Received: 7 February 2023 | Revised: 16 December 2023 | Accepted: 5 January 2024

DOI: 10.1002/vetr.3895

Vet Record

ORIGINAL RESEARCH

Assessment of glial fibrillary acidic protein and anti-glial fibrillary acidic protein autoantibody concentrations and necrotising meningoencephalitis risk genotype in dogs with pug dog myelopathy

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Funding information
Sveland Foundation of Animal Health and Welfare; Thure F. and Karin Forsberg's Research Foundation

Abstract

Background: Pugs commonly present with thoracolumbar myelopathy, also known as pug dog myelopathy (PDM), which is clinically characterised by progressive signs involving the pelvic limbs, no apparent signs of pain and, often, incontinence. In addition to meningeal fibrosis and focal spinal cord destruction, histopathology has confirmed lymphohistiocytic infiltrates in the central nervous system (CNS) in a considerable number of pugs with PDM. Lymphohistiocytic CNS inflammation also characterises necrotising meningoencephalitis (NME) in pugs. This study aimed to investigate the potential contribution of an immunological aetiology to the development of PDM.

Methods: The concentrations of glial fibrillary acidic protein (GFAP) in serum and CSF and of anti-GFAP autoantibodies in CSF were measured with an ELISA. In addition, a commercial test was used for genetic characterisation of the dog leukocyte antigen class II haplotype, which is associated with NME susceptibility.

Results: This study included 87 dogs: 52 PDM pugs, 14 control pugs, four NME pugs and 17 dogs of breeds other than pugs that were investigated for neurological disease (neuro controls). Anti-GFAP autoantibodies were present in 15 of 19 (79%) of the PDM pugs tested versus six of 16 (38%) of the neuro controls tested ($p = 0.018$). All 18 PDM pugs evaluated had detectable CSF GFAP. Serum GFAP was detected in two of three (67%) of the NME pugs and in two of 11 (18%) of the control pugs but not in any of the 40 tested PDM pugs. Male pugs heterozygous for the NME risk haplotype had an earlier onset of clinical signs (70 months) compared to male pugs without the risk haplotype (78 months) ($p = 0.036$).

Limitations: The study was limited by the lack of healthy dogs of breeds other than pugs and the small numbers of control pugs and pugs with NME.

Conclusions: The high proportion of PDM pugs with anti-GFAP autoantibodies and high CSF GFAP concentrations provide support for a potential immunological contribution to the development of PDM.

INTRODUCTION

Progressive ataxia and paraparesis as a consequence of a thoracolumbar spinal cord disorder are common in pugs and appear worldwide.[1–11] Consistent clinical and pathological findings suggest that most of these disorders are caused by a specific condition; until now, only reported in pugs.[11] The terminology used to describe this condition has varied between publications, and has included constrictive myelopathy[4,7] and meningeal fibrosis.[11] However, constrictive myelopathy is associated with the

presence of articular process abnormalities and does not include all pugs with this specific thoracolumbar myelopathy.[9] The use of a pathological term, meningeal fibrosis, is also not ideal when describing a clinical presentation. Therefore, the term pug dog myelopathy (PDM) will be used in this study to describe pugs clinically characterised by progressive signs involving the pelvic limbs, no apparent signs of pain, commonly observed incontinence and magnetic resonance imaging (MRI) confirmation of intradural compression and/or focal spinal cord T2W hyperintensity[3,7] or pathological confirmation of meningeal fibrosis and focal spinal cord destruction.[11]

Although a number of putative causes, including meningeal as well as vertebral column pathologies, have been proposed,[1,3–7,10,12–15] the pathophysiologies of parenchymal spinal cord lesions in PDM have not been determined. A previous pathology study found that a significant proportion (43%) of pugs with PDM showed varying degrees of inflammation in the central nervous system (CNS). CNS inflammation, indicated by lymphohistiocytic infiltrates, was in some but not all cases spatially related to the parenchymal spinal cord lesion.[11] The specific type of cell infiltration found in the CNS of pugs with PDM is also a predominant feature of necrotising meningoencephalitis (NME).[16,17] NME in pugs has been described as an immune-mediated condition with clinical signs reflecting the pathological predisposition of the disease to the cerebral hemispheres.[18] The major histocompatibility complex (MHC) genes (human leukocyte antigen and dog leukocyte antigen [DLA]) play a major role in autoimmune disease in both people[19] and dogs.[20–29] NME in pugs[30] has been associated with DLA class II, and a high-risk haplotype (S/S) (dogs with this haplotype being approximately 13 times more likely to develop NME in their lifetimes) with a relatively high heritability has been identified.[25,26,29]

Astrocytes are specialised glial cells responsible for a wide variety of complex and essential functions in the CNS.[31] Astrocyte integrity is maintained by glial fibrillary acidic protein (GFAP), an intermediate filament protein that is released from astrocytes into the cerebrospinal fluid (CSF) after CNS injury.[32] As GFAP is almost uniquely found in astrocytes of the CNS, GFAP in body fluids, including serum and CSF, has been suggested to be a sensitive and specific biomarker of CNS disorders in people.[33,34] GFAP concentrations in serum and CSF have also been described for dogs with and without neurological disease,[35–38] with high concentrations of CSF GFAP suggested to be specific for NME.[38]

Furthermore, GFAP may induce an immune-mediated reaction in both people and dogs, with the development of anti-GFAP autoantibodies.[16,17,38–42] The presence of anti-GFAP autoantibodies is used to confirm a specific meningoencephalomyelitis and 'astrocytopathy' as having autoimmune aetiology in people.[39,43] For canine NME, proposed as the equivalent of human autoimmune GFAP meningoencephalomyelitis,[43] the diagnostic sensitivity and specificity of anti-GFAP autoantibodies have been reported to be 91% and 73%, respectively.[38]

However, whether the presence of an inflammatory reaction contributes to the development of PDM is largely unknown. Due to the presence of lymphohistiocytic infiltrate in the CNS of many PDM pugs, this should be further explored. By investigating pugs diagnosed with PDM in a similar way as previously described for NME pugs (by analysing concentrations of GFAP in serum and CSF and anti-GFAP autoantibodies in CSF and assessing the NME risk genotype of affected and unaffected pugs), this study aimed to investigate a potential inflammatory disease reaction contributing to the development of PDM. We hypothesised that PDM pugs would show increased CSF GFAP and anti-GFAP autoantibodies. We also wanted to explore whether the NME susceptibility DLA class II haplotype is associated with PDM-affected pugs.

MATERIALS AND METHODS

The study was approved by the local Swedish Ethical Committee. Informed client consent was obtained from all owners before inclusion of their dogs in the study. Blood and CSF samples were collected from client-owned dogs between 2013 and 2021 at Albano Animal Hospital, Danderyd, and at the University Animal Teaching Hospital, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

Dogs

The study comprised of four groups of dogs: (1) pugs diagnosed with PDM (PDM pugs), (2) pugs without neurological deficits (control pugs), (3) pugs diagnosed with NME (NME pugs), and (4) dogs of breeds other than pugs being investigated for various neurological diseases (neuro controls).

PDM pugs were included if they presented with clinical signs, including ataxia and paraparesis with a duration of 1 month or more, and had undergone MRI confirming intradural compression and/or focal spinal cord T2W hyperintensity[3,7] and/or, in the event the dog was euthanased, pathology confirmed meningeal fibrosis and focal spinal cord destruction.[11] Anamnestic and signalment data, including sex, weight, colour and age of onset of clinical signs and age at sampling, were collected for control and PDM pugs. A subset of the recruited dogs had also been included in two previous studies investigating the presence of vertebral malformations and pathology in pugs with PDM.[10,11] Control pugs were included if they presented without signs of neurological deficits and were older than 6 years. Control pugs were recruited through the Swedish breed club by inviting owners to participate in the study. PDM pugs, control pugs and neuro controls were prospectively recruited for this study, while NME pugs were retrospectively recruited.

The clinical phenotype was determined based on physical examination by the first author. Neuro

controls included dogs of any breed other than pugs for which a serum and a CSF sample were taken as part of the routine diagnostic workup at the neurology unit at Albano Animal Hospital, Danderyd, Sweden. Pugs with NME included those that had been diagnosed with this condition by MRI and CSF,[44–46] and had blood (serum and/or whole blood) and/or CSF available for analysis.

Pathology was not performed for any of the control pugs, pugs with NME or dogs of breeds other than pugs being investigated for various neurological diseases.

Blood and cerebrospinal fluid samples

Blood (serum and whole blood) and CSF were sampled from PDM pugs and neuro controls on the day of diagnostic workup. Control pugs were sampled on the day of phenotypic confirmation, while blood and CSF from pugs with NME had been previously sampled as part of their diagnostic workup and stored. Blood was collected from the cephalic vein into 5 mL EDTA tubes (Vacuette vacuum tubes EDTA K2) for genetic testing, and into 5 mL serum tubes (Vacuette vacuum tubes, serum Sep Clot Activator) for GFAP analyses. CSF samples were collected into 3.5 mL plastic tubes (Ellerman tubes) during anaesthesia or directly following euthanasia for the control pugs. In general, CSF was collected from the lumbar area in all the dogs that underwent routine diagnostic work-up and from the cerebellomedullary cistern in all the dogs that underwent euthanasia. After collection, whole blood and serum samples were centrifuged, and along with the CSF samples, were immediately frozen and stored at −70°C until analysis.

ELISA analyses

GFAP in serum and cerebrospinal fluid

The GFAP concentrations in serum and CSF were determined in duplicate using the commercial, canine-compatible, human GFAP ELISA (RD192072200R; BioVendor Laboratory Medicine) according to the manufacturer's instructions. This assay has previously been used for canine samples.[35–38] All the kits used had the same lot number. Cut-off values for separating normal and abnormal GFAP concentrations in the serum and CSF of dogs have previously been described.[37,38]

Anti-GFAP autoantibodies in cerebrospinal fluid

Anti-GFAP immunoglobulin G (IgG) antibodies were measured with an ELISA, essentially performed as described previously in dogs.[38] Briefly, 96-well microtiter plates (MS-8508 M, previously named MS-3508F; Sumitomo Bakelite Co.) were coated with 100 ng/well of purified bovine GFAP (62207; Progen Biotechnik) in phosphate-buffered saline (PBS) pH 7.4 at 4°C for 24 hours. After coating, the plates were washed three times with 300 µL of PBS supplemented with 0.05% Tween-20 (PBST). The plates were blocked with PBST containing 1% bovine serum albumin (A3803; Sigma–Aldrich Co.) for 60 minutes and then washed as previously. The CSF samples were diluted 1:10 with PBS (pH 7.4), and 100 µL of the diluted samples were added in duplicate to the corresponding wells and incubated at room temperature for 60 minutes. Two CSF samples with confirmed high and low values were added in duplicates as controls. The two samples were from a 2.5 year old French bulldog (high value) and a 4.5 year old mixed breed (low value). The French bulldog presented with neurological signs of a multifocal brain localisation, with MRI and CSF abnormalities suggesting NME. The mixed breed dog presented with a 3 year history of non-progressing behavioural problems, no neurological deficits, and unremarkable MRI of the brain and CSF. Samples from these two dogs had been used in a test run of the ELISA prior to the analyses of the entire study population. The CSF samples from these two dogs were not included in the study population. Also, as a background control, 100 µL of PBS was added in duplicate. After three washes with PBST containing 1% bovine serum albumin, 100 µL of goat anti-dog IgG-horseradish peroxidase (A40-123P; Bethyl Laboratories) diluted 1:1000 in PBS was added to each well and the mixture was incubated at room temperature for 60 minutes. The plates were washed three times with PBST and developed with tetramethylbenzidine (20-2629; Mercodia AB) at room temperature for 20 minutes. After adding Stop Solution (20-2693; Mercodia AB), the absorbance was measured at 450 nm on a plate reader (Multiscan EX; Thermo Labsystems). The absorbance value of the background control on each plate was subtracted from each sample value. Cut-off values in dogs separating normal and abnormal anti-GFAP autoantibody concentrations in CSF have previously been described.[38]

NME risk genotype assessment

Genomic DNA was extracted from 1 mL of whole blood on a QIAsymphony SP instrument and a QIA symphony DNA kit (Qiagen) to a concentration of 5–10 ng/µL before being shipped to the Veterinary Genetics Laboratory at UC Davies. The NME risk genotype was determined using a linked short tandem repeat marker test.[26] The DLA class II susceptibility variants were interpreted as described previously: N/N haplotype—low risk of developing NME, N/S—low risk of NME and S/S—increased risk of NME (N: normal haplotype, S: susceptibility variant haplotype).[25]

Statistics

Statistical analyses were performed using a commercially available statistical software program (JMP Pro v. 15.2.0). The data were analysed using descriptive as

TABLE 1 Signalment and clinical variables of pugs with pug dog myelopathy (PDM) and control pugs

Variable	Information rate	PDM pugs ($n = 52$)	Control pugs ($n = 14$)
Sex	100%		
Male		36 (69%)	7 (50%)
Female		16 (31%)	7 (50%)
Coat colour	100%		
Fawn		39 (75%)	14 (100%)
Black		13 (25%)	
Median weight (kg)	76%		
Male		9.1 (IQR 8.5–10.3)	9.9 (IQR 8.8–10.7)
Female		7.7 (IQR 7.3–8.4)	7.6 (IQR 7.4–7.7)
Median age (months) at sampling	100%	90 (IQR 69–102)	120 (IQR 95–138)
Male		86 (IQR 69–102)	130 (IQR 77–155)
Female		96 (IQR 63–110)	116 (IQR 99–125)
Median duration of clinical signs (months) before sampling	88%	4.5 (IQR 2.0–9.5)	
Male		4.0 (IQR 2.3–6.0)	
Female		7.0 (IQR 2.0–13.3)	

Abbreviation: IQR, interquartile range.

well as inferential statistics. Continuous variables were presented as medians and interquartile range (IQR). The chi-squared test and Fischer's exact test were used to test for differences in proportions concerning categorical data. Differences in continuous anamnestic, signalment and laboratory variables between groups were investigated using the non-parametric Wilcoxon signed rank test. The association between concentrations of CSF anti-GFAP autoantibodies and CSF GFAP in PDM pugs was tested using linear regression. The level of statistical significance was set at a p-value of less than 0.05.

RESULTS

Study population

In total, 87 dogs were included in the study: 52 PDM pugs, 14 control pugs, four NME pugs and 17 neuro controls. Of the PDM pugs, 34 were confirmed by MRI and 25 by pathology (+/− MRI). Information about the signalment and clinical variables of PDM and control pugs is presented in Table 1. The group of NME pugs included three males and one female. CSF was available for three NME pugs, and serum was also available for three. At the time of sampling, two of the NME pugs were on treatment with immunosuppressant and were neurologically stable. The group of neuro controls included 10 male and seven female dogs. The diagnoses of the neuro controls were: meningoencephalitis of unknown origin (MUO) ($n = 4$), steroid-responsive meningitis-arteritis (SRMA) ($n = 3$), myoclonus/paroxysmal dyskinesia ($n = 2$), cranial neuropathy ($n = 2$), lumbosacral stenosis ($n = 2$), spinal pain without a confirmed diagnosis ($n = 2$), neoplasia brain ($n = 1$) and cerebellitis ($n = 1$).

GFAP in serum

The sera of 69 dogs were analysed for the presence of GFAP, which included 40 PDM pugs, 11 control pugs, three NME pugs and 15 neuro controls (Figure 1). Four pugs showed elevated serum concentrations of GFAP; two control pugs and two NME pugs. The two female control pugs with positive serum GFAP concentrations (2.7 and 13.5 ng/mL, respectively) were sampled at 117 and 125 months of age and carried DLA class II haplotype S/S and N/S, respectively. CSF was unavailable for analysis from the two control pugs with detectable GFAP in serum. One of the two female pugs was euthanased, without obvious neurological deficits, 29 months later. The second female control pug showed no obvious neurological deficits 38 months following sampling.

One of the two NME pugs with a positive serum concentration of GFAP was sampled during initial examination (0.5 ng/mL). The second (0.1 ng/mL) and third (undetectable serum GFAP concentrations) NME pugs were sampled 2 weeks and 2 years after being diagnosed, respectively. Both of these pugs were medicated using immunosuppressants and were neurologically stable at the time.

GFAP in cerebrospinal fluid

The CSF of 40 dogs was analysed for the presence of GFAP, which included 18 PDM pugs, four control pugs, two NME pugs and 16 neuro controls (Figure 1). GFAP was detected in the CSF of all dogs (Figure 2).

The dogs with the highest CSF GFAP concentrations included one NME pug (21.0 ng/mL), four PDM pugs (16.0, 19.1, 23.8 and 25.0 ng/mL) and four neuro

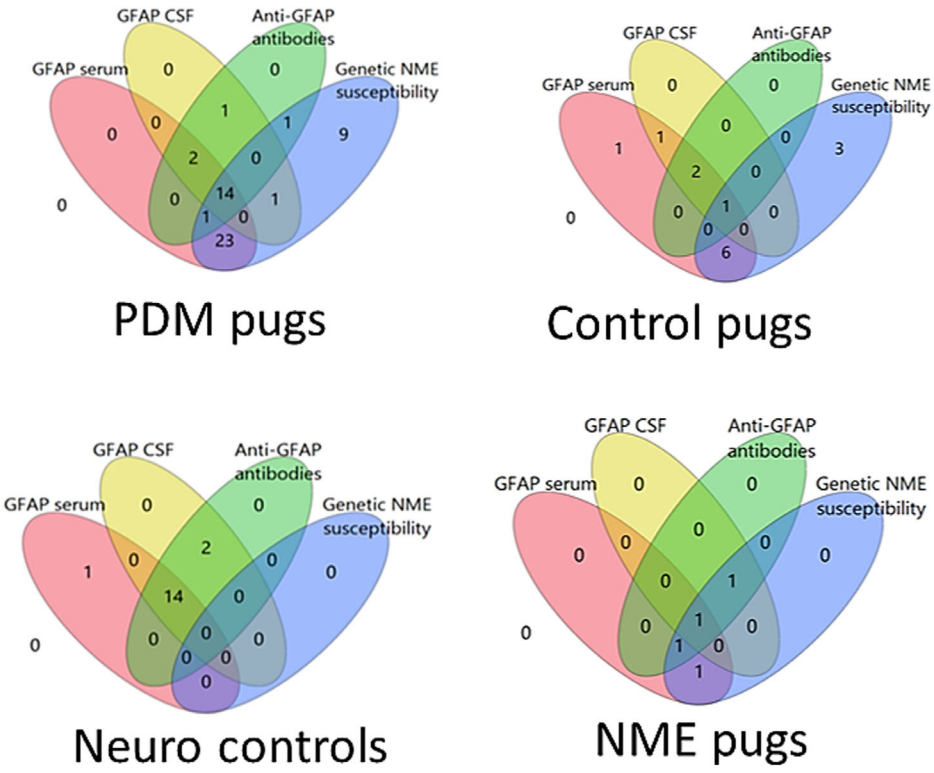
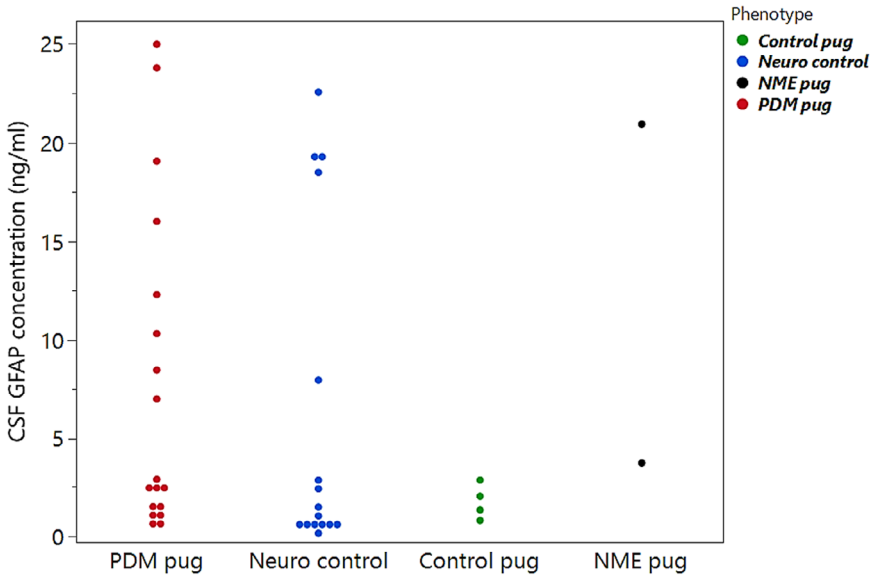


FIGURE 1 Venn diagrams showing the overlap and proportions of 87 dogs that underwent analysis of glial fibrillary acidic protein (GFAP) in serum, GFAP in cerebrospinal fluid (CSF) and anti-GFAP autoantibodies in CSF, and genetic testing for necrotising meningoencephalitis (NME) susceptibility. PDM, pug dog myelopathy.

FIGURE 2 Glial fibrillary acidic protein (GFAP) concentration in the cerebrospinal fluid (CSF) of pugs with pug dog myelopathy (PDM) ($n = 18$), neuro controls ($n = 16$), control pugs ($n = 4$) and pugs with necrotising meningoencephalitis (NME) ($n = 2$). Each point represents the mean concentration of duplicate assays.



controls. The neuro controls included two dogs with MUO (19.4 and 19.41 ng/mL), one dog with cranial neuropathy (22.6 ng/mL) and one dog with spinal pain but without a confirmed aetiological diagnosis (18.5 ng/mL).

The median CSF GFAP concentration for samples obtained from the cerebellomedullary cistern of PDM pugs ($n = 14$) was 2.5 ng/mL (IQR 1.2–13.2), and that of samples obtained from the lumbar cistern ($n = 4$) was 7.7 ng/mL (IQR 3.4–16.4).

Anti-GFAP autoantibodies in cerebrospinal fluid

The CSF of 41 dogs was analysed for the presence of anti-GFAP autoantibodies (Figure 1), which included 19 PDM pugs, three control pugs, three NME pugs and 16 neuro controls. Anti-GFAP autoantibodies were detected in the CSF of 31 (75.6%) dogs (Figure 3). Fifteen of the PDM pugs (78.9%) and six neuro controls (37.5%) showed an elevated CSF anti-GFAP

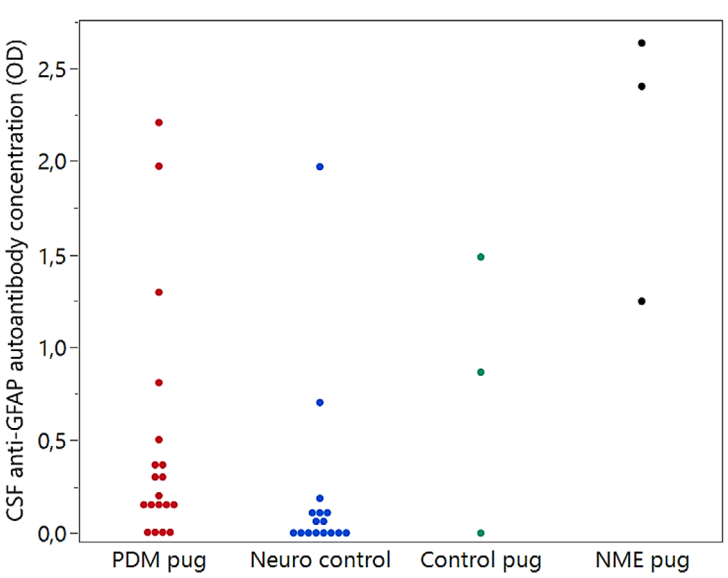


FIGURE 3 Anti-glial fibrillary acidic protein (GFAP) autoantibody concentrations in the cerebrospinal fluid (CSF) of pugs with pug dog myelopathy (PDM) ($n = 19$), neuro controls ($n = 16$), control pugs ($n = 3$) and pugs with necrotising meningoencephalitis (NME) ($n = 3$). Each point represents the mean optical density (OD) of duplicate assays. Cut-off value 0.10 OD.

TABLE 2 Distribution of sex and age at onset of clinical signs of pugs with pug dog myelopathy by N/N and N/S necrotising meningoencephalitis susceptibility dog leukocyte class II haplotype in 56 affected and control pugs

Variable	N/N haplotype	N/S haplotype	Median onset of clinical debut (months) for pugs with the N/N haplotype	Median onset of clinical debut (months) for pugs with the N/S haplotype	*p*-Value
Affected pug	24 (49.0%)	23 (46.9%)	84 (IQR 72–102)	72 (IQR 54–90)	0.082
Affected pug, male	19 (55.9%)	14 (41.2%)	78 (IQR 72–100)	70 (IQR 24–86)	0.036
Affected pug, female	5 (33.3%)	9 (60.0%)	84 (IQR 66–116)	84 (IQR 64–96)	0.546
Control pugs	7 (70.0%)	2 (20.0%)			

Abbreviations: IQR, interquartile range; N, normal haplotype; S, susceptibility variant haplotype.

autoantibody concentration ($p = 0.018$). The six neuro controls showing elevated CSF anti-GFAP autoantibody concentrations included two dogs with MUO (optical density [OD] 0.19 and 1.97), two dogs with SRMA (OD 0.13 and 0.70) and two dogs presenting with spinal pain without a confirmed aetiological diagnosis (OD 0.10 and 0.10). One of the three control pugs (OD 0.37) and the three NME pugs (OD 1.25, 2.4 and 2.64) presented with elevated CSF anti-GFAP autoantibody concentrations. There was no correlation between the CSF GFAP and CSF anti-GFAP autoantibody concentrations in PDM pugs.

The median CSF anti-GFAP autoantibody concentration for samples obtained from the cerebellomedullary cistern of PDM pugs ($n = 15$) was 0.29 OD (IQR 0.15–0.50), and that for samples obtained from the lumbar cistern ($n = 4$) was 0 OD (IQR 0–0.97).

NME risk genotype assessment

A total of 59 pugs, including 49 PDM and 10 control pugs, were characterised according to their NME susceptibility DLA class II haplotype (Figure 1). The N/N haplotype was present in 24 (49.0%) and seven (70.0%) pugs, the N/S haplotype was present in 23 (46.9%) and two (20.0%) pugs, and the S/S haplotype was present in two (4.1%) and one (10.0%) PDM and control pug, respectively (Table 2).

DISCUSSION

Despite being a common disease with severe morbidity, the aetiopathogenesis of PDM is largely unknown. The finding of anti-GFAP autoantibodies and high CSF GFAP concentrations in PDM pugs in the present study adds support to a suspected immunological contribution to the development of PDM.

As hypothesised, PDM pugs presented with increased CSF GFAP concentrations compared with the control pugs; indeed, more than one-third of the PDM pugs presented with increased CSF GFAP. In addition, a proportion (17%) of PDM pugs had CSF GFAP concentrations corresponding to those of the NME pugs. Acute CNS conditions such as trauma or vascular insults are followed by the release of GFAP, which normalises within a few days.[47] High CSF GFAP concentrations are observed in patients with autoimmune astrocytopathies in the acute phase of the disease and at relapse but with normal levels of GFAP in between.[33,38] Similarly, an increase in CSF GFAP in dogs with NME occurs only in the early stages of the disease.[38] The increased CSF GFAP concentrations in PDM pugs therefore suggest that they may have

active, ongoing, CNS affection despite chronic clinical signs. Microtrauma, as a result of instability, related or unrelated to articular process abnormalities,[4,48,6] CNS inflammation[38] and intervertebral disc herniation,[5,7,9] could, by itself or in combination, be potential drivers of astrocyte damage and the continuous release of GFAP.

The presence of anti-GFAP autoantibodies in PDM pugs was significantly more common than in dogs of other breeds investigated for neurological diseases, many with inflammatory CNS disease. The pathogenicity of anti-GFAP autoantibodies for astrocytes is not entirely clear, and any astrocyte activation in response to CNS injury may trigger the development of anti-GFAP autoantibodies.[49] However, in people, anti-GFAP autoantibodies in CSF have a high sensitivity and specificity for autoimmune GFAP astrocytopathy.[39,50] Anti-GFAP autoantibodies have also been studied in dogs with inflammatory CNS disease,[17,38,51,52] and a high diagnostic sensitivity and specificity has been shown for canine NME.[38] Unlike GFAP concentrations, anti-GFAP autoantibodies are detectable in both the acute and chronic stages of autoimmune GFAP astrocytopathy in people and in canine NME.[38,53] The majority of PDM pugs do not present histopathological evidence of CNS inflammation,[11] and PDM pugs without CNS inflammation present a longer duration of clinical signs before euthanasia than PDM pugs with CNS inflammation.[11] It has therefore been suggested that PDM pugs present with an early inflammatory phase and a later chronic proliferative state in which fibrosis and adhesions become permanent.[11] Theoretically, the early phase could include any spinal cord effect that causes the release of GFAP and triggers an immunological response, including the production of anti-GFAP autoantibodies. As the disease becomes chronic, the concentrations of GFAP would return to normal, while anti-GFAP autoantibody concentrations remain high. Although speculative, this reasoning could explain why only 43% of histopathologically investigated pugs showed CNS inflammation, while 79% of ELISA-analysed PDM pugs presented with CSF anti-GFAP autoantibodies.

None of the PDM pugs presented with elevated serum GFAP concentration, but two healthy control pugs (117 and 125 months of age) and two out of three NME pugs did. The results for the two healthy pugs corresponded to serum concentrations previously reported for dogs developing progressive myelomalacia after severe spinal cord injury.[35] The cause of the high serum GFAP concentrations of these two healthy pugs, which far exceeded the serum concentrations of the two NME pugs that were expected to show elevations, is difficult to explain. The finding of elevated GFAP in the CSF of healthy pugs has previously been described, and has been suggested to represent an inherent fragility of the pug's astrocytes, which makes them prone to 'leak' GFAP.[38] However, for GFAP to be released into the blood, an acute insult with loss of the integrity of the astrocyte and a damaged blood–brain barrier is required.[32] Increased serum GFAP has been described in people with clinically silent CNS injury but requires the use of highly sensitive assays.[47] Therefore, the majority of acute and chronic neurological diseases in people do not cause detectable GFAP concentrations in the blood.[47,54] The possibility of false-positive ELISA results needs to be considered[36,55]; however, none of the other dogs analysed (53 PDM pugs and neuro controls) showed positive serum GFAP concentrations. The two female pugs were followed for a considerable length of time after sampling without apparent development of any neurological dysfunction. Although considered highly specific for neuronal damage, being expressed in astrocytes and Schwann cells, GFAP can also occasionally be found in tissues outside the nervous system.[56] These tissues include Kupffer cells in the liver and interstitial cells in the pituitary gland and paraganglions.[56] GFAP is also expressed in osteocytes of mature bones and chondrocytes from epiglottal, bronchial and tracheal cartilage.[57–59] The results of this study could suggest that other, non-neuronal, routes for elevated serum GFAP concentrations in pugs may need to be considered.

Due to the presence of lymphohistiocytic inflammation in the CNS of PDM pugs similar to that in NME pugs, we wanted to explore whether the presence of the NME susceptibility DLA class II haplotype was associated with PDM. Male PDM pugs with the NME susceptibility class II risk haplotype N/S presented with clinical signs of PDM at a significantly younger age (70 months) than male pugs with the N/N haplotype (78 months). Although a genetic study recently showed that multiple loci, including those involved in inflammatory responses, were associated with PDM, the study did not provide specific support for the involvement of the DLA class II.[60] Therefore, the potential role of the MHC genes in the development of PDM, including the importance of an earlier clinical development of PDM in male pugs with the risk allele, needs to be further explored.

This study aimed to investigate the potential contribution of an immunological aetiology to the development of PDM in pugs by examining PDM pugs in a similar way to what had previously been done in NME pugs.[25,26,37,38,42] However, other methods for investigating the immunological contribution to PDM could be considered. A recent study investigated systemic inflammatory cytokine profiles in asymptomatic NME pugs.[61] The same study, using a slightly different protocol to identify genetic risk markers and describe pugs as having a low, medium or high risk for NME, suggested the presence of alterations in systemic immune variables in asymptomatic dogs at genetic risk for NME.[62] However, despite the presence of low- to medium-risk haplotypes, some pugs presented a strong inflammatory cytokine profile. Among the pugs with a strong inflammatory cytokine profile but a low clinical risk for the development of NME were a few older dogs with ataxia and paraparesis, potentially suggestive of PDM. Based on the study by Windsor et al.,[61] it would be of interest to investigate if alterations in systemic

immune variables, unrelated to the NME risk haplotype, might be considered for the development of PDM.[61] In humans, there is robust evidence for a link between obstructive sleep apnoea syndrome (OSAS) and systemic inflammation,[63,64] and in mice, intermittent hypoxia has been shown to upregulate GFAP in the brain.[65] Brachycephalic airway syndrome (BOAS), common in pugs,[66] causes naturally occurring hypoxia and resembles OSAS.[67] Because a previous study found an association between dyspnoea and gait abnormalities in pugs,[68] the role of chronic hypoxia in the development of PDM should be further investigated. Hypoxia could potentially contribute to the PDM pathophysiology by driving inflammation, specifically neuroinflammation, in BOAS-affected dogs.

GFAP was detected in the CSF of all analysed dogs, including control pugs, in our study. GFAP can be detected in the CSF under normal conditions, albeit at very low concentrations. However, the mechanism behind these basal levels is unknown.[69]

A CSF GFAP cut-off value of 2.0 pg/mL was reported in a previous study in dogs, in which the diagnostic sensitivity and specificity for NME were 53% and 84%, respectively.[38] In the present study, we used a similar commercial ELISA kit for human GFAP (Biovendor). Due to GFAP CSF concentrations ranging between 0.16 and 25.0 ng/mL, we were not able to apply the previously suggested cut-off value. The limit of detection of the assay, provided by the manufacturer, is described as 0.045 ng/mL, and in view of this, the previously suggested cut-off value may be questioned. The 'limit of detection', or preferably the 'analytical sensitivity', asserts the smallest amount of an analyte that can be measured correctly in an assay, which for GFAP measured by ELISA has been reported as 78.0 pg/mL.[34,70] Normal reference intervals for CSF GFAP concentrations analysed by ELISA are described for healthy people at various ages[69,71] and should also be determined for healthy dogs.

As indicated by our results, the location of sampling for CSF GFAP could be of importance and should preferably be standardised for future studies. While lumbar punctures are routine in human medicine, both cerebellomedullary and lumbar sampling are used routinely in veterinary medicine. The preference is often based on the location of the lesion but considerations related to the risk of sampling at the cerebellomedullary cistern and the technical difficulty of lumbar sampling are also taken into consideration.The limitations of this study included the absence of CSF and blood samples from healthy dogs of breeds other than pugs and the low number of CSF samples from control and NME pugs. However, our control pugs were comparably old, which reduced their risk of developing PDM at a later stage. Sampling of CSF from healthy individuals requires anaesthesia and thus brings a certain risk for the patient; therefore, it is difficult to ethically justify. Sampling in association with euthanasia is an alternative; however, it requires the owner's consent. Another limitation was the overlap between sampled dogs; for example, not all dogs had the same tests performed on blood and CSF. The unfortunate loss of samples stored in a freezer that broke down during the study largely contributed to the lack of consistency in sampled material. Further limitations were the lack of histopathology to confirm the clinical phenotype of NME pugs and neuro controls.

CONCLUSION

In this study, a potential immunological contribution to the development of PDM was supported by the high proportion of PDM pugs with anti-GFAP autoantibodies and high GFAP concentrations in the CSF. In addition, an earlier onset of clinical signs was associated with the N/S haplotype than with the N/N haplotype in male PDM pugs. The results of this study could guide future research into the potential role of the immunological system in the development of PDM.

AUTHOR CONTRIBUTIONS

Contributions to the concept and design of the study were made by Cecilia Rohdin, Ingrid Ljungvall, Karin Hultin Jäderlund and Jens Häggström. Data acquisition was performed by Cecilia Rohdin. Data analysis was performed by Anna Svensson. Interpretation of data for the article was performed by Cecilia Rohdin, Ingrid Ljungvall, Karin Hultin Jäderlund, Jens Häggström and Kerstin Lindblad-Toh. All the authors contributed to authorship and approved the final version of the manuscript.

ACKNOWLEDGEMENTS

We thank Monika Spång for her kind help with the sampling of dogs, Susanne Gustafsson for help with the preparation of samples and Haleh Yazdan Panah for her meticulous handling of the ELISAs. Thanks to the generosity of the dogs and owners enrolled in this study. This study was funded by the Sveland Foundation of Animal Health and Welfare and the Thure F. and Karin Forsberg's Research Foundation.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are archived at the Anicura Albano Small Animal Hospital and are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical approval was obtained from the Animal Ethics Committee of Sweden (Uppsala Djurförsöksetiska Nämnd C202/2014b and Stockholms Djurförsöksetiska Nämnd Dnr4599-202).

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How to cite this article: Rohdin C, Ljungvall I, Jäderlund KH, Svensson A, Lindblad-Toh K, Häggström J. Assessment of glial fibrillary acidic protein and anti-glial fibrillary acidic protein autoantibody concentrations and necrotising meningoencephalitis risk genotype in dogs with pug dog myelopathy. Vet Rec. 2024;e3895. https://doi.org/10.1002/vetr.3895