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STANDARD ARTICLE

A potential early clinical phenotype of necrotizing meningoencephalitis in genetically at-risk pug dogs

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Abstract

Background: Necrotizing meningoencephalitis (NME) in the pug dogs is a fatal neuro-inflammatory disease associated with rapid progression and poor response to conventional immunosuppressive therapy. Diagnosis is typically made after severe neurological abnormalities have manifested.

Hypothesis/Objective: Pug dogs at genetic risk for NME might manifest neurological abnormalities before developing pathognomonic clinical signs of NME.

Animals: Thirty-six pug dogs less than 4 years of age asymptomatic for NME.

Methods: Prospective observational cohort study with germline genome-wide genotyping. Neurological examinations were performed 4 weeks apart to document reproducible findings of central nervous system disease. Magnetic resonance imaging, cerebrospinal fluid analysis, and testing for infectious diseases were performed in all pugs with reproducible abnormalities detected on neurological examination.

Results: The overall risk allele frequency in this cohort was 40%; 5 (14%) dogs were high risk, 19 (53%) dogs were medium risk, and 12 (33%) dogs were low genetic risk for NME. Reproducible abnormalities detected on neurological examination were identified in 8/24 (33%) genetically at-risk dogs and 0/12 (0%) low risk dogs. Clinical abnormalities included multifocal spinal pain in 8/8, reduced menace response in 5/8, and lateralizing postural reaction deficits in 5/8 pugs. There was a strong association between genotype risk and the presence of this clinical phenotype ($P = .03$).

Conclusions and Clinical Importance: Our findings suggest the presence of a novel early clinical phenotype of NME in apparently asymptomatic genetically at-risk pugs which might be used to plan early diagnostic and therapeutic clinical trials.

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; DLA, dog leukocyte antigen; FLAIR, fluid attenuated inversion recovery; GME, granulomatous meningoencephalitis; GRE, gradient echo; MHC, major histocompatibility; MRI, magnetic resonance imaging; MS, multiple sclerosis; NME, necrotizing meningoencephalitis; PDE, pug dog encephalitis; SARDS, sudden acquired retinal degeneration syndrome; SNPs, single nucleotide polymorphisms; TKI, tyrosine kinase inhibitors.

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KEYWORDS

encephalitis, immune, inflammatory, meningitis

1 | INTRODUCTION

Necrotizing meningoencephalitis (NME) is a devastating fatal neuro-inflammatory disease described approximately 30 years ago in pugs,¹ hence the term pug dog encephalitis (PDE). NME occurs in several breeds including the yorkshire terrier,² maltese,³ chihuahua,⁴ pekingese,⁵ papillon,⁶ shih tzu,⁶ coton de tulear,⁶ and brussels griffon.⁶ Neurological signs of NME are believed to arise rapidly and include seizures, circling, visual deficits, behavior change, and lethargy.^{1,7} These conventional signs typically manifest before 6 years of age with a median onset of 1.5 to 2.5 years.^{7,8} Antemortem diagnosis is typically achieved via magnetic resonance imaging (MRI), which classically demonstrates loss of gray/white matter distinction, parenchymal inflammatory/necrotic lesions, and leptomeningeal contrast enhancement.⁹⁻¹¹ Cerebrospinal fluid (CSF) is abnormal in many of these dogs and typically includes a lymphocytic inflammation.^{4,7,12}

Genetic risk for NME has been linked to chromosome 12 within the dog leukocyte antigen (DLA) major histocompatibility (MHC) II complex^{8,13,14} with additional association to chromosome 15 in the pug and chromosome 4 in the maltese.¹⁵ MHC II gene mutations are also associated with increased risk for multiple sclerosis (MS) in humans, and NME in pugs mimics the clinical, imaging, and pathological characteristics of the acute fulminant or nonprototypic forms of MS.¹⁶ The incidence of NME in pugs is approximately 1% to 2%, whereas the incidence of the high-risk haplotype in a homozygous state has a reported range of 6% to 18%, suggesting a multifactorial disease.^{13,16,17}

There are systemic inflammatory changes in asymptomatic but genetically at-risk pugs.¹⁷ This led to the hypothesis that young asymptomatic genetically at-risk pugs might have subclinical brain inflammation associated with the systemic inflammatory signature. Furthermore, we hypothesized that these young genetically at-risk pugs might have subtle abnormalities on neurological examination that precede the conventionally recognized clinical signs typically associated with NME. Accordingly, we conducted a prospective cohort study of young, apparently asymptomatic pugs to define genomic risk and evaluate if concurrent blinded neurological examination would identify subtle abnormalities in the at-risk genotypes.

2 | MATERIALS AND METHODS

Pug dogs less than 4 years of age that were described as neurologically normal by their owners participated in a prospective observational cohort study. After obtaining informed owner consent, dogs were enrolled in the study, which had received animal care and use committee approval (Animal Clinical Investigation ACUC, Chevy Chase, Maryland).

Historical information including location of adoption and any previous medical background was obtained from the owners. On the day

of the initial examination, whole blood was collected and transferred to 2 tubes containing EDTA (for CBC and germline risk assessment) and 1 serum separator tube (for serum biochemistry). Post hoc genotyping confirmed pug breed in all dogs in this prospective cohort.

All pugs received 2 physical and neurological examinations, spaced 4 to 6 weeks apart, by a board-certified veterinary neurologist who was blinded to the genotype risk. All abnormalities on neurological examination were recorded and a neuroanatomical localization was determined when applicable. Pugs with a cerebral or multifocal central nervous system (CNS) localization with reproducible abnormalities on serial neurological examinations were selected for further evaluation by MRI, CSF analysis, and infectious disease testing and underwent a third neurological examination on the day the dog was admitted for advanced diagnostics.

2.1 | NME risk genotype assessment

Genomic DNA was isolated from frozen anticoagulated whole blood collected in K₂EDTA coated tubes (BD, Franklin Lakes, New Jersey) via magnetic bead-based purification using the Chemagic 360 (PerkinElmer, Waltham, Massachusetts) and the DNA Blood 400 Kit H96 (PerkinElmer, catalog #: CMG-1091). Briefly, whole blood was thawed on ice, gently mixed, and 400 μ L was pipetted into individual wells of a 96-deep-well plate. Protease (PerkinElmer, part of the kit: catalog #: CMG-1091) was then added to each well before being processed on the Chemagic 360 using the 96-rod head according to the manufacturer's protocol. DNA was eluted in \sim 150 μ L of Elution Buffer (PerkinElmer) and concentration and quality were assessed. The average DNA yield reported via NanoDrop One (ThermoFisher, Waltham, Massachusetts) was 65.9 ng/ μ L, the average 260 : 280 wavelength ratio was 1.91, and the average 260 : 230 wavelength ratio was 2.40. Seven randomly selected DNA samples were further evaluated using Genomic Tape (Agilent, Santa Clara, California) on the TapeStation 4200 instrument (Agilent). Each sample was considered high quality as the average DNA integrity number (DIN) across the 7 samples was 8.93.

According to the manufacturer's protocol, approximately 200 ng of DNA from all samples was processed for genotyping using the CanineHD Whole-Genome Genotyping BeadChip (Illumina, San Diego, California). BeadChips were scanned on the iScanSystem (Illumina) to generate IDAT files. Genotype calling was carried out using GenomeStudio (Illumina) per the manufacturer's recommendations. The resulting genotype data were exported to PLINK format according to the *CanFam3.1* assembly and was quality controlled to include single nucleotide polymorphisms (SNPs) with cross-cohort missingness \leq 95% and samples with a genotyping rate of \geq 90%.

Principal components analysis was performed to identify non-purebred dogs, and cohort-wide relatedness was assessed using a

pi-hat metric to identify any cryptic relatedness (defined as a pi-hat ≥ 0.2). After all quality control metrics were applied, no animals were removed from the analysis yielding a final cohort size of 36 animals. To identify an NME risk-associated haplotype, we extracted SNPs from the pug NME GWAS.¹⁸ We selected SNPs that were located within a 500 kb region centered on the top significant SNP association, BICF2P194998.¹⁸ This yielded a total of 25 SNPs each from 22 cases and 86 controls after selecting for samples and SNPs with a genotyping rate $>95\%$, and SNPs with a Hardy-Weinberg equilibrium $P > 1.0E-05$, and MAF > 0.05 .¹⁸ Linkage Disequilibrium (LD) blocks were estimated in this entire cohort of samples using Gabriel's method¹⁹ in *Haploview* v4.2.²⁰ We identified an LD block of 146 kb encompassing 7 SNPs including the top pug NME GWAS SNP, BICF2P194998.¹⁷ After conducting haplotype association analysis in *Haploview*, we found 5 haplotypes within this LD block in the Barber et al cohort: 1 associated with increased NME risk (88.6% frequency in cases, 27.1% frequency in controls; $P = 1.3E-13$), and the remaining haplotypes were associated with decreased NME risk.^{13,16,17} To assess NME risk in this study cohort, we extracted the same 7 SNPs from the Illumina Canine HD BeadChip data and haplotypes were phased using the default options in Beagle 5.2.²¹ Dogs in the cohort are defined as having low genomic risk (no genomic risk allele identified) and at genomic risk (presence of 1 or 2 risk alleles).

2.2 | Magnetic resonance imaging

Magnetic resonance imaging was acquired in pugs determined to have reproducible signs on neurological examination using a 1.5 Tesla MRI (Siemens MAGNETOM Symphony). MRI sequences of the brain included T1-weighted pre and postcontrast sagittal, transverse, and dorsal planes, T2-weighted sagittal and transverse planes, and fluid attenuated inversion recovery (FLAIR) and gradient echo (GRE) transverse planes. Cervical and thoracolumbar spinal cord imaging was obtained using T2-weighted and short tau inversion recovery (STIR) sagittal and transverse planes and postcontrast T1-weighted sagittal plane. Magnetic resonance imaging was reviewed and reported independently by 2 board-certified neurologists, 1 of whom was blinded to abnormalities on neurological examination. Identical abnormalities detected by MRI were reported by both neurologists.

2.3 | Cerebrospinal fluid analysis and infectious disease testing

Cerebrospinal fluid was collected aseptically from the cerebellomedullary cistern using a 22 gauge 1.5 in. spinal needle in all 8 dogs undergoing MRI. A sample of CSF was submitted for cytological analysis and clinical pathologist review to a commercial laboratory (Zoetis Reference Laboratory). A neurological polymerase chain reaction (PCR) panel on CSF was submitted to detect the presence of *Anaplasma*, *Blastomyces*, *Bartonella*, *Cryptococcus*, *Ehrlichia*, *Histoplasma capsulatum*, *Canine Distemper Virus*, *Lyme disease*, *Neospora caninum*, and *Rickettsia rickettsii* deoxyribonucleic acid (DNA).

2.4 | Statistical analyses

Statistical analyses were conducted using GraphPad Prism 9.2.0. Pugs were divided into 2 groups: low genomic risk (no genomic risk allele) and at genomic risk (presence of 1 or 2 risk alleles). Distributions of continuous variables (age, weight, CBC abnormalities) within each treatment group were assessed by visualizing Q-Q plots and performing tests for normality (Anderson-Darling test, D'Agostino-Pearson test, Shapiro-Wilk test, Kolmogorov-Smirnov test). Variables that failed multiple tests for normality within at least 1 of the groups were analyzed using nonparametric Mann-Whitney tests, while variables that did satisfy criteria for a normal distribution were analyzed using 2-sample *T*-tests. Categorical variables (sex and neurological examination findings) were compared using Fisher's exact tests.

3 | RESULTS

3.1 | Haplotype-based NME risk profile

A total of 36 pugs were enrolled into the study. Using the haplotype information obtained for each dog at the chromosome 12 NME risk locus,^{8,13,15} we found that 5 (14%) were homozygous/high risk, 19 (53%) were heterozygous/medium risk, and 12 (33%) were low genetic risk, resulting in a risk haplotype frequency of 40% in this cohort with 24 dogs having increased genomic risk and 12 dogs having low genomic risk. No differences were observed between medium and high risk pugs with respect to any of variables included in this study (data not shown).

3.2 | Signalment characteristics

The age and weight findings of the dogs are described in Table 1. There were a higher number of males (11/12, 91.7%) than females (1/12, 8.3%) in the low risk group ($P = .01$). There were 11 males and 13 females in the at-risk group.

3.3 | Clinical pathology findings

CBC results were abnormal in 19 dogs and included mild neutrophilia in 10 dogs, monocytosis in 6 dogs, lymphocytosis in 5 dogs, and thrombocytosis in 4 dogs. There were no differences in the CBC

TABLE 1 Age and weight characteristics

Variable	Group	N	Mean	SD	P-value T-test
Age (years)	Low risk	12	1.86	0.98	.92
	At-risk	24	1.83	0.90	
Weight (kg)	Low risk	12	8.33	1.3	.17
	At-risk	24	9.17	1.8	

TABLE 2 CBC abnormalities

Variable	Group	N	Mean	SD	P-value T-test
Neutrophils (10 ³ /μL)	Low risk	12	7107	998	.66
	At-risk	24	7407	2222	
Lymphocytes (10 ³ /μL)	Low risk	12	3866	1757	.42
	At-risk	24	3405	1515	
Monocytes (10 ³ /μL)	Low risk	12	720	315	.81
	At-risk	24	693	321	
Platelets (10 ³ /μL)	Low risk	12	332 333	75 746	.67
	At-risk	24	343 042	68 092	

TABLE 3 Neurological examination findings

	Group		P-value Fisher's exact test
	Low risk	At-risk	
Menace deficit			
No	12 (100%)	19 (79.2%)	.15
Yes	0 (0%)	5 (20.8%)	
Right-sided	0	4	
Left-sided	0	1	
Spinal pain			
No	12 (100%)	16 (66.7%)	.03
Yes	0 (0%)	8 (33.3%)	
CP deficits			
No	12 (100%)	19 (79.2%)	.15
Yes	0 (0%)	5 (20.8%)	
Pelvic limbs only	0	2	
Thoracic and pelvic limbs	0	3	
Ataxia			
No	12 (100%)	20 (83.3%)	.28
Yes	0 (0%)	4 (16.7%)	
General proprioceptive	0	3	
Pelvic limb	0	1	

abnormalities between the genomic risk groups (Table 2). Abnormalities on biochemistry included abnormally high BUN in 8 dogs, abnormally high ALT and AST in 2 dogs, and abnormally high ALP in 1 dog.

3.4 | Physical and neurological examination findings

General physical examinations were normal in all pugs other than mild stertor in 6 pugs. No apparent ocular abnormalities were identified in any of the pugs. Twenty-one of 36 (58%) pugs had no neurological abnormalities on either examination, including 12/12 (100%) dogs in the low genetic risk group and 9/24 (38%) dogs in the at-risk group. Seven (29%) of the at-risk pugs had abnormalities on the first

neurological examination that were not present on the second examination which included mild pelvic limb proprioceptive ataxia in 1 pug, mild tetraparesis and generalized proprioceptive ataxia in 2 pugs, lateralizing pelvic limb conscious proprioceptive (paw placement) deficits in 3 pugs, and mild spinal pain in 1 pug.

Neurological examinations were reproducibly abnormal on 2 serial examinations in 8/36 (22%) at-risk pugs compared to 0/12 pugs in the low genetic risk group ($P = .03$). The neurological examination abnormalities are summarized in Table 3. The only abnormality on neurological examination that met significance between the groups was the identification of multifocal spinal hyperesthesia ($P = .03$). However, the multifocal phenotype (characterized by any reproducible or progressive neurological examination finding) was more common in the at-risk genetic group compared to the low risk genetic group ($P = .03$).

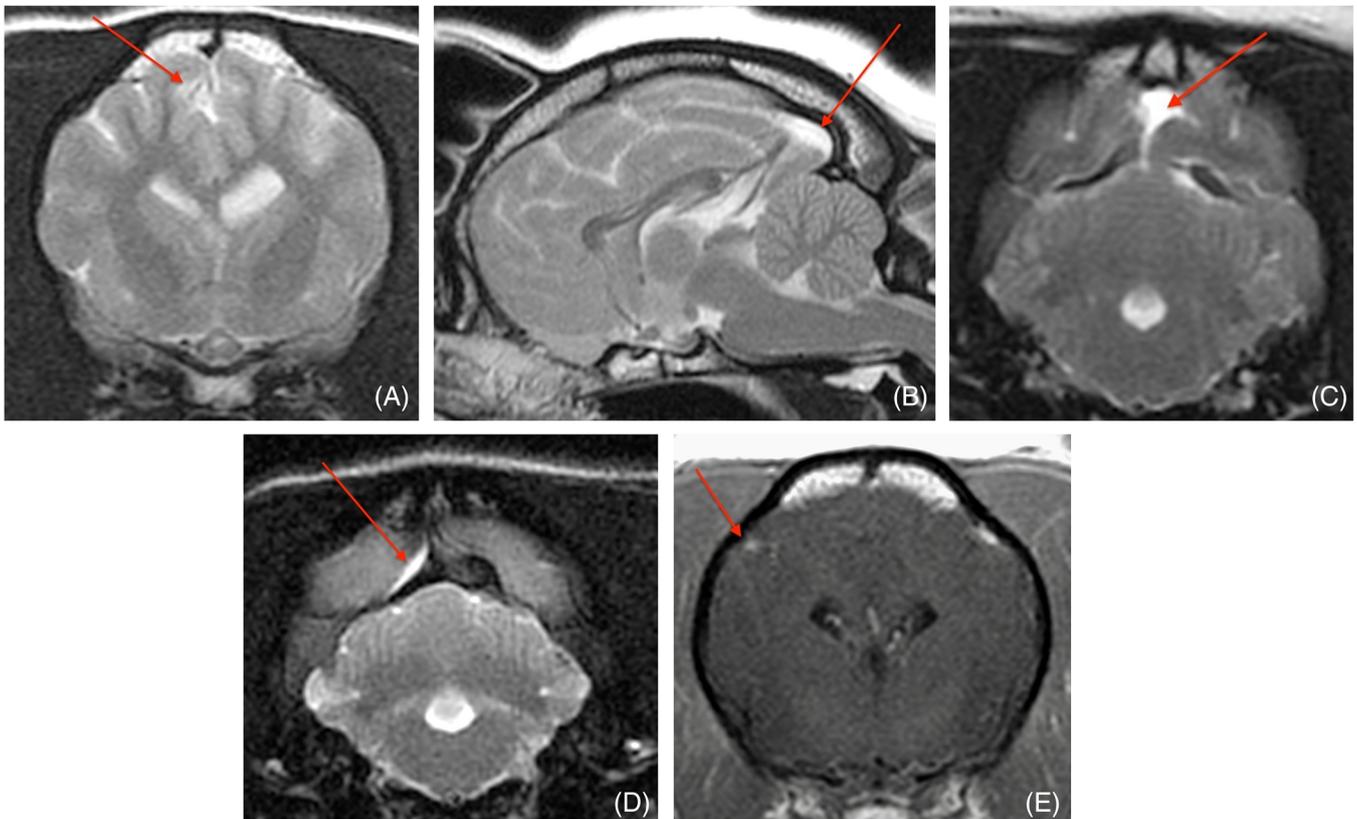


FIGURE 1 Examples of MRI parenchymal lesions from 4 dogs. (A) T2W hyperintense (pictured), T1W/FLAIR hypointense, mildly T1W contrast enhancing lesion in right parietal lobe. (B) Sagittal and (C) transverse: T2W hyperintense (pictured), FLAIR/T1W hypointense, mildly peripherally contrast enhancing lesion in the left occipital lobe. (D) T2W hyperintense (pictured), T1W/FLAIR hypointense, mildly T1W contrast enhancing lesion in right occipital lobe. (E) T1W contrast enhancing lesion in right parietal lobe

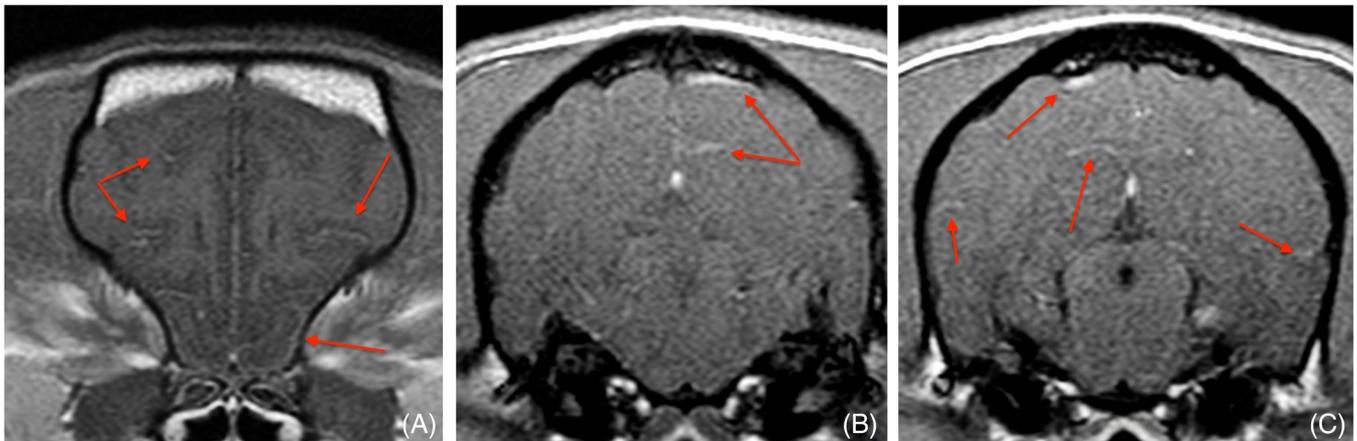


FIGURE 2 Examples of meningeal enhancement in 3 dogs. (A) Multifocal areas of pachymeningeal, leptomeningeal, and parenchymal enhancement throughout the frontal lobes. (B) Pachymeningeal and leptomeningeal enhancement in the right parietal lobe. (C) Multifocal areas of pachymeningeal, leptomeningeal, and parenchymal enhancement throughout the parietal and temporal lobes

3.5 | Magnetic resonance imaging results of neurologically abnormal pugs

MRI abnormalities potentially suggestive of early inflammatory lesions were noted in 6/8 dogs. The most consistent findings were focal areas

of T2-weighted hyperintensity, T1-weighted, and FLAIR hypointensity with variable mild T1-weighted contrast enhancement in the parietal lobes of 4 dogs and occipital lobes of 3 dogs (Figure 1A-E). Meningeal enhancement was present in the pachymeninges of 5 dogs and leptomeninges of 3 dogs and was most common in the parietal and

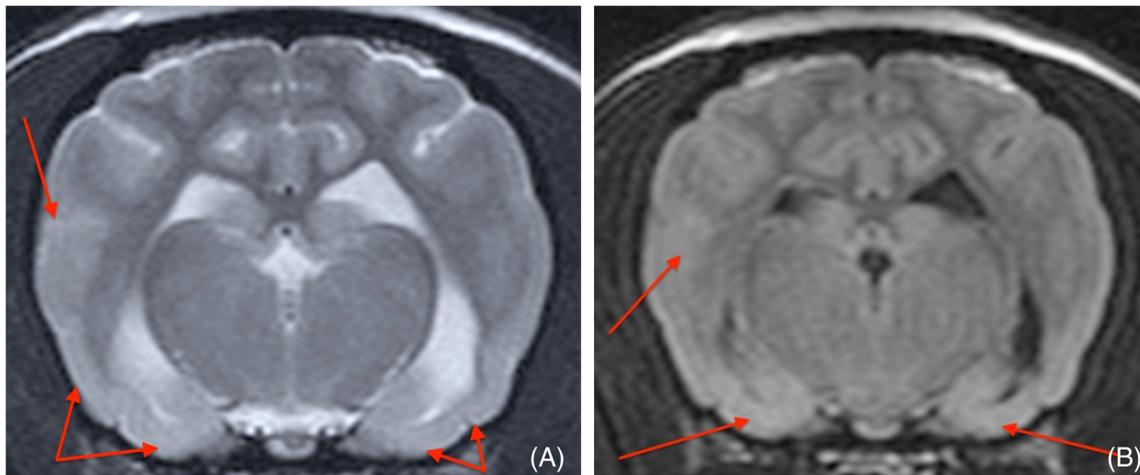


FIGURE 3 (A) T2W and (B) FLAIR images demonstrating diffuse T2W/FLAIR hyperintensity within the hippocampus, patchy T2W/FLAIR hyperintensity in the right parietal lobe with loss of gray/white matter distinction in 1 dog

occipital lobes (Figure 2A-C). One dog had mild diffuse T2W/FLAIR hyperintensity throughout the hippocampus bilaterally and mild patchy T2W/FLAIR hyperintensity in the right parietal lobe with loss of gray/white matter distinction (Figure 3A,B). There were no structural malformations in the brain to contribute to lateralizing menace or postural reaction deficits. Although all dogs had progressive multifocal spinal pain, no dog had any structural vertebral or spinal cord abnormalities such as congenital vertebral anomalies, intervertebral disc disease, subarachnoid diverticula, articular process dysplasia, syringomyelia or any overt evidence of spinal cord edema or fibrosis.

3.6 | Cerebrospinal fluid analysis

Abnormal results on CSF analysis were observed in 3 of the 8 pugs with neurological abnormalities. One dog had mild lymphocytic inflammation, another dog had an abnormally high protein concentration, and a third dog had a basophil in the CSF, which is a rarely reported cell type in the CSF of dogs. Infectious disease PCR testing was negative in all dogs.

4 | DISCUSSION

These findings suggest a potential early phenotype of NME in genetically at-risk pugs not exhibiting conventional clinical signs of NME. Necrotizing meningoencephalitis classically shows a poor response to immunosuppressive therapy and initiating treatment when conventional (advanced) signs manifest is typically associated with limited benefit, with maximum reported survival times of 6 to 7 months.^{7,22,23} Our ability to predict the likelihood of developing disease has until now been dictated by genetic testing alone. Assessing genetic predisposition is extremely valuable; however, genetic risk alone does not adequately predict the probability of developing disease. Accordingly our finding of an early clinical phenotype in genetically at-risk dogs

might provide opportunities for preventative care clinical trials for NME. The allele frequency reported here is higher than previous studies,⁸ likely due to a higher geographical distribution of NME in the western United States⁷ which might reflect breeding populations within the area. Accordingly, the value of the early clinical phenotype assessment might not be equal in all geographic regions.

Abnormalities on neurological examination were consistent among the pugs with signs of neurological disease, increasing our confidence that these could indicate an early inflammatory phenotype. Progressive diffuse cervical and thoracolumbar spinal pain was most consistent, present in all 8 pugs with signs of neurological disease. Given the absence of any structural spinal abnormalities on MRI, it is reasonable to attribute the origin of the pain to diffuse meningeal inflammation. Cervical rigidity is a reported sign of NME,^{1,24} but more diffuse cervical and thoracolumbar spinal pain has not been described. Spinal hyperesthesia was easy to assess in the mentally appropriate and responsive pugs in this study, whereas pugs with fulminant NME who present with seizures and severe obtundation often have reduced responsiveness to stimuli. Many histopathological NME studies make no comment on sampling the spinal cord parenchyma or meninges, making it possible that only the brain was examined.^{1,7,11,25-27} It is possible that spinal cord parenchymal and meningeal lesions also exist but have not been extensively evaluated given the technical challenge and time required to perform full CNS histopathological assessment.

MRI abnormalities in these pugs were identified most commonly in the parietal and occipital lobes, which is consistent with other reports documenting the prevalence of prosencephalic NME lesions in pugs.^{10,11,28} Proprioceptive somatosensory information is processed in the parietal lobes while visual information is processed in the occipital lobes. This correlates well with the abnormalities on neurological examination of lateralizing postural reaction deficits in 5/8 dogs and visual processing deficits in 5/8 dogs. Similar asymmetrical deficits occur with NME.⁵ Common ophthalmological diseases including corneal melanosis and sudden acquired retinal degeneration syndrome

(SARDS)²⁹ were ruled out during physical examination, making cortical blindness the likely cause for the reduced vision. Visual hemifield loss has been reported in a pug with NME weeks before onset of seizures, and there was histopathologic confirmation of NME several months later in the visual striated cortex of the right cerebrum.³⁰ Occipital and parietal lobe meningeal inflammation and necrosis are common with NME.^{1,10,11,26} Therefore, it is reasonable to assume that chronic subclinical visual and proprioceptive deficits might occur before developing seizures and other more severe signs of NME, making menace response and paw placement evaluation useful early screening tools.

Meningeal enhancement was identified in 5 dogs and occurs in the dura mater and leptomeninges of approximately half of dogs with NME.^{10,11} This meningeal enhancement correlates with histopathologically confirmed inflammatory infiltrates in the sulci and is noted most prominently in the cerebral sulci and longitudinal fissure.^{1,11} Contrast enhancement within parenchymal lesions is variable and mild.¹⁰ One dog in this study at high genetic risk for NME showed diffuse T2W/FLAIR hyperintensity, which correlates with histologically confirmed areas of inflammation or microscopic liquefaction.¹⁰

Histopathologic evaluation in dogs with NME reveals a large and more diverse distribution of inflammation than can be identified with MRI,^{7,10} therefore it is plausible that the MRI abnormalities identified in most dogs with fulminant NME are a more apparent representation of global brain lesions. Normal MRIs have been described in dogs that were subsequently diagnosed with NME histopathologically.⁴ There are sparse reports of serial MRI in dogs with NME, and description of MRI findings in early stages of NME is limited.^{7,27} An isolated report documented a subtle MRI lesion similar to those reported here that ultimately manifested as a pronounced necrotic lesion 48 months later.³¹ Histopathology is necessary to confirm that the MRI changes noted in these dogs represent an early manifestation of NME, however, the distribution of the lesions and their correlation to reported MRI and histopathologic findings in pugs with NME makes it a reasonable possibility. Histopathologic confirmation via stereotactic brain biopsy might have helped validate early inflammatory changes in the brain but given the focal and subtle nature of the MRI findings and potential risk associated with brain biopsy, it was not attempted in these pugs that were considered clinically normal to the owners. Necropsy on these dogs will ultimately help determine the presence or absence of focal or global CNS inflammatory changes. MRI in the abnormal pugs in this study most importantly allowed us to rule out any congenital malformation/structural abnormalities that could have contributed to the abnormal neurological examination findings.

CSF analysis was normal in the majority of the pugs reported here, however normal CSF is possible with NME, particularly in the early stages.^{4,10,12,31} Lymphocytic inflammation is most commonly reported^{1,7} and was evident in 1 of the pugs in this cohort. Another dog had an abnormally high protein concentration with normal cell count (albuminocytological dissociation), which has been reported with NME in pugs and other breeds.⁴ One dog had a basophil in the CSF, which is a rare finding and has been reported in humans with various inflammatory conditions.^{32,33}

Conventional therapy of steroids and other immunosuppressives including cytosine arabinoside, azathioprine, lomustine, procarbazine, mycophenolate mofetil, leflunomide, and cyclosporine is ineffective to cure NME.^{7,9,23,34} The potential early phenotype for NME in pugs identified in this study might be used in conjunction with genetic risk testing to enroll dogs into early therapeutic trials. There is an increasing interest in human and veterinary medicine to use immunomodulatory therapies including mesenchymal stem cells³⁵ and tyrosine kinase inhibitors (TKI)³⁶ to treat immune-mediated CNS disease. Altered tyrosine kinase expression is present in dogs with granulomatous meningoencephalitis (GME) and NME and various tyrosine kinase inhibitors are currently employed in human trials for treatment of neuroinflammatory disorders including MS.³⁶ Studies are underway to determine whether early therapeutic intervention can reverse the neurological examination and MRI abnormalities identified here and potentially change the course of disease.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approved by Ethos Veterinary Health and Ethos Discovery.

HUMAN ETHICS APPROVAL DECLARATION

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

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