PAPER

Serum anti-GM2 and anti-GalNAc-GD1a IgG antibodies are biomarkers for acute canine polyradiculoneuritis

S. K. Halstead*, D. S. Gourlay*, J. Penderis†, E. Bianchi ‡, M. Dondi‡, A. Wessmann§, M. Musteata¶, M. Le Chevoir‖, L. Martinez-Anton**, S. F. M. Bhatti††, H. Volk‡‡, I. Mateo§§, A. Tipold‡‡, E. Ives¶¶, A. Pakozdy‖‖, R. Gutierrez-Quintana***, J. Brocal†††, Z. Whitehead‡‡‡, N. Granger§§§,¶¶¶, P. Pazzi‡‡‡, T. Harcourt-Brown‖‖‖, R. José-López***, S. Rupp****, H. C. Schenk††††, P. Smith‡‡‡‡, G. Gandini §§§§, M. Menchetti¶¶¶¶, V. Mortera-Balsa‖‖‖‖, C. Rusbridge *****,†††††, A. Tauro **, F. Cozzi‡‡‡‡‡, M. Deutschland§§§§§, F. Tirrito‡‡‡‡‡, P. Freeman ¶¶¶¶¶, M. Lowrie‖‖‖‖‖, M. R. Jackson******, H. J. Willison* and A. Rupp [1,***]

*Neuroimmunology Laboratory, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow G12 8TA, UK
†Vet Extra Neurology, Broadleys Veterinary Hospital, Stirling FK7 7LE, UK
‡Department of Veterinary Science, University of Parma, 43126, Parma, Italy
§Neurology and Neurosurgery Service, Pride Veterinary Centre, Pride Park, Derby DE24 8HX, UK
¶Neurology Service, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Iași 700489, Romania
‖Department of Neurology and Neurosurgery, University of Melbourne, Werribee, Victoria, 3030, Australia
**Chestergates Veterinary Specialists, Telford Court, Chestergates CH1 6LT, UK
††Small Animal Department, Small Animal Teaching Hospital, Faculty of Veterinary Medicine, Ghent University, 9820, Merelbeke, Belgium
‡‡Department of Small Animal Medicine and Surgery, University of Veterinary Medicine Hannover, 30559, Hannover, Germany
§§Servicio de Neurología, Hospital Clínico Veterinario – Universidad Alfonso X el Sabio, Madrid, Spain
¶¶Anderson Moores Veterinary Specialists, Hursley, Winchester SO21 2LL, UK
‖‖University Hospital for Small Animals, University of Veterinary Medicine, Vienna, Austria
***School of Veterinary Medicine, University of Glasgow, Glasgow G61 1QH, UK
†††Wear Referrals Veterinary Hospital, Bradbury, Stockton-on-Tees TS21 2ES, UK
‡‡‡Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa
§§§The Royal Veterinary College, University of London, Hatfield, Hertfordshire, UK
¶¶¶CVS Referrals, Bristol Veterinary Specialists at Highcroft, Bristol, UK
‖‖‖Langford Veterinary Services, School of Veterinary Sciences, University of Bristol, Lower Langford BS40 5DU, UK
****Tierklinik Hofheim, 65719, Hofheim, Germany
††††Tierklinik Lüneburg, 21337, Lüneburg, Germany
‡‡‡‡Hamilton Specialist Referrals, Cressex Business Park, High Wycombe, HP12 3SD, UK
§§§§Department of Veterinary Medical Sciences, University of Bologna, 40064, Ozzano dell'Emilia, Italy
¶¶¶¶Neurology and Neurosurgery Division, San Marco Veterinary Clinic, Veggiano, Italy
‖‖‖‖North Downs Specialist Referrals, 3&4 The Brewerstreet Dairy Business Park, Bletchingley, Surrey RH1 4QP, UK
*****Neurology Section, Fitzpatrick Referrals, Godalming, Surrey GU2 7AL, UK
†††††School of Veterinary Medicine, Faculty of Health & Medical Sciences, University of Surrey, Guildford, Surrey GU2 7AL, UK
‡‡‡‡‡Clinica Neurologica Veterinaria, 20148, Milan, Italy
§§§§§Neurological Referral Service, Berlin, Germany
¶¶¶¶¶The Queen's Veterinary School Hospital, Cambridge CB3 0ES, UK
‖‖‖‖‖Dovecote Veterinary Hospital, Castle Donington, Derby DE74 2LJ, UK
******Institute of Cancer Sciences, University of Glasgow, Bearsden G61 1QH, UK

[1]Corresponding author email: angelikafrances.rupp@glasgow.ac.uk

Objectives: **A previous single-country pilot study indicated serum anti-GM2 and anti-GA1 anti-glycolipid antibodies as potential biomarkers for acute canine polyradiculoneuritis. This study aims to validate these findings in a large geographically heterogenous cohort.**

Materials and Methods: **Sera from 175 dogs clinically diagnosed with acute canine polyradiculoneuritis, 112 dogs with other peripheral nerve, cranial nerve or neuromuscular disorders and 226 neurologically normal dogs were screened for anti-glycolipid antibodies against 11 common glycolipid targets to**

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determine the immunoglobulin G anti-glycolipid antibodies with the highest combined sensitivity and specificity for acute canine polyradiculoneuritis.

Results: Anti-GM2 anti-glycolipid antibodies reached the highest combined sensitivity and specificity (sensitivity: 65.1%, 95% confidence interval 57.6 to 72.2%; specificity: 90.2%, 95% confidence interval 83.1 to 95.0%), followed by anti-GalNAc-GD1a anti-glycolipid antibodies (sensitivity: 61.7%, 95% confidence interval 54.1 to 68.9%; specificity: 89.3%, 95% confidence interval 82.0 to 94.3%) and these anti-glycolipid antibodies were frequently present concomitantly. Anti-GA1 anti-glycolipid antibodies were detected in both acute canine polyradiculoneuritis and control animals. Both for anti-GM2 and anti-GalNAc-GD1a anti-glycolipid antibodies, sex was found a significantly associated factor with a female to male odds ratio of 2.55 (P=0.0096) and 3.00 (P=0.0198), respectively. Anti-GalNAc-GD1a anti-glycolipid antibodies were more commonly observed in dogs unable to walk (odds ratio 4.56; P=0.0076).

Clinical Significance: Anti-GM2 and anti-GalNAc-GD1a immunoglobulin G anti-glycolipid antibodies represent serum biomarkers for acute canine polyradiculoneuritis.

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INTRODUCTION

Acute canine polyradiculoneuritis (ACP), characterised by acute onset of rapidly progressive lower motor neuron flaccid para/tetraparesis, potentially progressing to tetraplegia and frequently associated with dysphonia (Cuddon 2002, Martinez-Anton *et al.* 2018), is a common sporadic peripheral neuropathy in dogs (Olby 2004, Hirschvogel *et al.* 2012). Due to its similarity both in the clinical presentation and pathological findings (Cummings & Haas 1967, Northington & Brown 1982) to the human autoimmune neuropathy Guillain-Barré syndrome (GBS) (Elf *et al.* 2014), it is considered to represent the canine equivalent to GBS (Cuddon 2002). In human GBS, serum anti-glycolipid antibodies (AGAbs) are frequently present in certain clinical subtypes, such as acute motor axonal forms or Miller Fisher syndrome, where certain AGAbs are seen in 55.9% (individual AGAb) to 86.3% (AGAb panel) and 89.2% of cases, respectively (Halstead *et al.* 2016, Yoshikawa *et al.* 2018). This prompted our previous study conducted in a small cohort of Italian dogs clinically and electrophysiologically diagnosed with ACP in which we identified ACP-specific serum AGAbs in 60% of cases (Rupp *et al.* 2013). In contrast to human GBS where autoantibodies to many different sialylated glycolipids (gangliosides) are present, ACP-dogs exhibited a very distinct immunoglobulin G (IgG) AGAb profile directed against GM2 ganglioside. GM2 ganglioside is an uncommon AGAb target in human GBS (<10% of cases), with AGAbs typically of IgM subtype and linked to a previous infection with Cytomegalovirus (Khalili-Shirazi *et al.* 1999, O'Hanlon *et al.* 2000, Caudie *et al.* 2002).

The diagnosis of ACP is based on the typical clinical presentation supported by salient electrodiagnostic findings, unremarkable laboratory data and cerebrospinal fluid (CSF) analysis (other than albuminocytologic dissociation), and potential muscle/nerve biopsies, combined with the exclusion of other clinically similar diseases including botulism, myasthenia gravis, tick paralysis, snake envenomation and organophosphate toxicity (Cuddon 2002, Olby 2004). Fatal respiratory paralysis and/or concurrent aspiration pneumonia may occur, however, typically is rare (Cummings & Haas 1967, Northington & Brown 1982, Cuddon 2002, Rupp *et al.* 2013). Generally, the prognosis for the majority of dogs affected with ACP is good, provided they receive appropriate intensive nursing and physiotherapy, and spontaneous recovery occurs over a number of weeks to months (de Lahunta & Glass 2009, Hirschvogel *et al.* 2012). Therefore, a biomarker with high sensitivity and specificity would represent a useful supplement to other diagnostic investigations both for veterinarians and owners in order to direct appropriate care and prognostication. This validation study aimed to provide general relevance to our previous finding of AGAbs in Italian ACP-dogs by determining the seroprevalence of AGAbs in a geographically diverse population of dogs clinically diagnosed with ACP and in comparison, to both dogs diagnosed with other neuromuscular, peripheral nerve and cranial nerve disorders and neurologically normal control dogs.

MATERIALS AND METHODS

Sample submission

Over 3 years (2014 to 2016), a national (UK) and international call for serum samples from dogs clinically diagnosed with ACP was sent out to selected board-certified veterinary neurologists.

In addition to the pre-selected group of neurologists, other veterinary surgeons and neurologists contributed ACP cases and samples to this study either following word of mouth advertisement or on hearing presentation of prior data at conferences. This study was ethically approved by the University of Cambridge (CR101) and the University of Glasgow (Ref 14a/16).

The diagnosis of ACP was based on the presence of rapid onset (2 to 4 days) flaccid, lower motor neuron para/tetraparesis progressing to maximal severity within 2 weeks of onset, and exhibiting the potential progression to tetraplegia and variable hyperaesthesia (Hirschvogel *et al.* 2012, Anor 2014, Laws *et al.* 2017), supported by additional investigations as seen fit by the collaborator, results of which included inconspicuous serology and biochemistry, consistent CSF changes and electrodiagnostic findings (Cuddon 1998) comprising (delayed) spontaneous myofibre activity on electromyography, decrease, delay or absence of F-waves, decreased compound muscle action potential amplitudes and variably decreased motor nerve conduction velocity, and combined with a history of lack of toxin exposure, snake bites or presence of ticks. Investigators were also asked to collect serum samples from dogs presenting with other cranial nerve, peripheral nerve or neuromuscular disorders (ONM) and/or age-, sex- and breed matched samples from neurologically normal dogs (CTRL), when possible. All contributors were provided with sample submission guidelines, owner information sheets, consent forms and a questionnaire addressing epidemiological data (signalment, date of disease onset, presentation and sampling), clinical features, preceding events (within 3 weeks of disease onset) and additional investigations (such as electrophysiology, CSF-examination, serology, biochemistry, imaging etc).

Serum sample submission directly corresponded to the guidelines of international, serological investigations conducted in GBS [IGOS – International GBS outcome study (Jacobs *et al.* 2017)] and required the submission of frozen sera shipped on dry ice, or for fresh serum samples submitted from within the UK at ambient temperature.

Upon receipt, all serum samples and questionnaires were blinded by coding and sera were stored at −80°C until use.

Sample screening and determination of assay cut-off values

All samples were initially screened in triplicate for the presence of AGAbs against a broad panel of 10 glycolipids using combinatorial glycolipid microarrays as previously described (Halstead *et al.* 2016) and in parallel with positive and negative quality controls (dog sera with and without AGAbs). Based on results from this screen, a refined panel of four key glycolipid antigens was selected for a further single round of screening. In brief, panels of either 10 (sulphatide, GM1, GM2, GA1, GD1a, GD1b, GT1b, GD3, GalC and SGPG) or four glycolipid antigens (GM1, GM2, GA1 and GalNAc-GD1a), in addition to their 1:1 (v:v) heteromeric complexes, each at 200 µg/mL, were printed in duplicate onto glass slides coated in low fluorescence polyvinylidene difluoride membrane. After blocking in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), dog serum (diluted 1:50 in 1% BSA in PBS) was applied to each array. Anti-glycolipid IgG binding was detected using fluorescence-conjugated, isotype-specific, anti-dog IgG antibody (3 µg/mL; Jackson ImmunoResearch Laboratories) and the median fluorescence signal associated with each antigen spot was quantitated (Genepix 4300A microarray scanner, Molecular Devices). Following subtraction of the local fluorescence background signal, the mean for the duplicate antigens spots was calculated and expressed as fluorescence intensity unit (FIU).

Data analysis and statistics

The optimal FIU cut-off value for serological diagnosis of ACP was determined for each target by plotting the ROC curve (MedCalc software) and then using the Youden index (J) method (Youden 1950), which calculated the optimal threshold value based on each biomarker's ability to differentiate between ACP and ONM groups when equal weight is given to sensitivity and specificity. For the comparison of paired ROC curves, the DeLong method was applied (DeLong *et al.* 1988). Heat maps created in MeV (MultiExperiment Viewer software; version 4.9.0) in the TM4 software suite, and employing the rainbow scale, were used for graphical display of array FIU values.

Following thresholding of antibody into positive/negative, the relevant sampling, epidemiological and clinical parameters included in a generalised linear model (logit link) were determined separately for each antibody by minimisation of the Akaike information criterion. These were ambulatory status, sex and onset season in the case of anti-GM2 positivity (fitted to 156 observations, 5 degrees of freedom), and ambulatory status, sex, onset season, time from onset to sampling, involvement of CNs V and VII and dysphonia for anti-GalNAc-GD1a positivity (fitted to 119 observations, 9 degrees of freedom). The significance of the fitting was confirmed by comparison with the null model using likelihood ratio testing. The effect sizes of statistically significant predictors ($P \leq 0.05$) are presented as odds ratios with a 95% confidence interval. Analysis was performed using R (3.6.3, R core Team 2021; https://www.R-project.org/).

RESULTS

Sample submission, epidemiological and clinical data

In total, 513 samples were submitted by 27 veterinarians from 21 institutions across nine temperate countries worldwide (Table 1). Out of these, 448 samples (87.3%; ACP: 159; ONM: 105; CTRL: 184) were submitted by board-certified neurologists or veterinarians under their supervision, and 420 samples (81.9%; ACP: 159; ONM: 96; CTRL: 165) were submitted under optimal temperature conditions. There was no overlap between samples submitted from non-boarded submitters and samples submitted under suboptimal conditions. Some samples from both diseased and control groups had previously been included in our preliminary study (n=38) (Rupp *et al.* 2013) or other studies associated with ACP (n=48) (Martinez-Anton *et al.* 2018).

Signalment was available for 506/513 dogs (98.6%) and 162/175 (92.6%) of ACP-samples were accompanied by clinical

Table 1. Sample submission and epidemiological data

	ACP	ONM	CTRL
Total samples (%)	175 (34.1)	112 (21.8)	226 (44.1)
Italy	63	30	41
UK	42	45	101
Australia (Melbourne)	22	1	25
Germany	14	13	6
Belgium	9	7	5
Spain	10	9	0
South Africa	7	2	8
Austria	6	1	6
Romania	2	4	34
Male (%)	100 (57.1)	69 (61.6)	124 (54.9)
Female (%)	75 (42.9)	43 (38.4)	101 (44.7)
Age mean (median), years	7.5 (8)	6.8 (7)	7.2 (7.5)
Most common breeds (%)			
- X-breed	38 (21.7)	25 (22.3)	50 (22.1)
- Jack Russell Terrier	14 (8.0)	2 (1.8)	13 (5.6)
- Labrador Retriever	13 (7.4)	11 (9.8)	20 (8.8)
- Cocker Spaniel	12 (6.9)	1 (0.9)	13 (5.6)
- Poodle (including Miniature)	9 (5.1)	1 (0.9)	7 (3.1)
- Collie (including Border)	8 (4.6)	4 (3.6)	3 (1.3)
- West Highland White Terrier	6 (3.4)	0 (0.0)	6 (2.7)
- Maltese	5 (2.9)	1 (0.9)	1 (0.4)
- Shih-tzu	5 (2.9)	0 (0.0)	5 (2.2)

ACP Acute canine polyradiculoneuritis, ONM Other peripheral nerve, cranial nerve or neuromuscular disorders, CTRL Neurologically normal dogs
Other ACP-breeds: Bichon Frise (4), Fox Terrier (including Wire-haired; 4), German Shepherd (4), Griffon Bruxellois (4), Siberian Husky (4), Golden Retriever (3), Pug (3), Yorkshire Terrier (3), Australian Shepherd (2), Beagle (2), Dachshund (2), Parsons Russell Terrier (2), Schnauzer (including miniature; 2), Springer Spaniel (2), Bearded Collie (1), Boerbol (1), Bolognese (1), Boxer (1), Bracco Italiano (1), Bracke (1), Bull Terrier (1), Chihuahua (1), Clumber Spaniel (1), Epagneul Breton (1), Flat-Coated Retriever (1), Italian Hound (1), Italian Spinone (1), Jagtterrier (1), Lhasa Apso (1), Newfoundland (1), Patterdale Terrier (1), Polonka Zwetna (1), Portuguese Waterhound (1), Silky Terrier (1), Keeshond (1), Spanish Waterdog (1), Staffordshire Bullterrier (1)

Table 2. Clinical features of acute canine polyradiculoneuritis (ACP)-dogs (n=175)

	Known	Present (%)
Ambulatory status	162	
Ambulatory paraparesis		4 (2.5)
Ambulatory tetraparesis		31 (19.1)
Non-ambulatory paraparesis		2 (12.3)
Non-ambulatory tetraparesis		105 (64.8)
Paraplegia		1 (0.6)
Tetraplegia		19 (11.7)
Hyperaesthesia	148	34 (23.0)
Hyporeflexia	155	135 (87.1)
Areflexia	146	61 (41.8)
Involvement of CNs	160	
CNV		10 (6.3)
CNVII		44 (27.5)
CNXII		7 (4.4)
Dysphonia	150	93 (62.0)
Megaoesophagus	157	1 (0.6)
Aspiration pneumonia	155	6 (3.9)
Respiratory compromise	158	23 (14.6)
Preceding events	149	
Vaccination within 3 weeks		8 (5.4)
Vaccination within 4 to 6 weeks		3 (2.0)
Gastrointestinal upset within 3 weeks		13 (8.7)
Respiratory symptoms within 3 weeks		4 (2.7)

questionnaires, indicating that in 96 dogs (59.3%) the clinical examinations had been supported by electrophysiologic examinations, in 55 dogs (34.0%) by CSF-examination, in 105 dogs (64.8%) by other laboratory investigations, in 63 dogs (38.9%) by imaging procedures and in 15 dogs (9.3%) by muscle/nerve biopsies. Ages of ACP-dogs ranged from 2 months to 15 years and all sizes of dogs were represented (Table 1).

In those ACP-dogs where the month of disease onset was known (158/175; 90.3%), the highest proportion of dogs (31%) exhibited an onset over the winter months (December, January and February for Northern Hemisphere; June, July and August for Southern Hemisphere), followed by summer (25.9%), autumn (24.1%) and spring (19.0%).

The most common presentation for ACP-dogs was non-ambulatory tetraparesis (105/162; 64.8%), hyporeflexia (135/155; 87.1%) and dysphonia (93/150; 62.0%). If other cranial nerves were involved, this was most frequently CNVII (44/160; 27.5%). Respiratory compromise was present in 14.6% (23/158) of dogs. Preceding events in ACP-dogs (18.8%) comprised vaccination over the last 6 weeks (11/149; 7.4%), or gastrointestinal signs (13/149; 8.7%) or respiratory signs (4/149; 2.7%) over the preceding 3 weeks (Table 2). Vaccinations administered and when reported (6/11), comprised the core vaccines CDV, CPV, CAV-2 and various combinations of non-core vaccines (canine parainfluenza virus, *Bordetella bronchiseptica* and *Leptospira* spp).

Diagnoses in the 112 ONM-dogs comprised 51 cases of peripheral neuropathies that were polyneuropathies with and without muscle involvement not considered compatible with ACP (n=20), chronic (n=15), degenerative (n=4), metabolic (n=2), breed-specific (Leonberger; n=2), paraneoplastic (n=2) or drug-induced (n=1), or mononeuropathies (n=5). Additionally, there were cranial neuropathies (n=41), myasthenia gravis (n=12), (poly-)myositis (n=5), botulism (n=2) and storage myopathy (n=1).

Serology

The preliminary antibody screen, directed against a broad panel of 10 glycolipid targets and their associated 1:1 heteromeric complexes, highlighted the three glycolipids GM1, GM2 and GA1 as the most frequent targets for IgG AGAbs in ACP-dogs. Other single or heteromeric glycolipid targets (as listed in Methods) did not yield any significant positive samples in ACP cases or controls. Subsequently, all sera were screened against a refined array containing these three glycolipids and additionally GalNAc-GD1a (and their 1:1 heteromeric complexes), since GalNAc-GD1a shares the terminal epitope GalNAcβ1-4(Neu5Acα2-3)Gal with GM2, an already known cross-reactive epitope for neuropathy-associated autoantibodies (Ilyas *et al.* 1988).

This refined four-glycolipid antigen screen (Fig 1) revealed that 156/175 (89.1%) ACP-dogs possessed serum IgG antibodies greater than the cut-off threshold against one or more of the single glycolipid targets. Anti-GM2 IgG AGAbs (Figs 2 and 3) were the most frequently detected AGAb in ACP samples (114/175; 65.1%), followed by anti-GalNAc-GD1a IgG AGAbs (108/175; 61.7%). One hundred ACP-sera (57.1%) were reactive (above threshold) against both GM2 and GalNAc-GD1a, with the

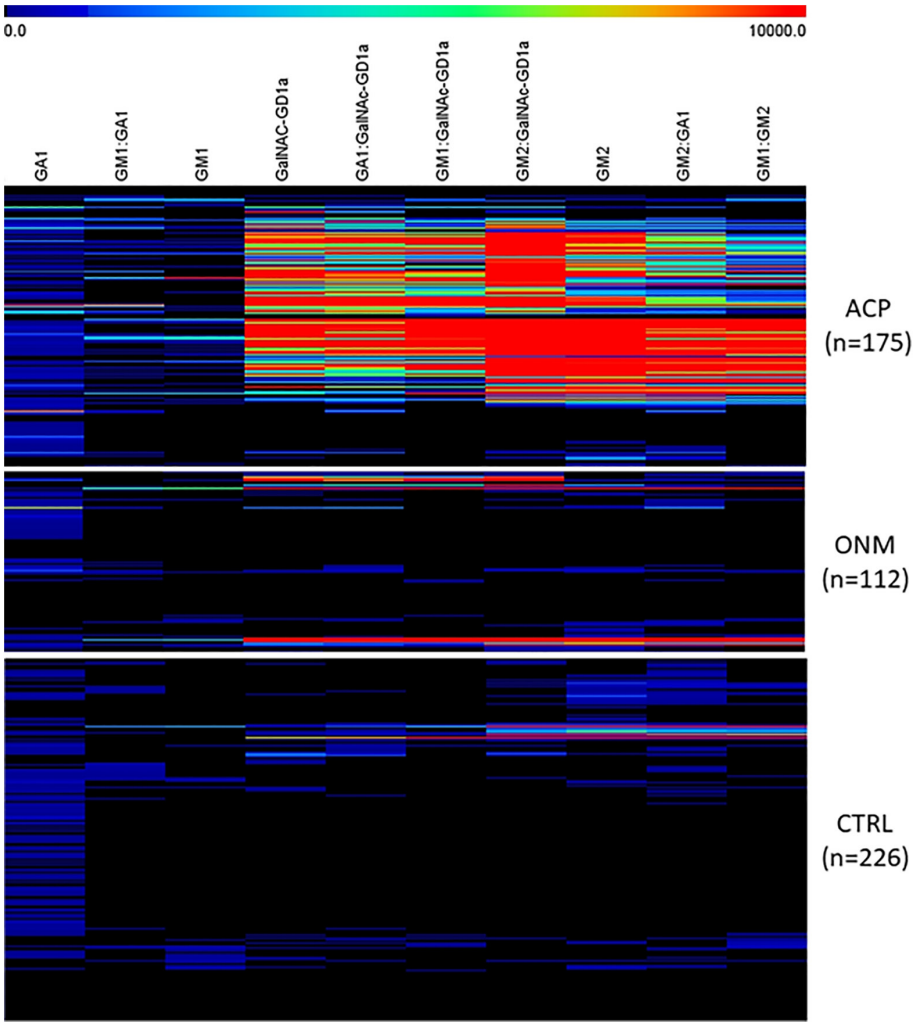

FIG 1. Heat map of IgG antibody binding intensity to four glycolipid targets (GM1, GM2, GalNAc-GD1a and GA1) and their 1:1 heteromeric complexes (n=10, refined screen) in dogs with acute canine polyradiculoneuritis (ACP; n=175), neurological controls (ONM; n=112) and non-neurological controls (CTRL; n=226). The rainbow scale indicates the intensity of antibody binding with black corresponding to no binding, blue to weak binding and red to strong antibody binding. Hierarchical clustering was applied to group samples with similar antibody binding patters. Serum samples are displayed in rows and antigen targets are displayed in columns. Whilst a large proportion of ACP-dogs exhibit strong AGAb binding against the glycolipid antigen targets assessed, these binding patterns are rarely detected in ONM- and CTRL-dogs

majority (66/100; 66.0%) exhibiting higher relative intensity binding to GM2. Anti-GM1 IgG AGAbs were less frequently observed (59/175; 33.7%) and were only very rarely present as the sole key glycolipid target (5/43; 11.6%; Fig 2). Finally, whilst anti-GA1 Ig AGAb was present in a high proportion of ACP samples (120/175; 68.6%) and was the most common solitary AGAb (26/120; 21.7%) present in ACP serum, anti-GA1 Ig AGAb was not a specific marker of ACP, as both ONM and CTRL sera also frequently contained this AGAb (51/112 (45.5%) and 116/226 (51.3%), respectively; Fig 1).

When examined by ROC analysis which gives equal weight to both the sensitivity and specificity of an assay, anti-GM2 AGAbs reached the highest combined sensitivity and specificity, closely followed by anti-GalNAc-GD1a AGAbs (Fig 4 and Table 3). No statistically significant difference was observed when comparing anti-GM2 and anti-GalNAc-GD1a AGAbs (P=0.3750); however, the ROC curves for both of these were significantly different from anti-GM1 and anti-GA1 AGAbs (P<0.0001 in all cases).

Examining heteromeric complexes of two glycolipids as targets, GM2:GalNAc-GD1a (Fig 3) reached the highest combined sensitivity and specificity (sensitivity 66.9%, specificity 91.1%; Table 3) and also the combination of anti-GM2 and/or anti-GalNAc-GD1a as individual AGAbs gave the highest combined sensitivity and specificity (sensitivity: 69.7%; specificity: 86.6%). However, when additionally considering the presence of anti-GM1 and anti-GA1 AGAbs as markers, the overall performance of the assay decreased due to a loss in specificity.

Influence of sample submission variables, epidemiological and clinical data on serology

The majority of samples in this study were submitted by board-certified veterinary neurologists (or trainees under their direct supervision) and under optimal temperature conditions (87.3% and 81.9%, respectively). Most ACP-sera for which the sampling interval was known (n=135; overall range 1 to 130 days) were collected within 3 weeks of disease onset (85.9%; 116/135). Both for

anti-GM2 and anti-GalNAc-GD1a AGAb-positive samples, no significant effects associated with sample collection and handling, such as time taken from disease onset to sampling (including thresholding at 3 weeks) and sample thawing were observed. Importantly, there also was no evidence of an effect associated with the credentials of the sample contributor (board-certified neurologist or not).

AGAbs were more common in female dogs; 57% (57/100) of male and 76% (57/75) of female ACP-dogs had anti-GM2 AGAbs, giving a female to male odds ratio of 2.55 (1.27 to 5.31; P=0.0096), and 53% (53/100) of male and 73.3% (55/75) of female ACP-dogs had anti-GalNAc-GD1a AGAbs modelled with a female to male odds ratio of 3.00 (1.22 to 7.89; P=0.0198).

With respect to age distribution, none of the very young ACP-dogs (2 to 5 months; n=6) exhibited anti-GM2 or anti-GalNAc-GD1a AGAbs. Whilst most breeds of ACP-dogs and also cross-breeds exhibited anti-GM2 and/or anti-GalNAc-GD1a AGAbs, a small number of breeds, which included Poodles (9/9), West Highland White Terriers (6/6), Maltese (5/5), Griffon Bruxellois (4/4), Siberian Huskies (4/4) and Fox Terriers (4/4) attracted attention by the fact that the AGAbs under investigation were identified in all the ACP-dogs submitted from these breeds.

When examining the AGAb profile of non-ambulatory dogs, 67.7% (86/127) had anti-GM2 AGAbs compared with 48.6% (17/35) of ambulatory dogs. Likewise, anti-GalNAc-GD1a AGAbs were more frequently present in non-ambulatory ACP-dogs (65.4%; 83/127) than in ambulatory dogs (40%; 14/35); however, only for anti-GalNAc-GD1a AGAbs was the lack of ambulation significantly associated with the presence of AGAbs [modelled odds ratio: 4.56 (1.56 to 14.87); P=0.0076].

None of the other clinical or epidemiological parameters investigated, including hyporeflexia, areflexia, hyperaesthesia, involvement of cranial nerves (including comparison of specific nerves such as CNV, CNVII and CNXII), dysphonia, respiratory compromise, aspiration pneumonia, presence of megaoesophagus, season of onset or preceding events were found to be significantly associated with the presence of anti-GM2 or anti-GalNAc-GD1a AGAbs.

With respect to preceding events, four of the 11 dogs which had been vaccinated within 6 weeks of disease onset had both anti-GM2 and anti-GalNAc-GD1a AGAbs, with a fifth dog having anti-GM2 AGAbs only. These five dogs ranged in age from 8 to 11 years. In contrast, no ACP-dogs vaccinated under the age of 6 months (n=5) and who all developed ACP within 3 weeks of vaccination had detectable AGAbs. For ACP-dogs with preceding gastrointestinal (n=13) and respiratory (n=4) signs, 8/13 (61.5%) and 3/4 (75.0%) exhibited AGAbs (concurrent anti-GM2 AGAbs and anti-GalNAc-GD1a AGAbs), respectively.

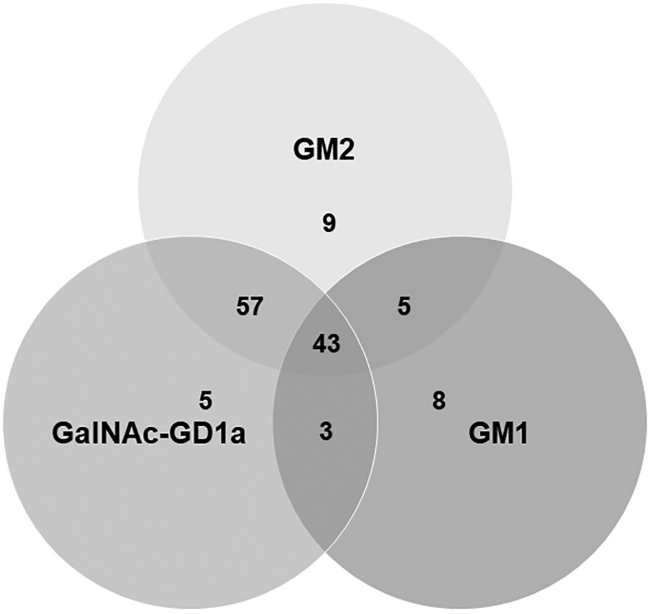

FIG 2. Venn diagram depicting the number of ACP-dogs exhibiting IgG antibody binding intensities greater than the cut-off threshold for single glycolipid targets GM2, GalNAc-GD1a and/or GM1. These are present in various overlapping patterns, with the majority of ACP-dogs (100/175; 57.1%) exhibiting both GM2 and GalNAc-GD1a AGAbs, with or without concomitant anti-GM1 AGAbs. In total, 45/175 (25.7%) of ACP-sera were negative for these three antigen targets

Anti-GM2 and anti-GalNAc-GD1a AGAb-positive controls

Fifteen of the 112 ONM-dogs (13.4%) had either anti-GM2 AGAbs (3/15), anti-GalNAc-GD1a AGAbs (4/15) or AGAbs reactive against both targets (8/15). The reported diagnoses for these ONM dogs were axonal polyneuropathy (n=3), chronic polyneuropathy (n=3), and n=1 each of axonal polyneuropathy

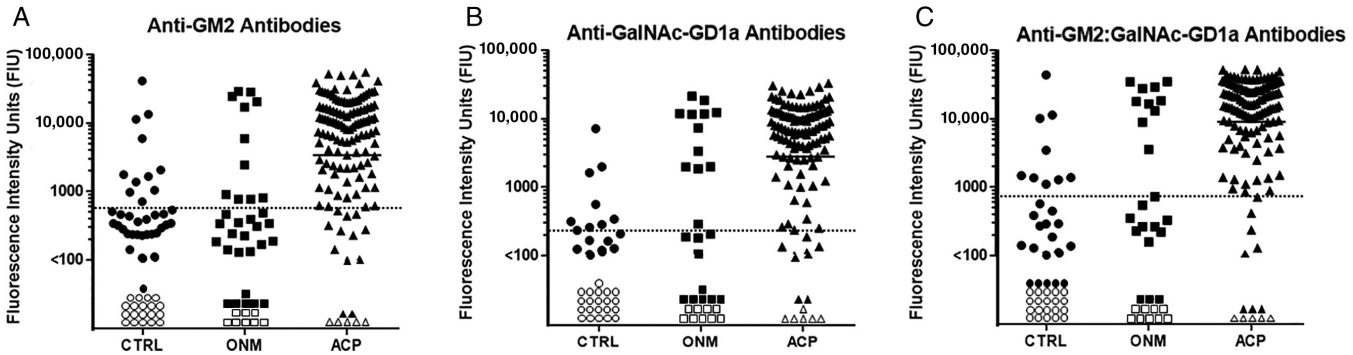

FIG 3. Dotplots displaying the fluorescence intensity unit (FIU) measurement for all serum samples [1 sample=solid black shape, groups of 10 samples=open white shape (used for values less than 100 FIU)] for the single glycolipid antigen targets GM2 (A) and GalNAc-GD1a (B), and heteromeric complex GM2:GalNAc-GD1a (C). The dashed lines depict the threshold cut-off values for each antigen (GM2: 542.0 FIU; GalNAc-GD1a: 217.0 FIU; GM2:GalNAc-GD1a: 754.5 FIU)

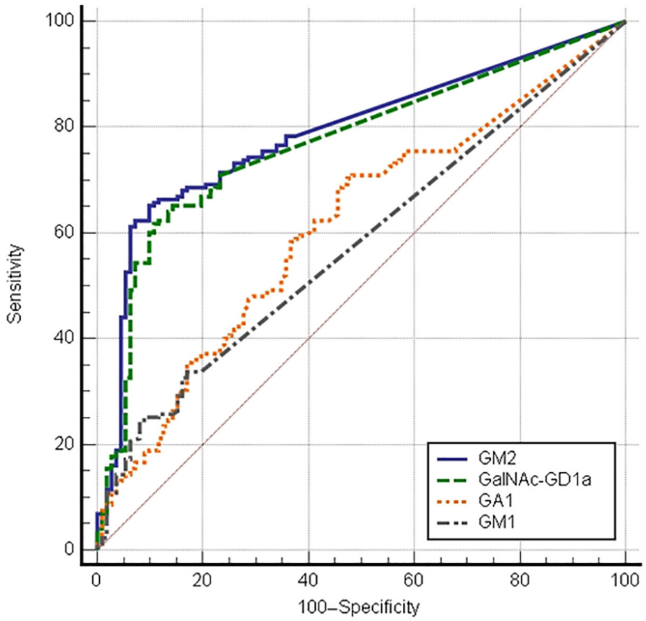

FIG 4. Receiver operating characteristic (ROC) curves to graphically display the trade-off between sensitivity (ACP) and specificity (1-ONM) across all cut-off values for each of the four single glycolipid targets. Anti-GM2 AGAbs (blue solid line) exhibit the highest combined diagnostic yield followed closely by anti-GalNAc-GD1a AGAbs (green dashed line)

Table 3. Anti-glycolipid serology for single glycolipid targets and their 1:1 heteromeric complexes (comparison of ACP and ONM groups)

	FIU cut off	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)
GM1	>2.5	33.7 (26.8 to 41.2)	83.0 (74.8 to 89.5)
GM2	**>542**	**65.1 (57.6 to 72.2)**	**90.2 (83.1 to 95.0)**
GA1	>41.5	68.6 (61.1 to 75.4)	54.5 (44.8 to 63.9)
GalNAc-GD1a	**>217**	**61.7 (54.1 to 68.9)**	**89.3 (82.0 to 94.3)**
GM1:GM2	>316	57.1 (49.5 to 64.6)	94.6 (88.7 to 98.0)
GM1:GA1	>84.5	26.3 (19.9 to 33.5)	91.1 (84.2 to 95.6)
GM1:GalNAc-GD1a	>108	59.4 (51.8 to 66.8)	89.3 (82.0 to 94.3)
GM2:GA1	>336.5	67.4 (59.9 to 74.3)	90.2 (83.1 to 95.0)
GM2:GalNAc-GD1a	**>754.5**	**66.9 (59.4 to 73.8)**	**91.1 (84.2 to 95.6)**
GA1:GalNAc-GD1a	>319	62.9 (55.2 to 70.0)	88.4 (81.0 to 93.7)

Note: The bold values are the antibodies considered.
FIU Fluorescence intensity unit, CI Confidence interval

without preferential nerve root involvement, bilateral facial and vestibular neuropathy, unilateral abducens neuropathy, proximal demyelinating polyneuropathy, polyneuropathy without recovery, polyneuromyopathy, myasthenia gravis with concurrent polyneuropathy, neuromuscular syndrome with concurrent hypothyroidism and caudal brachial plexus avulsion.

Finally, 16 of the 226 CTRL dogs (7.1%) exhibited anti-GM2 AGAbs (8/16), anti-GalNAc-GD1a AGAbs (4/16) or both these AGAbs (4/16).

DISCUSSION

This study, examining sera for AGAbs from a large, geographically heterogenous group of dogs of various different breeds and ages clinically diagnosed with ACP, confirmed and extended previous results showing anti-GM2 IgG antibodies as potentially useful biomarkers for ACP in a smaller group of dogs from Italy (Rupp *et al.* 2013). In addition, we confirmed the salient clinical and seasonal features described in the previous literature (Hirschvogel *et al.* 2012, Laws *et al.* 2017, Martinez-Anton *et al.* 2018).

In comparison with human GBS, in which AGAb profiles are heterogenous according to clinical subtypes (Goodfellow & Willison 2016), ACP-dogs examined for the same antigenic targets as human GBS-patients exhibit a more homogenous AGAb profile with predominant binding to the glycolipids GM2 and/or GalNAc-GD1a. Co-existence of these two AGAbs in a single serum is frequently observed, either representing two distinct antibody species each recognising unique molecular components on the gangliosides GM2 and GalNAc-GD1a, or more likely a single antibody species which binds to the shared terminal trisaccharide moiety present on both gangliosides (Ilyas *et al.* 1988, Santafe *et al.* 2005). In human neuropathy subjects, the co-existence of anti-GM2 and anti-GalNAc-GD1a (albeit IgM) AGAbs is seen in GBS-patients with a predominantly demyelinating neuropathy characterised by sensory loss, frequent facial nerve deficits and only mild weakness (Kaida *et al.* 2001), and has also been reported in chronic sensory demyelinating neuropathies (Lopate *et al.* 2002) and a chronic motor demyelinating neuropathy (Ortiz *et al.* 2001). The anti-GM2 AGAb association with predominantly demyelinating features may also correlate with the demyelination seen in ACP (Cummings & Haas 1967, Northington *et al.* 1981) and is further supported by immunostaining studies that localise GM2 in canine peripheral nerve to the abaxonal Schwann cell surface and less commonly to axons themselves (Rupp *et al.* 2013). However, AGAbs that bind the shared terminal moiety common to GM2 and GalNAc-GD1a have also been observed binding murine motor nerve terminals and Schwann cells overlying the motor nerve terminal, suggesting that distal nerve structures might also be targeted in disease (Santafe *et al.* 2005). Research has demonstrated that one mechanism by which AGAbs can lead to injury of structures is by activation of the complement cascade and formation of a membrane attack complex (Halstead *et al.* 2005). This results in pathological changes to and dysfunction of the structures targeted (O'Hanlon *et al.* 2001, Halstead *et al.* 2004, McGonigal *et al.* 2010, Rupp *et al.* 2012), with complement inhibitors in turn being able to abrogate injury and dysfunction (Halstead *et al.* 2008) and such treatment now being assessed alongside immunoglobulin treatment of GBS-patients (Davidson *et al.* 2017). Damage to Schwann cells, axons and motor nerve terminals could lead to the mixed axonal and demyelinating (electrophysiological) phenotype described in ACP-dogs (Cuddon 1998, Hirschvogel *et al.* 2012, Rupp *et al.* 2013), where it has been suggested that whilst electrophysiological changes are reported both in the nerve roots and along the entire peripheral nerve, distal motor axonal changes may mask the ability to detect demyelination along the length of the nerve (Cuddon 1998).

Only a small proportion of ACP-dogs (7.4%; n=11) had been vaccinated in the 6 weeks preceding disease onset, which either indicates that post-vaccination onset of ACP is extremely rare (Olby 2004) or that there actually is no such association. Interestingly, all six

ACP-puppies (dogs younger than 6 months), five of which in this study were reported to have developed disease within 3 weeks of vaccination, were devoid of both anti-GM2 and anti-GalNAc-GD1a AGAbs. In contrast to this, the majority of the six older dogs presenting with ACP post-vaccination, commonly with slightly longer timeframes (up to 6 weeks) developed anti-GM2 and anti-GalNAc-GD1a AGAbs. The reason for this is unclear, considering that vaccine components for standard vaccination of dogs should not differ between puppies and adult dogs, whilst the results for the combined age groups are less supportive of a reliable association between vaccination and ACP. Unfortunately, exact vaccination protocols were un-retrievable for the majority of dogs, but these interesting observations could be explored in future studies, also to determine a true risk for post-vaccination ACP, especially in puppies.

Gastrointestinal and respiratory upset preceding disease onset within 3 weeks were reported in a relatively low number of ACP-dogs (combined total of 11.4%) and were not associated with the presence or absence of AGAbs. Recent research describes the consumption of raw chicken, associated with a potential mild clinical or subclinical infection with *Campylobacter* spp as a risk factor for the development of ACP (Martinez-Anton *et al*. 2018). Campylobacter infection is commonly associated with human GBS and considerable data indicate that molecular mimicry between Campylobacter lipo-oligosaccharides and gangliosides is the underlying mechanistic driver for the development of AGAbs and consequent GBS (Willison & Yuki 2002). It is possible that a similar mechanism might be present in canine ACP, at least that associated with AGAbs and perhaps associated with a clinically silent *Campylobacter* spp infection.

In several dog breeds, all ACP-dogs exhibited the AGAbs under investigation. This may echo a greater risk described for West Highland White Terriers to develop ACP (Laws *et al*. 2017), and Maltese and Poodles representing the most commonly affected breeds in a different study (Martinez-Anton *et al*. 2018). Furthermore, Poodles, Siberian Huskies, West Highland White Terriers and Maltese represent breeds described or suspected to be predisposed to autoimmune-mediated diseases in general (Abramson 2004, Bergvall 2012, White & Hohenhaus 2012, Dodi 2015). Whilst the relatively small sample group size did not allow for further analyses of these observations, examination of larger groups of these dogs could potentially highlight involved susceptibility genes, in turn shedding light on pathomechanisms.

A small proportion of dogs in the two control groups had anti-GM2 and/or anti-GalNAc-GD1a AGAbs, as we previously described (Rupp *et al*. 2013) and as occasionally also is observed in human non-neurological serum control samples (O'Hanlon *et al*. 2000). Considering 93/112 ONM samples (83.0%) represented various forms of peripheral and cranial neuropathies, this further supports that the AGAbs under investigation indeed are most likely to be directly associated with and specific to ACP and do not represent unrelated AGAbs, *i.e*. bystanders for example developing as a consequence to nerve damage. In the ONM-group, the AGAb-positive cases included six dogs affected with acute peripheral polyneuropathies deemed clinically or electrophysiologically incompatible with ACP and nine dogs with various other diagnoses. Whether especially these first six dogs represent misdiagnoses of variants or formes frustes of ACP or true false positives, or a combination of both is presently unknown. All efforts were made to standardise case ascertainment and sample submission/preparation and we have no evidence to indicate diagnostic acumen or sample preparation, both of which represent the most significant limitations in this large multi-centre study, to have affected our results. In regard to logistical aspects of sample handling, our analysis indicates that specimen submission at room temperature or accompanied by an icepack (if previously frozen) is sufficient for the determination of AGAbs. Finally, the overall seropositivity of 60 to 65% for the AGAbs under investigation may reflect that ACP may have varying underlying immunological causes, some of which are associated with distinct, yet so far unidentified biomarkers, and that similar to human GBS, a number of different AGAbs may play a role in supporting the clinical diagnosis (Kusunoki *et al*. 2021), overall warranting further research in this field.

In summary, the results of this study confirm the value of examining dogs clinically diagnosed with ACP for the presence of serum IgG AGAbs to support the clinical diagnosis, at the same time bearing in mind that a negative AGAb-result does not categorically rule out the presence of ACP. Taking into account the substantial overlap of anti-GM2 and anti-GalNAc-GD1a AGAbs, the slightly higher combined sensitivity and specificity for anti-GM2 AGAbs when compared with anti-GalNAc-GD1a AGAbs, and glycolipid availability and cost, we conclude that anti-GM2 AGAb measurement is the most convenient single supportive biomarker for ACP.

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Conflict of interest

No conflicts of interest have been declared.

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