PAPER

# Serological markers of gluten sensitivity in Border terriers with gall bladder mucocoeles

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**Objectives:** To evaluate serological markers of gluten sensitivity in conjunction with cholecystokinin measurement in Border terriers with gall bladder mucocoeles.

**Materials and Methods:** Medical records from two referral hospitals were obtained between 2011 and 2019 to identify Border terriers with gall bladder mucocoeles, non-Border terriers with gall bladder mucocoeles and control Border terriers with non-biliary diseases. Enzyme-linked immunosorbent assays were performed on stored fasted serum samples for anti-gliadin IgG, anti-canine transglutaminase-2-IgA autoantibodies and cholecystokinin. Statistical analysis was performed using the Kruskall-Wallis test to identify differences between the groups.

**Results:** Fifteen Border terriers with gall bladder mucocoeles, 17 non-Border terriers with gall bladder mucocoeles and 14 control Border terriers with non-biliary diseases were recruited. Median transglutaminase-2-IgA autoantibodies in Border terriers with gall bladder mucocoeles was 0.73 (range: 0.18 to 1.67), which was significantly greater than in control Border terriers at 0.41 (0.07 to 1.14). Median cholecystokinin concentration in Border terriers with gall bladder mucocoeles was 13 pg/mL (6 to 45 pg/mL), which was significantly lower than in control Border terriers at 103 pg/mL (9 to 397 pg/mL). There was no difference in the anti-gliadin IgG between these groups. There was no difference observed in the non-Border terriers with gall bladder mucocoeles with either of the other groups.

**Clinical Significance:** Reduced cholecystokinin and increased transglutaminase-2-IgA autoantibodies was detected in Border terriers with gall bladder mucocoeles; which is in part homologous to gall bladder disease identified in human coeliac disease. The results suggest an immunological disease with impaired cholecystokinin release may be affecting gall bladder motility and possibly contributing to mucocoele formation in Border terriers.

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There is no off-label antimicrobial use.

## INTRODUCTION

Gall bladder mucocoele (GBM) is a biliary disorder recognised in dogs and is characterised by an abnormal accumulation of semi-solid bile or mucus that results in varying degrees of distension of the gall bladder and biliary duct obstruction (Aguirre *et al.* 2007).





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Reports of GBM have recently increased, suggesting an emerging phenomenon, and with no comparable condition in humans, this has prompted investigations into the pathogenesis of GBM in dogs (Pike *et al*. 2004, Gookin *et al*. 2015, Kesimer *et al*. 2015). Previously proposed associations in GBM formation have included hyperlipidaemia (Kutsunai *et al*. 2014), hyperadrenocorticism, hypothyroidism (Mesich *et al*. 2009), increased serum leptin (Lee *et al*. 2017), neonicotinoids (Gookin *et al*. 2015) and a possible genetic mutation in Shetland sheepdogs that has been subsequently disputed (Mealey *et al*. 2010, Cullen *et al*. 2014). These factors may increase the risk of GBM, possibly by altering gall bladder motility (Tsukagoshi *et al*. 2012) or altering the composition of bile and mucin within the gall bladder (Kook *et al*. 2012). Some purebreed dogs are at increased risk of GBM formation, including Shetland sheepdogs, miniature schnauzers, Pomeranians, Chihuahuas, American cocker spaniels, Affenpinschers and Border terriers (BTs) (Aguirre *et al*. 2007, Kutsunai *et al*. 2014, Allerton *et al*. 2018).

Breed associated disease studies in BTs have supported gluten sensitivity as the cause for paroxysmal gluten-sensitive dyskinesia (PGSD), a movement disorder homologous to paroxysmal non kinesigenic dyskinesia observed in people (Jankovic & Demirkiran 2002, Black *et al*. 2014, Lowrie et al. 2015, 2016). Investigators established this link by documenting serum anti-gliadin IgG (AGA IgG) and anti-canine transglutaminase-2-IgA (TG2 IgA), (markers of gluten sensitivity in humans) were increased in BTs with PGSD and subsequently decreased after transition to a gluten free diet (Lowrie *et al*. 2015, 2018). These authors proposed that PGSD in dogs is part of a syndrome of gluten sensitivity in the BT breed, including gastrointestinal and dermatological signs, representing a systemic gluten associated disease (Lowrie *et al*. 2016, 2018).

In coeliac disease in humans, gall bladder sludge formation can be identified due to a reduction in gall bladder motility (Wang *et al*. 2017) and this hypomotility is attributed to a reduction in cholecystokinin (CCK) secretion (Maton *et al*. 1985, Upp *et al*. 1987, Fraquelli 1999, Wang *et al*. 2017). CCK is an endogenous peptide that stimulates pancreatic enzyme release and gall bladder contraction and delays gastric emptying (Hall *et al*. 2016). In humans suffering from coeliac disease it has been demonstrated that transition to a gluten free diet results in increased CCK concentration and subsequently increased gall bladder contractility (Maton *et al*. 1985).

Based on this link and the known history of gluten sensitivity in BTs, it was hypothesized that GBM formation in BTs is part of the proposed systemic gluten associated disease. Therefore, the primary aim of this study was to determine if serological indices of gluten sensitivity (increased AGA IgG and TG2 IgA) are significantly associated with decreased CCK concentrations in BTs with GBM.

## MATERIALS AND METHODS

### Study population

For this pilot study, medical records were retrospectively reviewed to identify all dogs with a diagnosis of GBM presented to Langford Vets Small Animal Referral Hospital (LVS) between January 1, 2011 and January 1, 2018. Ethical approval was granted by the Animal Welfare and Ethical Review Body (VIN/17/038) and Faculty Health Sciences Research Ethics Committee (FREC) at the University of Bristol. Dogs were identified using the keywords "mucocoele" and "mucocele." From January 2, 2018 to January 7, 2019, prospective enrolment was undertaken for dogs similarly presenting with GBM to LVS and Willows Veterinary referrals, to appropriately increase the sample size calculated using the online programme http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize. This sample size calculation used a power of 0.8 and significance level of 0.05. A previous paper looking at CCK in humans with coeliac disease gave a reference standard deviation of 2.4 pg/mL (Fraquelli *et al*. 1999). This sample size calculation, based on an expected difference of 2.4 in the mean concentration of CCK, determined the minimum number of dogs required in each group to detect that difference would be 15. A control group of BTs with non-biliary tract disorders presenting to LVS was also recruited for inclusion, retrospectively within the same study period January 1, 2011 to January 1, 2018, and prospectively from January 2, 2018 to January 7, 2019.

A "Biobank" store at LVS was searched for residual fasted serum samples for analysis from both GBM and control dogs. For canine cases enrolled prospectively, residual fasted serum was collected and stored within that "Biobank" at −80°C until analysis. All samples were derived from residual material after routine clinical investigations with owner permission obtained via a consent form, signed on admission to the hospital.

Medical records were searched, and information collected, to include where available: breed, age, sex, neutered status, bodyweight, current and historical dietary history. For GBM cases, additional data recorded included histopathology results from the gall bladder and liver; culture results for bile, liver and gall bladder wall; the presence or absence of gall bladder rupture (as recorded in the ultrasound or surgical reports). We also looked to identify and describe the presence of underlying endocrinopathies, results for endocrine testing for hypothyroidism, diabetes mellitus and hyperadrenocorticism were assessed. Results were collected from either historic or current medical records including: blood glucose, thyroxine and thyroid stimulating hormone (T4/TSH), adrenocorticotrophic hormone (ACTH) stimulation test and low dose dexamethasone suppression test (LDDST).

For inclusion in the GBM group, dogs required: an abdominal ultrasound or CT scan reviewed by a board-certified radiologist or supervised resident in training with a diagnosis of GBM as per previously described criteria; immobile bile with a striated or stellate pattern (Choi *et al*. 2014). All dogs required an imaging confirmed diagnosis of GBM, and for dogs where histopathology was performed, evidence of mucinous hyperplasia was required to confirm the imaging diagnosis.

For inclusion in the control group, dogs required an abdominal ultrasound or CT, reviewed by a board-certified radiologist or resident in training, with no evidence of biliary disease; all of the animals in this control group were BTs. Criteria for no evidence of biliary disease included: a normal biliary tree on ultra-

sound showing a normal gall bladder wall thickness (<2 mm, (Nyland & Hager 1985)) and no evidence of gall bladder wall oedema (thickened gallbladder wall with a layered appearance and hypoechoic central layer) and on CT or ultrasound, no evidence of choleliths (hyperechoic structure causing acoustic shadowing), no evidence of biliary sludge (hyperechoic floccules within the gall bladder) and no evidence of a GBM (based on the prior inclusion criteria listed). These animals had this imaging performed within 48 hours of their blood sampling.

All animals also required a dietary history for review and no previous history compatible with PGSD (clinical signs of a movement disorder suspected to be consistent with PGSD, with no evidence of pre or post-ictal signs and involuntary movement of one or more limbs whilst remaining conscious) recorded in their clinical and historical notes.

### Serology

All samples were measured for concentrations of anti-gliadin IgG (AGA IgG), anti-canine transglutaminase-2-IgA autoantibodies (TG2 IgA) and CCK. These were determined by enzyme linked immunosorbent assay (ELISA) according to manufacturer's guidelines (AGA IgG and TG2 IgA: ZEDIRA GmbH Darmstadt, Germany, CCK: Biomatik™, Ontario, Canada) and as previously detailed (AGA IgG and TG2 IgA: Lowrie *et al.* 2015, CCK: Noh *et al.* 2016). All serum samples were stored at −80°C until they were assayed in duplicate, alongside controls and standards, and their average value reported for analysis. All washing steps were performed by a BioTek 50 TS automated microplate washer and optical density measurements determined by a Thermo Scientific Multiskan GO spectrophotometer.

Briefly, for anti-canine TG2 IgA detection, the microtiter plate was coated with canine TG2 antigens and the surface blocked with bovine serum albumin prior to use with canine sera and control samples. Bound antibodies against canine TG2 were detected by incubation with peroxidase-conjugated secondary antibody against canine IgA. The peroxidase converts a substrate (tetramethylbenzidine) into a blue product that with the addition of the stop product (0.2 M $H_2SO_4$) turns yellow. Negative control values were recorded using a buffer and conjugate.

For IgG anti-gliadin antibodies, a purified gliadin-coated microtiter plate was used. Detection of bound anti-gliadin antibodies resulted from incubation with peroxidase-conjugated secondary antibody against canine IgG, conversion of substrate and addition of stop solution as described above for anti-canine TG2 antibody. Negative control values were recorded using a buffer and conjugate.

CCK was measured via competitive inhibition ELISA using pre-coated microplates containing a monoclonal antibody specific for CCK. Samples, standards and blanks were incubated alongside biotin labelled CCK so that, with the addition of peroxidase-conjugated avidin, the subsequent conversion of substrate (tetramethylbenzidine) was inversely proportional to sample CCK concentration. Using a commercial software programme (CurveExpert Basic 2.0) a standard curve was produced using regression analysis, from which serum CCK concentrations could be extrapolated, supplementary file Appendix 1.

### Statistical analysis

Data were analysed using a commercial computer software programme (Microsoft Excel™) and commercial statistical package (SPSS statistics, v.26 IBM corps). Results were identified to be non-normally distributed using Shapiro-Wilks test. The Independent Samples Kruskall-Wallis test was used to assess if there was a statistical difference in the TG2 IgA, AGA IgG and CCK values as well as the ages, between BTs with GBM, non-BTs with GBM and the control group of BTs. Pairwise comparisons were then performed, with significant values adjusted by the Bonferroni correction for multiple tests, with statistical significance set at $P < 0.05$.

## RESULTS

### Non-BTs with GBM

Seventeen non-BTs with GBM were identified and met the inclusion criteria. These dogs were a variety of breeds including: Jack Russell terrier (3), Shetland sheepdog (3), miniature schnauzer (2), bichon frise (2), Crossbreed (1), English bull terrier (1), Siberian husky (1), poodle (1), Pomeranian (1), shih-tzu (1) and Cavalier King Charles spaniel (1). There were 5 neutered females, 1 intact male and 11 neutered males. The median age at diagnosis was 9.5 years (range 2.3y –14.1y), median weight was 11.4 kg (range 2.7–30.1 kg). All dogs in this group were fed a commercial dog food.

### BTs with GBM

Fifteen BTs with GBM were identified who met the inclusion criteria. There were two intact females, six neutered females, two intact males and five neutered males. The median age at diagnosis was 10.4 years (range 5.1y–13.3y), median weight was 9.0 kg (range 7.0–12.0 kg). All dogs were fed a commercial dog food.

### Control BTs

Fourteen control BTs were diagnosed with other conditions, with no evidence of biliary disease on abdominal imaging. Other conditions included gastrointestinal disease [acute gastroenteritis (2), gastric foreign body (1), pancreatitis (1) and chronic enteropathy (1)], liver disease [acute hepatitis (2) and portosystemic shunt (1)], neurological disease (4), pyometra (1) and syncope (1). There was one intact female, four neutered females, one intact male and eight neutered males. The median age at diagnosis was 6.4 years (range 1.6–13years) median weight was 8.3 kg (range 6.5–10.1 kg). All dogs in this group were fed a commercial dog food.

Between the three groups, there was a significant difference between the ages of the control and BT GBM group ($P = 0.045$) and control and non-BT GBM group ($P = 0.021$).

### Gall bladder histopathology

In total, 18 of 32 dogs had gall bladder histopathology performed with all of these dogs having a histopathological diagnosis consistent with a GBM, 7 of 17 non-BTs and 11 of 15 were BTs. In the remainder of dogs, histopathology was not performed and the diagnosis of GBM was made on imaging confirmation alone.

Gall bladder rupture was identified on abdominal ultrasound or recorded in the surgical report in 4 of 16 BTs and 8 of 17 non-BTs.

## Liver histopathology

Liver histopathology was performed in 5 of 17 non-BTs with GBM, and identified: portal fibrosis with bile duct proliferation and parenchymal necrosis (n = 1), biliary hyperplasia and portal and pericellular fibrosis (1), extrahepatic cholestasis (1), mild lymphoplasmacytic cholangiohepatitis (1), and severe chronic hepatitis with fibrosis (1).

Liver histopathology was perfomed in 6 of 15 BTs with GBM group, and identified: neutrophilic cholangiohepatitis (1), mixed lymphoplasmacytic and neutrophilic cholangiohepatitis with cholestasis (2), chronic extrahepatic cholestasis and pericellular fibrosis (1), nodular hyperplasia with mixed neutrophilic, lymphoplasmacytic cholangiohepatitis and portal fibrosis (1) and multifocal hepatocyte necrosis with patchy, mild pericellular fibrosis and extrahepatic cholestasis (1).

## Culture results

Gall bladder wall or bile culture was performed. In BTs with GBM, 11 of 15 had culture performed but a positive culture was only identified in one dog; a positive growth of *Escherichia coli* in both the bile and gall bladder wall in this dog. In non-BTs with GBM, 9 of 17 had culture performed and a positive culture was found in 4 of 9 of these dogs. Cultures were positive for *Escherichia coli* in two dogs, one from the gall bladder wall and one from the bile. One dog had a positive culture of *Lactobacillus* from the gall bladder wall and one dog had a positive culture of *Serratia marcescens* from the gall bladder wall.

Liver culture was performed. In BTs with GBM, 5 of 15 BTs had culture performed and no positive cultures were identified. In non-BTs with GBM, 5 of 17 had culture performed and one dog had a positive culture with a growth of *Clostridia spp*.

## Serum testing

Values of TG2 IgA for control BTs ranged from 0.07 to 1.14 (median 0.41), for BTs with GBM from 0.18 to 1.67 (median 0.73) and for non-BTs with GBM from 0.1 to 1.64 (median 0.60). There was a significant difference in TG2 IgA between the groups, and on pairwise comparison, the TG2 IgA was significantly increased in BTs with GBM compared to control BTs, P = 0.015 (Fig. 1), the P value was still significant when adjusted for multiple comparison (P = 0.045).

Concentrations for CCK in control BTs ranged from 9 to 397 pg/mL (median 103 pg/mL), in BTs with GBM from 6 to 45 pg/mL (median 13 pg/mL) and for non-BTs with GBM from 6 to 306 pg/mL (median 45 pg/mL). There was a significant difference in CCK between the groups, and on pairwise comparisons, the CCK was significantly lower in BTs with GBM compared to control BTs, P = 0.007 (Fig. 2), the P value was still significant when adjusted for multiple comparison (P = 0.016).

Values of AGA IgG for control BTs ranged from 0.08 to 1.89 (median 0.45), for BTs with GBM from 0.11 to 3.16 (median 0.36) and for non-BTs GBM from 0.08 to 0.27 (median 0.15). No statistically significant difference was noted in AGA IgG between the groups, P = 0.58 (Fig. 3), multiple comparisons were not performed because the overall test did not show significance.

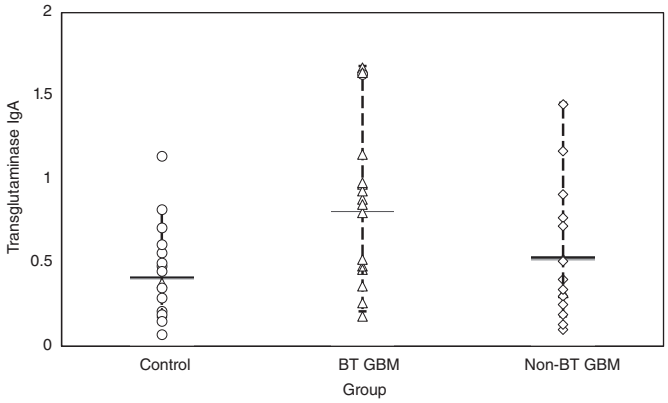


**FIG 1. Box plots demonstrating the transglutaminase-2 IgA (TG2 IgA) in each group of dogs. The solid line through represents the median. Control border terriers ○ (Control BT n = 14.) Border terrier gall bladder mucocoeles △ (BT GBM n = 15), Non-border terrier gall bladder mucocoeles ◇ (Non-BT GBM n = 17)**

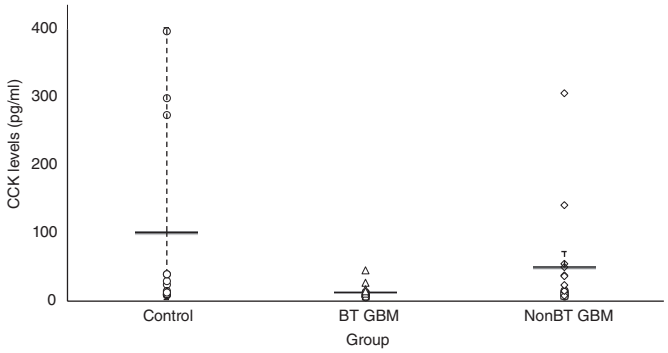


**FIG 2. Dot plots demonstrating the cholecystokinin (CCK) in each group of dogs. The solid line through represents the median. Control border terriers ○ (Control BT n = 14.) Border terrier gall bladder mucocoeles △ (BT GBM n = 15), Non-border terrier gall bladder mucocoeles ◇ (Non-BT GBM n = 17)**

Pearson's correlation coefficient found no significant correlation between values of CCK and either TG2 IgA (–0.05) or AGA IgG (–0.13), Figs. 4a, b.

## Endocrine disease

There were four dogs with diagnosed endocrinopathies. Two of which had multiple endocrinopathies; a non-BT GBM with concurrent hypothyroidism and hyperadrenocorticism, on treatment with thyroxine supplementation and not receiving treatment for hyperadrenocorticism at the time of referral, and a BT with GBM with concurrent hypothyroidism and diabetes mellitus, on treatment with thyroxine supplementation and insulin therapy. The remaining two dogs were one non-BT with GBM and one BT with GBM, and both presented with hyperadrenocorticism, and were diagnosed based on consistent clinical signs and one on ACTH stimulation test and the other on LDDST, neither dog was receiving treatment at the time of presentation (Table 1).

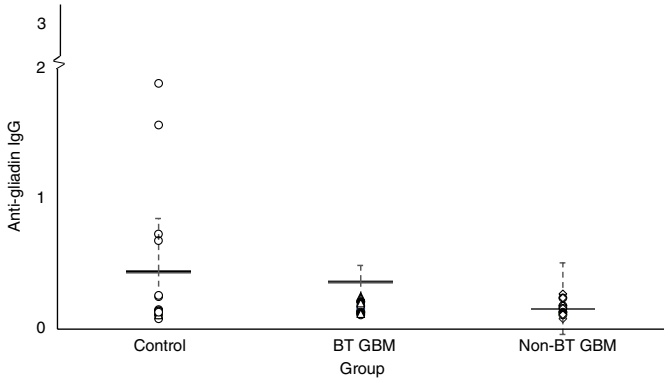


**FIG 3. Box plots demonstrating the anti-gliadin IgG (AGA IgG) in each group of dogs. The solid line through represents the median. Control border terriers ○ (Control BT n = 14.) Border terrier gall bladder mucocoeles △ (BT GBM n = 15), Non-border terrier gall bladder mucocoeles ◇ (Non-BT GBM n = 17)**

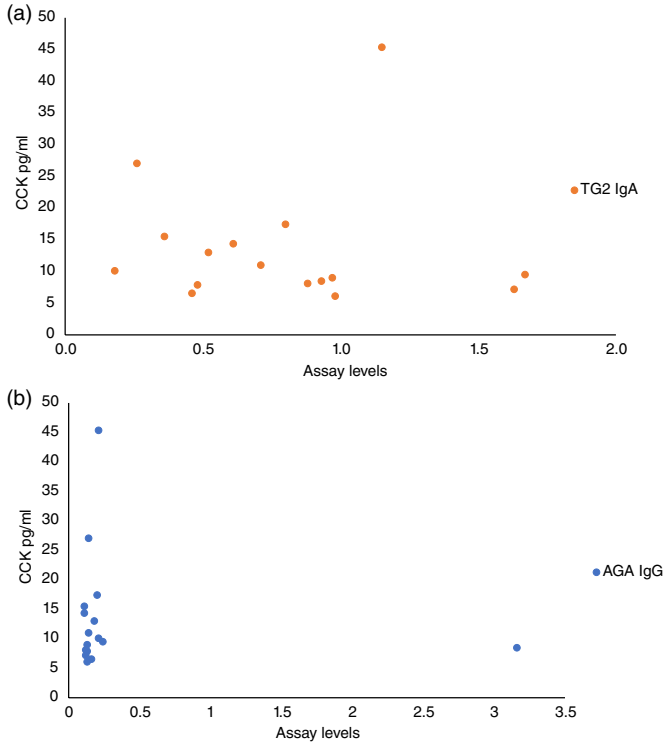


**FIG 4. (a) Scatter plot demonstrating (the correlation of) transglutaminase-2 IgA (TG2 IgA) values with the CCK (cholecystokinin) concentration in Border terriers with gall bladder mucocoeles (BT GBM). (b) Scatter plot demonstrating (the correlation of) anti-gliadin IgG (AGAIgG) values with the CCK (cholecystokinin) concentration in Border terriers cholecystokinin) concentration in Border terriers**

## DISCUSSION

This is the first study to evaluate BTs with GBM for markers of gluten intolerance, and may suggest a possible mechanism for GBM formation in the BT. Our study identified serological evidence of reduced CCK and increased TG2 IgA relatively in BTs with GBM compared to control BTs without any imaging evidence of biliary disease. These results are in part homologous to gall bladder disease identified in human coeliac disease, where decreased levels of CCK are seen, causing gall bladder hypomotility (Maton *et al.* 1985, Upp *et al.* 1987, Fraquelli 1999), and this has been associated with biliary sludge formation (Wang *et al.* 2017) suggesting that there could be an association between GBM formation and a similar gluten associated immunological disease process in the BT.

**Table 1. Number of previously diagnosed endocrinopathies**

| Cases of endocrine disease | BTs GBM n = 17 | Non-BTs GBM N = 15 | Control BTs N = 9 |
|---|---|---|---|
| Diabetes mellitus (% of all cases) | 1 (5%) | – | – |
| Hyperadrenocorticism (% of all cases) | 1 (5%) | 2 (13%) | – |
| Hypothyroidism (% of all cases) | 1 (5%) | 1 (6%) | – |

Gluten sensitivity in humans is an immunological response to the ingestion of gluten that can cause varying systemic diseases, including gastrointestinal and extra-gastrointestinal, which result in varied clinical signs including neurological and dermatological abnormalities. Diagnosis involves identifying serological evidence of antibodies to gliadin or auto-antibodies to transglutaminases (Lewis & Scott 2010). Gluten sensitivity in dogs is less well described, with gluten sensitive gastrointestinal disease identified in several breeds including the BT, soft-coated Wheaten terrier and Irish setter (Hall & Batt 1992, Vaden *et al.* 2000, Lowrie *et al.* 2016). Neurological and dermatological manifestations of gluten sensitivity appear to have only been identified in the BT (Lowrie *et al.* 2015, 2016). Our study has identified an increase in TG2 IgA to support serological evidence of autoantibodies to this transglutaminase in BTs with GBM and supporting the hypothesis that a similar immunological disorder may exist within BTs with GBM. However, we did not identify a significant difference within the AGA IgG between these groups, compared to previous studies assessing gluten sensitivity within the BT breed (Lowrie *et al.* 2018). This previous study used healthy controls so this may have affected the results we achieved. Additionally our knowledge of gluten sensitivity within dogs and specifically the BT breed is still limited, and subsequently this pilot study may have identified that there is variation in the accuracy of this ELISA between different gluten-related disease processes. Alternatively, this finding could represent a type II error, as our power calculation was based on a human study related to CCK, meaning this study could have been underpowered to identify a serological difference. Finally, the difference could relate to the sensitivity and specificity or predictive values of these antibody tests performed. In human coeliac disease, the single most sensitive and specific serological marker of coeliac disease is the IgA anti-transglutaminase 2 (IgA TG2), which was significantly increased in our GBM BT group. The anti-gliadin antibody (IgG AGA) has a lower sensitivity and specificity, leading to diminished utility of the marker for coeliac diagnosis in people, especially with the introduction of newer generations of anti-gliadin antibody tests that use disease specific deamidated gliadin peptides to optimise

test sensitivity and specificity (Briani *et al*. 2008). Therefore, this pilot study suggests that a gluten-related disorder could be involved in GBM formation in BTs, but further studies are warranted, including measurement of the deamidated gliadin in these patients too.

Gall bladder contraction is controlled by a number of neuropeptides including CCK (Krishnamurthy & Krishnamurthy 1997, Hall *et al*. 2016). Therefore, the identification of a significant reduction in CCK in BTs with GBM supports that gall bladder hypomotility could be involved in the pathophysiology of mucocoele formation in this breed. These results support previous studies which have hypothesized that GBM formation in dogs is due to a reduction in gall bladder motility (Tsukagoshi *et al*. 2012). The results from our study, suggest that GBM formation in the BT may have a similar pathophysiology to biliary disease identified in human coeliac disease, whereby a reduction in CCK levels causes gall bladder hypomotility. However, as significance was noted only within IgA TG2 it is possible another mechanism, unrelated to a gluten disorder is responsible for the reduction in GB motility identified. Further studies to examine standardised meal-induced gall bladder emptying in dogs with GBM and low serum CCK measurement are warranted to investigate this.

Interestingly in gluten sensitive BTs with PGSD, transition to a gluten free diet has been shown to cause an improvement in both clinical signs and a reduction in their serological antibody markers (Lowrie *et al*. 2018). In humans with coeliac disease, transition to a gluten free diet results in an increase in CCK levels and subsequently increased gall bladder contraction, causing a reduction in biliary sludge formation (Wang *et al*. 2017). Therefore, we propose that a dietary change to gluten free feeding could also be beneficial in the management of GBM formation in BTs, possibly due to a similar effect on gall bladder motility. However to truly explore the significance of a gluten free diet in GBM formation, a prospective randomised study assessing serological levels of TG2 IgA, AGA IgG and CCK in BTs with non-surgical GBM before and after transition to a gluten free diet, alongside direct assessment of gall bladder emptying, would need to be performed.

The significance of a reduction in CCK is important due to its role in gall bladder motility within the BT breed, although it is possibly relevant in other breeds presenting with GBM, despite non-BT not having a significant reduction in CCK in our study. Previous work has identified hyperadrenocorticism as an associative factor in GBM cases (Kim *et al*. 2017), and dogs with hyperadrenocorticism have been shown to have a significant reduction in both CCK and leptin levels (Cho *et al*. 2014, Noh *et al*. 2016). Therefore, the finding of decreased CCK in dogs within this study and the previous identification of reduced CCK (and leptin) in dogs with hyperadrenocorticism suggest the cause of GBM in dogs with hyperadrenocorticism could also relate to gall bladder hypomotility. However, the roles of leptin and CCK in dogs with both GBM and concurrent endocrinopathies may be relevant and something to be explored in future studies.

Our study has a number of limitations. First, the samples for this study were collected retrospectively and stored within a "Biobank," so sample quality and storage time varied between samples. The full effect of storage on conducting the ELISAs is unknown. However, as all samples were stored in the same manner, it was thought likely that storage would have similarly affected all the samples within the three comparative groups. Specific canine data on *in vitro* stability for these antibodies and CCK are unknown, but likely comparable to other proteins and human studies. They are therefore expected to be similar with acceptable sample storage in this study. The sample qualities (degree of haemolysis, icterus and lipaemia) were also similar between the groups. Another limitation of the study was within the control group, it was not possible to age match this group so there was a significant difference in the ages of the controls and GBM dogs, meaning this may have affected the significance of results achieved. Although not presenting with signs of PGSD, there is a possibility these control dogs would develop signs of PGSD or other gluten related diseases in the future, which could have adversely affected the results and their significance, however, the age for seroconversion is not known. Previously there has been some overlap in the measurements of assays for gluten intolerance between BTs with PGSD and gastrointestinal and dermatological signs (Lowrie *et al*. 2018), it is possible there some overlap has also occurred within the dogs in this study. Additionally it is known in humans that CCK will increase with age (MacIntosh *et al*. 1999), therefore, the finding of a higher CCK within our control group despite them being younger further supports this was a true finding and the significant age difference is unlikely to have affected this result. Also within the control group, some of the animals presented with gastrointestinal signs which may have affected the ELISA results, especially the CCK. However, the decision to leave these animals within the control group was made as the study aim was to assess a difference between dogs with GBM and those without, many of which would have complex GI diseases, so the control group including similar diseases was deemed an appropriate selection. Additionally we ran CCK on these samples, without a known history of fat content of the food or exact timing of feeding, however all ELISAs were run on samples which were starved for a minimum of 8 hours. A previous study showed dogs with GBM had significantly higher pre-prandial CCK than control dogs (Noh *et al*. 2016), and in humans lower basal CCK has been noted in patients with coeliac disease compared to controls (Thompson *et al*. 1982). Therefore it seemed appropriate to use pre-prandial, basal samples in these dogs, and these results were deemed to be accurate for CCK measurement. Finally, a significant difference was noted for CCK and TG2 IgA between the BTs with GBM and control BTs but no difference between the two GBM groups. These results suggest that these findings may not be specific to GBM within the BT breed, and could be contributing to GBM disease in other breeds. The assays performed for TG2 IgA and AGA IgG have only been described in BTs previously, so their differing interpretation in other breeds could have affected these results. Additionally it could be considered that a mechanism unrelated to gluten sensitivity, and solely affecting CCK and subsequently GBM motility may be an important factor for GBM formation. To investigate these possibilities further, larger and additional studies would be needed.

In conclusion, this study provides insights on the potential pathophysiology of GBM within the BT breed. This study has identified serological evidence of auto-antibody formation against TG2, alongside a reduction in CCK, as possible markers of risk for GBM disease in BT. The results from this study suggest a gluten-related disorder and reduction in gall bladder motility may then be associated with GBM formation in BTs. However, further prospective studies are needed to elucidate whether altered gall bladder motility has an effect on GBM formation, and whether feeding of a gluten free diet may then be beneficial in the successful management of non-surgical GBM disease in BTs.

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## Conflict of interest

There is no conflict of interest for this manuscript.

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## Supporting Information

The following supporting information is available for this article:

**Appendix S1**: Supporting Information