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Myenteric networks of interstitial cells of Cajal are reduced in horses with inflammatory bowel disease

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Summary

Background: Inflammatory bowel disease (IBD) is a well-recognised but poorly understood disease complex in the horse. Clinical signs may vary but often include weight loss, diarrhoea and colic. The effect this disease process may have on the gastrointestinal pacemaker cells (the interstitial cells of Cajal), enteric neurons and glial cells has not been previously evaluated in the horse.
Objectives: To compare the density of the interstitial cells of Cajal (ICC), enteric neurons and glial cells in horses with IBD to those of normal horses using immunohistochemical markers.
Study design: Retrospective, quantitative immunohistochemical study.
Methods: Ileal samples were collected during post-mortem examinations from 14 horses with a clinical and histopathological diagnosis of IBD and from eight normal controls. All horses were Standardbreds 1–15 years of age. Six of the IBD cases had eosinophilic gastroenteritis (EG) while the remaining eight had granulomatous enteritis (GE). Tissue sections were labelled with anti-CD117 (c-Kit), anti-TMEM16 (TMEM16), anti-protein gene product (PGP9.5) and anti-glial fibrillary acidic protein (GFAP) using standard immunohistochemical labelling techniques. Image analysis was performed to quantify the presence of ICC (CD117, TMEM16) as well as neuronal (PGP9.5) and enteroglial (GFAP) networks.
Results: Interstitial cells of Cajal networks were significantly reduced in the myenteric plexus (MP) region in IBD horses compared with the controls for both markers ($P<0.05$). There was no significant difference in the density of the neuronal or glial cell markers between the two groups ($P>0.05$).
Main limitations: The number of horses included in the study.
Conclusions: Disruption to ICC networks may contribute to the clinical signs of colic in some horses with IBD. Further studies are needed to establish the pathophysiological mechanisms involved and the functional effects of the reduced ICC networks.

Keywords: Horse; IBD; immunohistochemistry; intestine; ICC; neurons

Introduction

Inflammatory bowel disease (IBD) is a well-recognised though poorly understood disease complex in the horse. In the adult horse, the most typical forms of idiopathic and non-neoplastic IBD include eosinophilic gastroenteritis (EG), granulomatous enteritis (GE) and lymphocytic-plasmacytic gastroenteritis [1,2].

Even though these forms of IBD differ in the predominant cellular infiltrate and presumably underlying causes, many affected horses present with similar clinical signs including abdominal pain (colic). This may be directly related to an ongoing inflammatory process or to changes in the gut wall architecture caused by inflammation resulting in thickening and sometimes stricture and obstruction of the intestinal lumen [3].

Lindberg found that intestinal inflammation also involved the enteric nerve plexuses in horses with GE [4], indicating that motility disturbances could contribute to the clinical signs. Similarly, due to their close proximity to enteric neurons, the inflammatory process could also have a detrimental effect on the gastrointestinal pacemaker cells, the interstitial cells of Cajal (ICC). These cells generate pacemaker currents that result in phasic contractions of intestinal smooth muscle cells [5]. Factors that affect the activity of ICC networks may also influence the contractile activity of the intestinal tract.

Previous studies have indicated that local inflammatory processes in equine gastrointestinal disorders may have an adverse effect on enteric neurons and ICC networks [6–11]. Clinical reports in human patients with IBD have documented similar changes to both neuronal and ICC networks [12–14]. It is not clear whether both these cell populations are similarly affected in horses with IBD.

The purpose of this study was to evaluate immunohistological changes to ICC, enteric neurons and glial cells in the small intestine of horses with a confirmed IBD diagnosis. The hypothesis was that these cell populations would be reduced in IBD horses compared with normal animals.

Materials and methods

Horses

Ileal samples were collected immediately following euthanasia from 22 horses. Eight were subjected to euthanasia for reasons unrelated to the gastrointestinal tract (six for lameness, two for dental disorders) and served as the control population. They had no known history of current or previous intestinal disease. The remaining 14 horses all had clinical signs consistent with IBD, including weight loss and hypoalbuminaemia. Six horses also displayed intermittent clinical signs consistent with abdominal pain. The duration of illness varied from 3 weeks to 6 months. Two of the horses had received corticosteroid therapy prior to euthanasia. All horses had macroscopic changes in the intestinal tract and in some horses this involved multiple but different sites of both the small and large intestines. None of the EG cases had focal circumferential bands resulting in stricture and acute colic but rather diffuse focal or multifocal lesions. The GE cases varied and included both horses with diffuse areas of thickened small intestine or longer segments of affected intestine. In the majority of cases, the gross changes were circumferential. Two EG horses also had skin lesions, which could be consistent with a diagnosis of multisystemic equine epitheliotropic disease but unfortunately, this was not further investigated. Although some horses had lesions in other parts of the intestinal tract, ileal lesions were present in all horses so this anatomical site was selected in

order to standardise the study and making comparison possible. A diagnosis of IBD was confirmed histopathologically in all 14 horses, eight of which had GE while the remaining six had EG. The mean age of the control horses was 8 years (median 7.5 years, range 3–15 years) while the IBD group had a mean age of 2.1 years (median 2 years, range 1–4 years). The control horses consisted of four geldings, three mares and one intact male while the IBD population comprised eight mares and six intact males. All horses in the study were Standardbreds and all samples were collected with the owners' consent.

Immunohistochemistry and histopathology

Serial sections (4 μm) were cut from formalin-fixed paraffin-embedded samples. The first section in each series was stained for haematoxylin and eosin (HE) for histopathology and subsequent sections were used in indirect single immunohistochemistry for markers of enteric neurons (Protein Gene Product, PGP9.5[a]), enteric glial cells (anti-glial fibrillary acidic protein, GFAP[a]) and ICC (c-Kit, CD117[a] and TMEM16, TMEM16[b] respectively). Unless otherwise stated, sections were washed between steps in phosphate-buffered saline (PBS). The sections were dewaxed, then incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. An automated immunostainer (Lab Vision™ Autostainer 360[c]) was used for the immunohistochemical procedure. Nonspecific antibody binding was blocked by incubating in normal goat serum. The sections were incubated at room temperature with the primary antibody. The polyclonal rabbit anti-bovine PGP9.5 (1:500) antibody, polyclonal rabbit anti-bovine GFAP (1:1000) antibody and the polyclonal rabbit anti-human antibody CD117 (1:200) were diluted in 1% bovine serum albumin/Tris-buffered saline. The rabbit monoclonal anti-human TMEM16 antibody was diluted 1:100 in PBS. The tissue sections were incubated with the biotinylated secondary antibody (polyclonal: EnVision anti-rabbit kit[b] or monoclonal: ABC Vectastain Elite kit[d]) and then with ABC/PO reagent. Immunolabelling was revealed using the 3-amino-9-ethylcarbazole solutions (AEC from either the EnVision kit[b] or the Elite kit[d]). Sections were counterstained in haematoxylin and coverslips were mounted using a mounting medium (Aquatex[e]). For negative control, the primary antibody was replaced with an irrelevant antibody.

Western blot

As the rabbit monoclonal anti-human TMEM16 antibody has not previously been reported used on horse tissues, Western blot analysis (Supplementary Item 1) was performed on frozen intestinal tissue from a healthy horse to determine the molecular weight of the protein recognised by the antibody. Western blot analysis of equine intestinal tissue revealed a prominent band at around 100 kDa (Supplementary Item 2), which was consistent with the predicted molecular weight for the TMEM16A protein[b].

Image analysis

For each immunohistochemical section, five digital images of the myenteric plexus (MP) region were collected with a digital camera (Zeiss AxioCam ERc5s[f]) at 10× magnification. The digital image was centred on a ganglion of the MP with surrounding inner circular and outer longitudinal muscle layers. If more than five ganglia were present in a section, every second ganglion was selected. For every image, two regions of interest (ROI) were interactively defined (Fig 1) using image analysis software (ImageJ[g]) [15]. One ROI was drawn to select the MP plexus and ganglion between the two muscle layers. A second ROI was drawn to include the inner circular muscle layer present in the image. Colour deconvolution for haematoxylin and AEC was then performed on each image according to Ruifrok and Johnston (2001) [16]. Threshold settings and particle size settings for detection of immunolabelling were then determined using the Colour 2 binary image and area measurements within the ROIs of each image were performed. The percentage of immunolabelling (area of labelling per area of ROI) was calculated. For each horse and antibody, the average percentage of immunolabelling for the five images was used for further quantitative analyses. The collected data from horses were divided initially into two groups, IBD horses and healthy horses and then into three groups, the EG, GE and healthy horses.

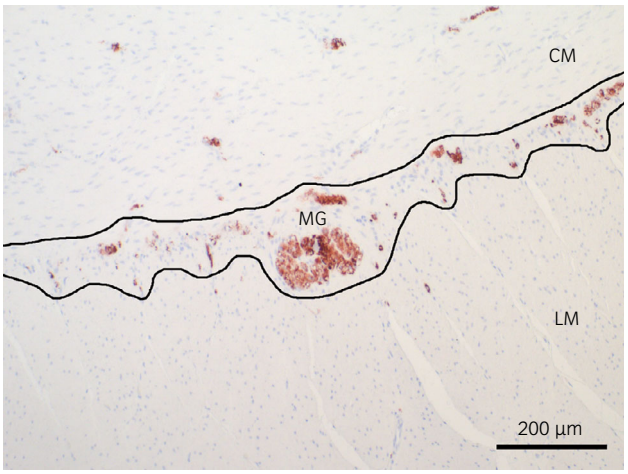

Fig 1: Immunohistochemical distribution of glial cells (GFAP) in the myenteric plexus of the ileum of a healthy horse, showing the myenteric plexus as an interactively defined region of interest (ROI, within black line). LM, longitudinal muscle; MG, myenteric ganglion.

Data analysis

The samples of the three groups were randomised prior to analysis to avoid selection bias. In addition, the reader (C.P.) was blinded to which of the groups were analysed. For group analysis, statistical software (GraphPad Prism® 7.0[h]) was used for calculation of statistical measures, including median values and interquartile ranges. The percentages of immunolabelling for the four cell markers in the two ROIs for the group of healthy horses (n = 8) and the group of IBD horses (n = 14) were compared using a Mann-Whitney test. For the comparison of the three groups of horses, healthy (n = 8), EG (n = 6) and GE (n = 8), a Kruskal-Wallis test was used. A Spearman's correlation test was used to evaluate areas of immunolabelling in the MP region and circular muscle layer. The level of significance was set to $P<0.05$.

Results

Histopathology

Typically, all IBD cases, irrespective of diagnosis, were characterised by villous atrophy and lymphoid hyperplasia of the mucosa and submucosa. In contrast, the myenteric ganglia typically appeared normal and it was rare to find inflammatory cell infiltrate in this region. The EG cases were further characterised by submucosal and sometimes granulomatous eosinophilic infiltrate, which would extend to a varying degree into the *muscularis externa*. The GE cases would frequently have granulomatous epithelioid macrophage clusters of the mucosa and/or submucosa and the lymphoid hyperplasia was often nodular. All control cases were histologically normal.

Immunohistochemistry

All horses showed immunohistochemical labelling of the MP region between the inner circular and outer longitudinal muscle layers of the ileum for all markers (Figs 2, 3 and 4). The MP ganglia and nerve fibres showed immunolabelling for PGP9.5 and strong labelling for GFAP. Although most labelling was restricted to this anatomical region, there was some labelling of nerve fibres in the inner circular muscle layer and limited labelling in the outer longitudinal muscle layer. CD117 (c-Kit) labelled a network of cells in the MP region that surrounded and included the ganglia of the MP. TMEM16 showed strong labelling of the network of cells in the MP region and surrounded the ganglia of the MP. TMEM16 also showed diffuse immunolabelling of smooth muscle cells and strong labelling of blood vessel endothelium.

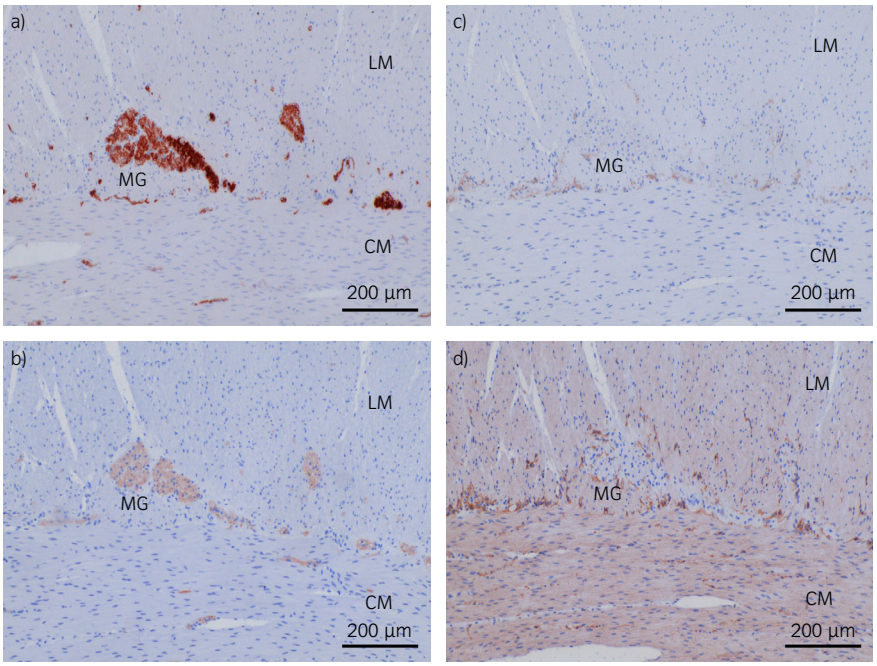

Fig 2: Immunohistochemical distribution in the myenteric plexus of the ileum of a healthy horse of markers for a) glial cells (GFAP); b) neurons (PGP9.5) and markers of interstitial cells of Cajal; c) CD117 (c-Kit) and d) TMEM16. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglion.

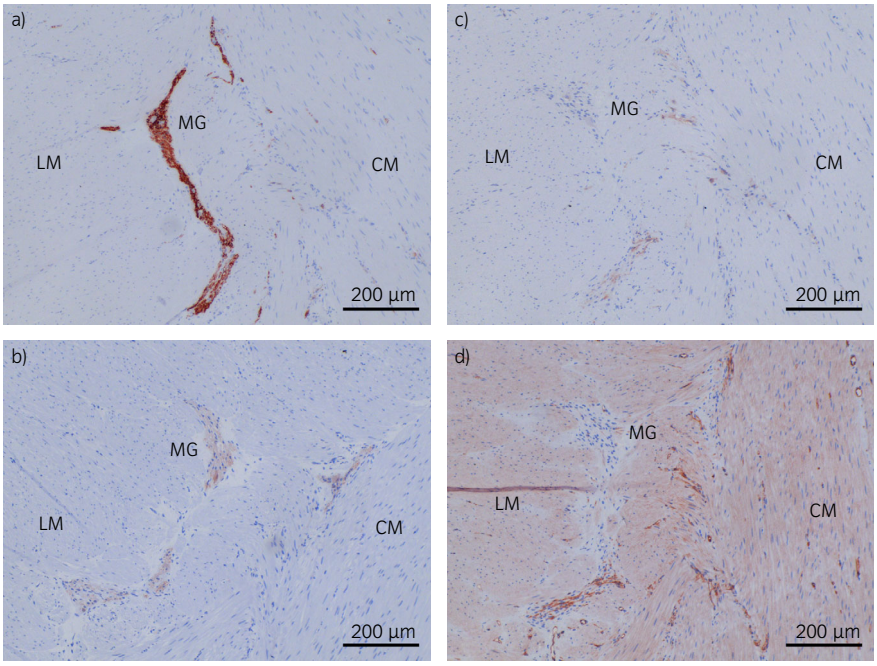

Fig 3: Immunohistochemical distribution in the myenteric plexus of the ileum of a horse with eosinophilic gastroenteritis of markers for a) glial cells (GFAP); b) neurons (PGP9.5) and markers of interstitial cells of Cajal; c) CD117 (c-Kit) and d) TMEM16. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglion.

Quantitative evaluation of immunolabelling in healthy and IBD horses

The percentage of immunolabelling in IBD horses was reduced for both markers of ICC in the MP region but not in the inner circular muscle layer compared with the control horses (Table 1). No significant reductions in immunolabelling for nerve cell and glial cell markers were detected in the MP region or inner circular muscle layer (Table 1).

To investigate whether the changes in immunolabelling were associated with the EG and GE forms of IBD, the material was divided into three groups (healthy, EG and GE) and a comparison of the percentage of immunolabelling for the four cell markers in the two tissue compartments

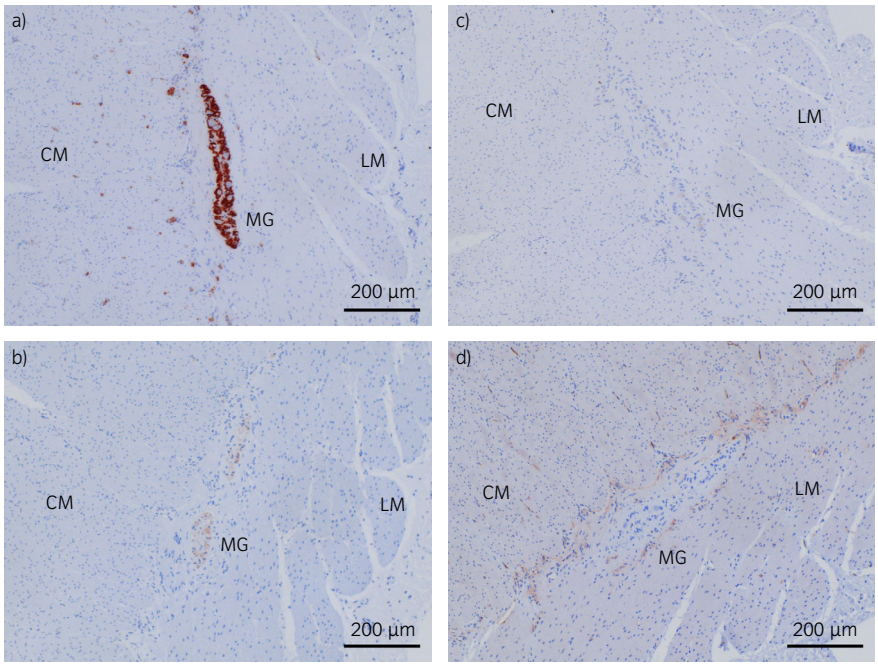

Fig 4: Immunohistochemical distribution in the myenteric plexus of the ileum of a horse with granulomatous enteritis of markers for a) glial cells (GFAP); b) neurons (PGP9.5) and markers of interstitial cells of Cajal; c) CD117 (c-Kit) and d) TMEM16. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglion.

TABLE 1: The median percentage of area immunolabelled for four cell markers in the myenteric plexus (MP) and inner circular muscle layer (IC) was determined for the control group and IBD horse group. The cell markers were glial fibrillary acidic protein (GFAP), neuronal protein gene product (PGP9.5), CD117 (c-Kit) and TMEM16 (TMEM)

Region of InterestCell marker	Healthymedian % area (interquartile range)	IBDmedian % area (interquartile range)	Mann-Whitney test (P value)
MP GFAP	12.62 (9.35)	13.57 (5.58)	0.5
MP PGP9.5	7.93 (2.70)	6.84 (7.97)	0.5
MP CD117	4.8 (1.97)	0.78 (0.63)	0.001***
MP TMEM16	8.77 (3.50)	3.63 (4.30)	0.005**
IC GFAP	0.21 (0.33)	0.22 (0.63)	0.5
IC PGP9.5	0.04 (0.52)	0.14 (0.37)	0.3
IC CD117	0.04 (0.03)	0.01 (0.02)	0.4
IC TMEM16	0.55 (0.55)	0.56 (0.72)	0.8

***P<0.001
**P = 0.005.

was performed (Figs 5 and 6). Both groups of horses showed a significant decrease in immunolabelling for CD117 (c-Kit) (P<0.001) and TMEM16 (P = 0.01) in the MP region. The immunolabelling for TMEM16 showed a larger variation in the MP region of the GE group of horses than in the EG horses (Fig 5d). There were no significant changes in immunolabelling in the inner circular muscle region of the EG or GE groups of horses for any of these two markers. Furthermore, there were no significant differences in the immunolabelling of the neuronal or enteroglial markers in any of the two anatomical regions evaluated.

There was a strong positive correlation between the cell markers for ICC (CD117/c-Kit and TMEM16) in the MP region (Spearman correlation coefficient r_s = 0.803; P<0.001). There was also a positive correlation between the glial cell and neuron markers (GFAP and PGP9.5) in the MP region (r_s = 0.4872; P = 0.03). In the inner circular muscle layer, there was a positive correlation between the percentage of area immunolabelled for the glial cell and neuron markers (r_s = 0.6015; P = 0.005) but not between the markers of ICC (r_s = 0.1188; P = 0.6).

Discussion

This report is the first study to assess ICC, enteric and neuroglial structures in a group of equine IBD cases. The study demonstrated that ICC immunoreactivity in the MP region of the ileum was significantly reduced in IBD horses compared with the control population. In contrast, the density of the neuronal and enteroglial cells was not significantly affected.

The histopathological findings were largely consistent with those previously described in horses with IBD [3,4,17,18]. However, in the current study, inflammatory cells were rarely present in the MP and myenteric ganglia were histopathologically normal. There could be several reasons for this. It is possible that any inflammatory cell infiltrate of the MP region was unevenly distributed in the tissue samples obtained and therefore not apparent in the sections examined. It is also possible that any inflammatory cells that were present in the initial stages of the disease process were no longer there as the duration of clinical symptoms ranged from weeks to months. However, all horses were subjected to euthanasia due to ongoing clinical disease and inflammatory changes were present at other sites in the ileal mucosa and submucosa.

Our immunohistochemical findings are in agreement with a previous study in a group of horses and donkeys with colic, which also used PGP9.5 as a neuronal marker [19]. In contrast, Pavone *et al*. [11] described a reduction in neuronal immunoreactivity in horses with colic using neuron-specific enolase and synaptophysin respectively as their markers although, as in the present study, no changes were observed for the glial cell marker GFAP. It is difficult to compare these results with those in the current investigation as the study populations and degree and duration of inflammation varied as did the methodologies. Similarly, clinical reports in human IBD patients have also documented a varying degree of changes to enteric neuronal networks [12–14,20].

The results in the current study nevertheless raise the question as to why a significant reduction in ICC immunoreactivity in the MP region was evident when the neuronal or enteroglial structures were not affected. It is currently unknown whether enteric neurons, glial cells and ICC are injured

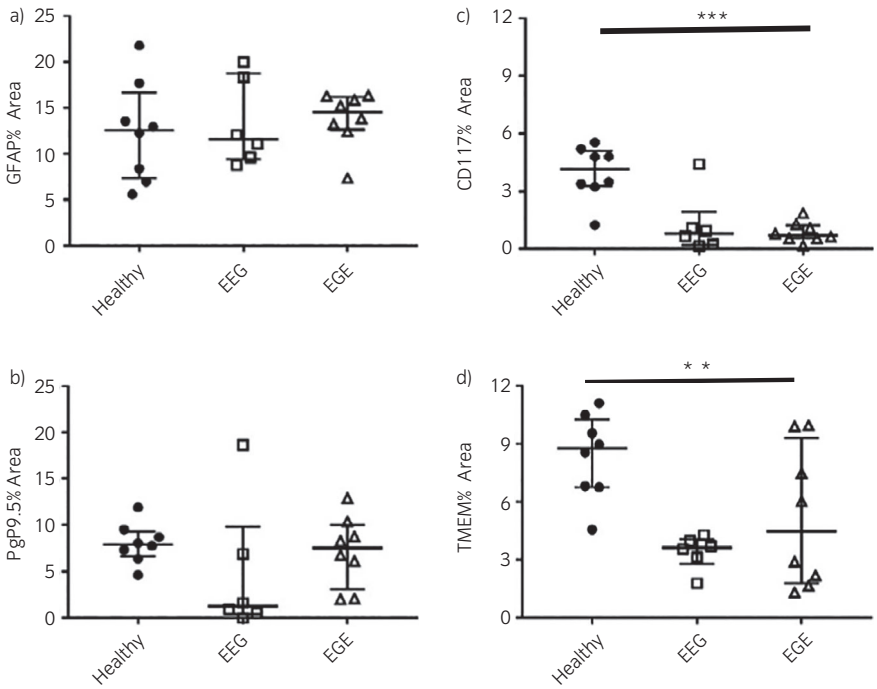

Fig 5: Percentage of immunolabelling (% area) in the myenteric plexus of the ileum of healthy, EG and GE horses assessed using a Kruskal-Wallis test for the cell markers a) GFAP; b) PGP9.5; c) CD117 (c-Kit) and d) TMEM16. ***P<0.001; **P = 0.01.

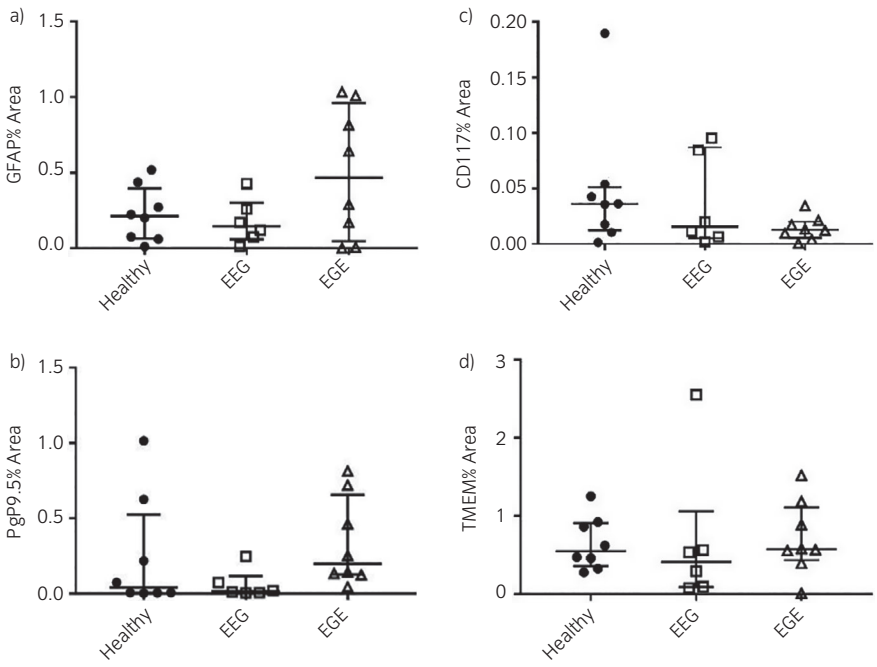

Fig 6: Percentage of immunolabelling in the inner circular muscle layer of the ileum of healthy, EG and GE horses assessed using a Kruskal-Wallis test for the cell markers: a) GFAP; b) PGP9.5; c) CD117 (c-Kit) and d) TMEM16. P>0.05.

by the same mechanisms and if they differ in their sensitivities to an inflammatory insult. Studies of ICC networks in experimentally induced small intestinal inflammation in mice have yielded contradictory results. Wang *et al*. [21] demonstrated ultrastructural changes to ICC but were unable to detect a significant reduction in c-Kit (CD117) immunoreactivity of the MP. These investigators concluded that the c-Kit receptor survived the effects of inflammation leaving the general ICC network intact even though cellular processes had been damaged [21]. Using a different experimental murine model, Kaji *et al*. [22] demonstrated that nitric oxide (NO)-induced oxidative stress directly resulted in immunohistochemical changes to ICC networks and raised the question as to whether these were associated with a change in cellular phenotype or with cell death. However, recent

work has failed to demonstrate detectable nitric oxide synthase 2 expression in both healthy equine intestinal tissue as well as in samples collected from horses with colic indicating that inflammatory mediators other than NO may be of importance in the horse [23]. The same study also demonstrated an upregulation of Il-6 and TNFα in the colic samples suggesting an involvement of these cytokines in intestinal inflammatory disease processes [23].

In the current study, ICC networks were clearly reduced and it is reasonable to speculate that this may have been caused by an inflammatory insult. Upregulation and increased production of cytokines such as Il-6 and TNFα, by mucosal immune cells such as macrophages, T cells and the recently discovered subsets of innate lymphoid cells has been demonstrated in human IBD patients as reviewed by Neurath [24]. Furthermore, this may result in injury to cell populations remote from macroscopically affected areas [20,24]. Considering the recent advances in equine intestinal immunology [23], this could offer an explanation as to why ICC networks of the MP region were injured in the current study in the apparent absence of local inflammatory cells. This is especially if ICC are more sensitive than neuroglial cells to such insults although this has to be proven.

It was also interesting to note that although both ICC markers were reduced in the MP region of the IBD horses, there was a greater variability in the TMEM16 immunolabelling in horses with GE compared with the eosinophilic enteritis cases. It is possible that different immunological and inflammatory disease pathways exist between the two conditions (GE and EG) but further studies are clearly needed to establish the pathological processes resulting in ICC injury.

Until recently, the only reliable ICC marker has been the c-Kit receptor (CD117). However, Espinosa *et al*. [25] developed a marker, ANO-1 (anoctamin-1 or TMEM16), initially intended for diagnosis of gastrointestinal stromal tumours, but noted a near identical staining pattern to c-Kit (CD117) of ICC. It was later established that TMEM16 (anoctamin-1) is a calcium-activated chloride channel and has been shown to be critical for the generation of slow waves by ICC [26]. TMEM16 is a marker independent of the Kit signalling pathway and thereby offers an alternative assessment of ICC networks [27]. The sequence similarity between human and horse of the region of anoctamin-1 used as immunogen for the anti-TMEM16 antibody is 97% and Western blot analysis revealed a prominent band at around 100 kDa, which was consistent with the reported molecular weight of the TMEM16 protein [28]. To the best of our knowledge, TMEM16 has not been previously used to evaluate ICC networks in the horse. The exclusion of the diffuse immunolabelling of smooth muscle and the avoidance of blood vessels allowed TMEM16 to identify a pattern of labelling that was similar to that of CD117 (c-Kit). This conclusion was supported by the strong correlation between areas of labelling for CD117 (c-Kit) and TMEM16 in the MP. Our findings with two separate ICC markers indicate that a genuine disruption to ICC networks was present and not merely a change in ICC phenotype. It was also interesting to note that the median percentage area labelled by TMEM16 and CD117 was consistently different between the two ICC markers. Although methodological aspects may have played a minor part, we believe that this does not entirely explain this difference. It is possible that a genuine difference in density between the CD117 receptor and TMEM16 of the ICC exists. This finding is in agreement with previous reports in humans and mice that have detected increased intensity of staining or numbers of TMEM16 compared with CD117 [27,29]. Furthermore, TMEM16 was found to be highly expressed in a genetic study [30], which again can help explain its high degree of immunolabelling. These findings further indicate that TMEM16 may be a more sensitive marker of ICC networks and their function than CD117 [27,29].

In the current study, immunolabelling was assessed in the MP region and in the inner circular muscle layer of the *muscularis externa*. This approach was based on previous studies in the horse that have described the distribution of ICC in the small intestine of both normal and diseased horses [10,31,32]. There was no significant reduction in the immunolabelling of the ICC in the inner circular muscle layer of IBD horses. This is in contrast to previous studies that have reported a reduction in c-Kit (CD117) immunoreactivity in the circular muscle layer of the ileum in horses with grass sickness [32] and in the colon of horses with colic [10] using semi-quantitative methods. However, the areas of immunolabelling detected by image analysis in the present study were very low in this anatomical region in both control and IBD horses. In the inner circular muscle layer, the ICC typically run parallel to the muscle fibres. Small deviations in orientation of the sections could introduce sufficient variation in area measurements to obscure a difference between the two groups, particularly given the small number of horses included in the study. We did not evaluate the outer longitudinal muscle layer as the density of ICC is relatively sparse and typically extends into this layer from the MP region [31,33].

The IBD horses included in the present study were diagnosed with either EG or GE, two subgroups in the equine IBD complex. A larger study population may have enabled difference to be detected between GE and EG groups of IBD horses and allowed comparisons on the basis of clinical signs and or duration of illness. Furthermore, although some horses had both macroscopic and microscopic lesions at anatomical regions other than the ileum, these were so few in numbers making comparison very difficult. Because all horses had histopathological lesions of the ileum, and because this is an anatomical area that has a high ICC density in the normal horse making comparison easier [31], this was the area that was selected for further investigation. Further studies are needed to investigate the other anatomical sites where inflammatory lesions are present.

In conclusion, this study has demonstrated a reduction in the ICC networks in the MP region of horses with IBD. Further studies are needed to explore the pathophysiological mechanisms of this disease and the effects on cellular networks required for normal intestinal function. An alternative ICC marker, TMEM16, may be of use in such investigations.

Authors' declaration of interests

The authors have no competing interests.

Ethical animal research

All samples were collected as part of a routine post-mortem examination and with the informed consent of the owners. All samples were obtained before 1 January 2017.

Owner informed consent

All samples were collected as part of a post-mortem investigation with the owners' informed consent.

Data storage and documentation

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authorship

All three authors contributed to the study design and execution. R. Lindberg performed histopathological assessment and diagnoses of all cases. C. McL. Press performed the image and statistical analyses and interpretation. C. Fintl produced the majority of the manuscript with

contributions from C. McL. Press. Both C. McL. Press and C Fintl had full access to all data and take responsibility for the integrity of the data and the accuracy of the data analysis. The manuscript was approved by all authors.

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[f]Zeiss, Oberkochen, Germany.
[g]ImageJ, NIH, Bethesda, Maryland, USA.
[h]GraphPad, La Jolla, California, USA.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Item 1: Western blot method.

Supplementary Item 2: Western blot of intestinal tissue from a healthy horse labelled using rabbit monoclonal antibody TMEM16A.