NE lesions compared with the GME lesions.

Some workers have reported an increase in serum D-dimer concentrations in patients with MS, which reflects an alteration in the coagulation system (Aksungar et al., 2008). Patients with relapsing-remitting

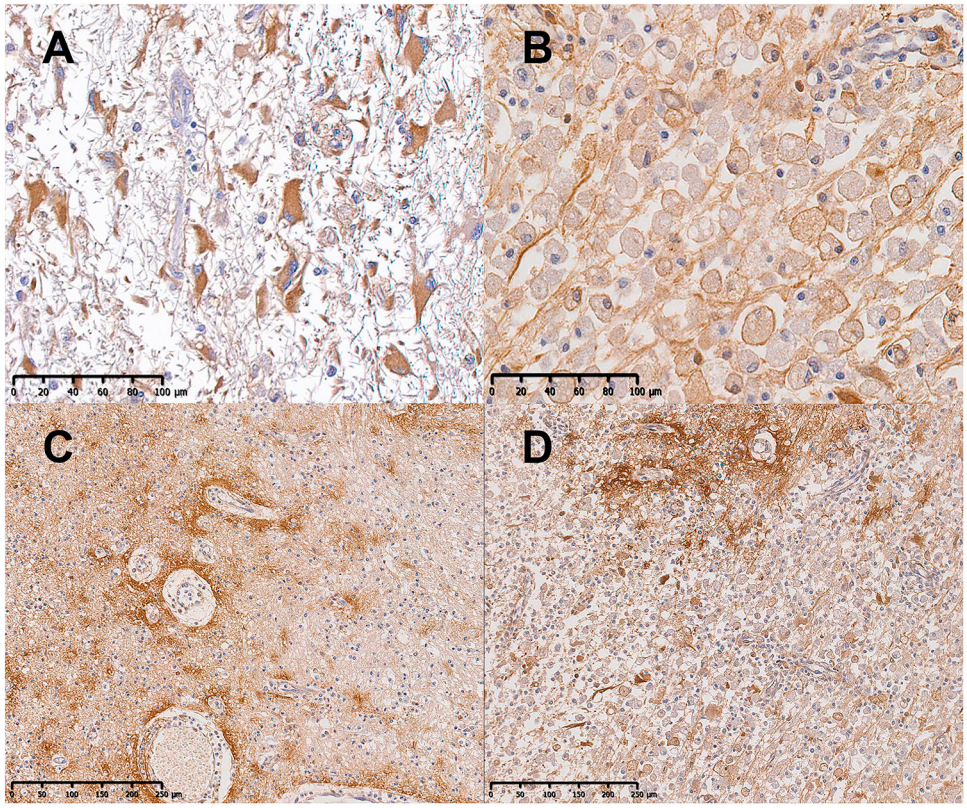

Fig. 1. Images showing fibrin/fibrinogen immunohistochemistry. Brown color indicates immunopositivity. (A) Fibrin/fibrinogen immunolabelling inside activated astrocytes in a necrotic lesion of a dog with necrotizing leukoencephalitis (NLE) (score 3). (B) Fibrin/fibrinogen deposition accumulated by gitter cells in a necrotic lesion of a dog with NLE (score 2). (C) Fibrin/fibrinogen immunolabelling with reticular patterns filling the extracellular space and surrounding the perivascular cuffs in a dog affected with granulomatous meningoencephalitis (score 3). (D) Fibrin/fibrinogen deposits in a dog affected with a necrotizing meningoencephalitis showing a mixed pattern, comprising a focal extracellular reticular pattern surrounding blood vessels and deposits inside gitter cells associated with a necrotic lesion (score 3).

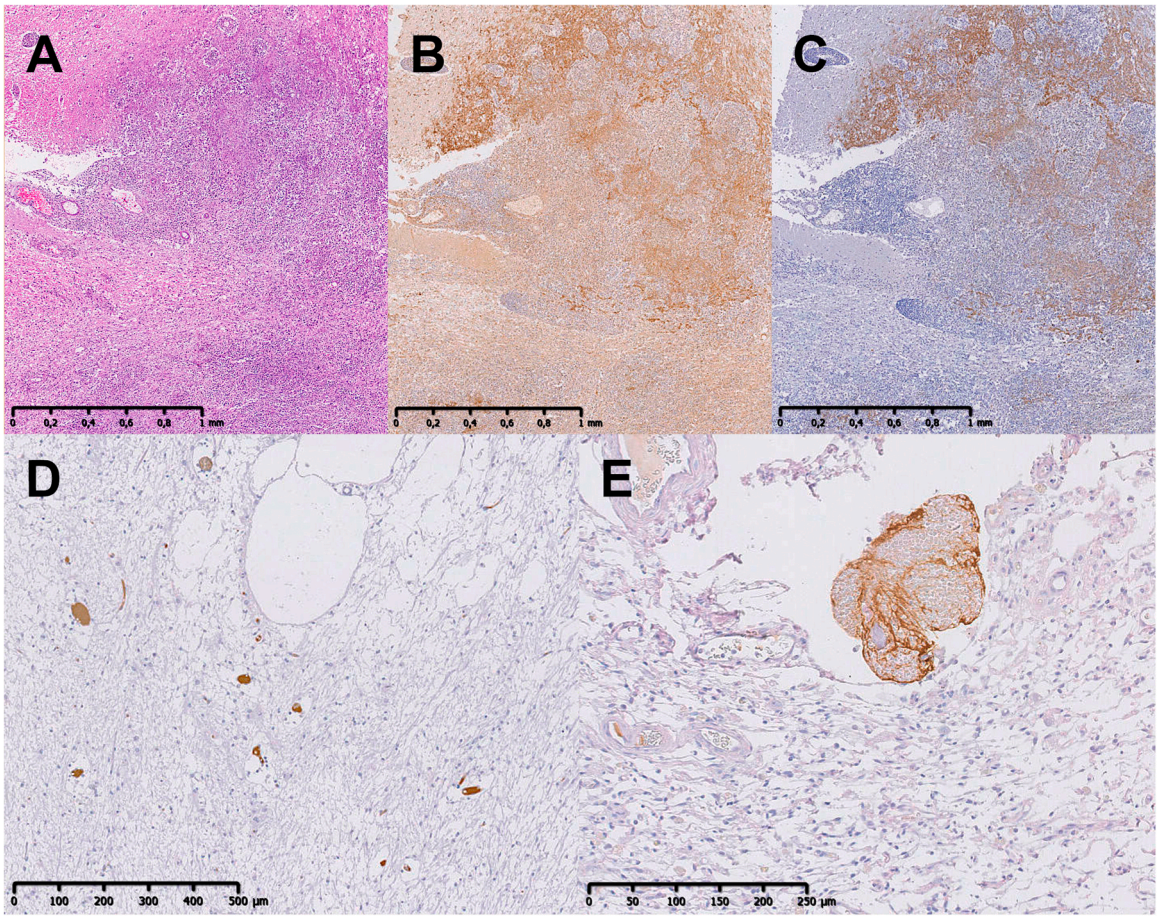

Fig. 2. (A) Hematoxylin and eosin staining, (B) fibrin/fibrinogen immunostaining, and (C) D-dimer immunostaining of the same region in a dog affected with granulomatous meningoencephalitis. Brown color indicates immunopositivity. Note the presence of both (fibrin/fibrinogen and D-dimer) deposits with a reticular pattern, but with more focal D-dimer immunostaining. (D) D-dimer immunohistochemistry in a dog with necrotizing meningoencephalitis, demonstrating D-dimer deposits inside blood vessels producing vascular occlusion close to a necrotic lesion (score 3). (E) Higher magnification of the dog in (D) with positive staining for D-dimer structure compatible with a thrombus in a larger vessel.

MS also have increased intrathecal CSF D-dimer concentrations, when compared with disease controls (Schaller-Paule et al., 2022), as was also noted in patients with other inflammatory diseases such as bacterial and viral meningitis and central nervous system neoplasia. Serum and CSF D-dimer concentrations have been measured in dogs with various neurological diseases (De la Fuente et al., 2012), with MUOs included in a diverse group of inflammatory CNS diseases that included bacterial meningomyelitis, distemper virus meningoencephalomyelitis, and acute idiopathic polyradiculoneuritis. Twenty of the 37 dogs in this group had detectable D-dimer concentrations in the CSF, but this was not significantly different from the control group. D-dimer immunostaining was found in two-thirds of the cases in our study, suggesting local activation of the coagulation and fibrinolytic system in GME and NE. Future studies could assess CSF D-dimer concentrations in MUO patients and compare the results between GME and NE patients.

The main histopathological difference between GME and NE is the presence of necrotic lesions in the latter. The main hypothesis to explain this is the chronicity of the inflammation in the NE, which entails phagocytosis of the affected nervous tissue forming cavitary lesions (Higgins et al., 2008). There are no other inflammatory CNS diseases with large necrotic areas as those seen in NE, regardless of the chronicity of the condition. Only chronic ischemic lesions show a microscopic appearance resembling necrotic NE lesions (Kuwamura et al., 2002; Vandevelde et al., 2012). IVT was detected in half of the samples in this study, and most of them were NE samples. This result indicates that abnormal thrombotic activity is present in dogs with inflammatory CNS disease, especially with NE, and this could explain the necrotic lesions being secondary to ischemic damage.

Most infarcts occur because of obstruction of a blood vessel, although some of them might be secondary to inflammatory lesions within the vessel wall (Vandevelde et al., 2012). Capillary damage caused by perivascular cuffs can be associated with the presence of thrombi, which can lead to ischemic insults and may explain the presence of necrotic lesions in NE. There has only been one case report of isolated angiitis in the CNS of a dog (Sasaki et al., 2003). In that case, the microscopic appearance of the lesions shared greater similarity with GME than NE. Lympho-histiocytic meningoencephalitis of unknown etiology with vasculitis of the small and medium vessels of the brain parenchyma has recently been described in three dogs, and is considered a new subtype of MUO (Zdora et al., 2022). The higher frequency of IVT in NE could indicate a greater impact on blood vessels than GME. However, endothelial / vascular damage in GME and NE samples was not investigated in this study, therefore no definitive explanation was elucidated for the origin of increased IVT in NE.

Recognition of the relationship between the hemostatic system and the development of MS lesions led to the investigation of different therapeutic targets to slow down progression of disease and thus improve prognosis. Fibrin depletion in transgenic mouse models of MS led to decreased inflammation and reduced demyelination (Akassoglou et al., 2004), and the use of recombinant activated protein C decreased the severity of lesions in EAE, due to its anticoagulant action (Han et al., 2008). In human medicine, people with MS are at increased risk of

cerebrovascular accidents, especially ischemic ones (Belliston et al., 2018; Hong et al., 2019). Treatment with acetylsalicylic acid shows positive effects, such as decreased possibility of ischemic stroke and thromboembolism or reduced inflammation, but also shows negative effects, such as increased risk of hemorrhagic stroke (Tsau et al., 2015). Our results suggest that the coagulation and fibrinolytic systems are activated in GME and NE, and this opens a field of research for future studies to elucidate potential therapeutic targets in the coagulation cascade.

There are some limitations associated with this study. Due to its retrospective nature, antemortem treatments were not documented and could have included glucocorticoids, cytosine arabinose, nonsteroidal anti-inflammatory drugs, and others that could have affected the microscopic findings and the immunostaining results. The subjective semi-quantitative nature of the assessment of the immunohistochemical results may lead to small inaccuracies. Additionally, due to the long period chosen to obtain the greatest number of cases possible, the preservation of the oldest samples may have influenced the immunohistochemical staining.

Conclusions

IVT is present in GME and NE, suggesting activation of the coagulation and fibrinolytic system in these diseases. This finding is especially evident in NE and could be due to ischemic damage causing the necrotic lesions present in these diseases. Future studies should be performed to elucidate the presence of vascular lesions in NE antemortem, and the hemostatic system as a potential therapeutic target in GME and NE.

Declaration of Competing Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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