HISTOPATHOLOGICAL CHARACTERISATION OF COLITIS IN WESTERN LOWLAND GORILLAS (*GORILLA GORILLA* SSP. *GORILLA*)

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Abbreviations

ACVP = American College of Veterinary Pathologists

AFHEA = Associate Fellow of the Higher Education Academy

API = Analytical Profile Index

ATP = Adenosine triphosphate

B. coli = *Balantoides coli*

BIAZA = British and Irish Association of Zoos and Aquariums

CD = Crohn's disease

CRH = Corticotropin-releasing hormone

EAZA = European Association of Zoos and Aquariums

EC = enterochromaffin cells

ECVP = European College of Veterinary Pathologists

ECVCP = European College of Veterinary Clinical Pathologists

ECZM = European College of Zoological Medicine

ESVP = European Society of Veterinary Pathologists

FELASA = Federation of European Laboratory Animal Science Associations

FISH = fluorescent in situ hybridization

GIT = gastrointestinal tract

HE = Haematoxylin and eosin

HPF = high power fields

IBD = Inflammatory Bowel Disease

ICD = idiopathic chronic diarrhoea

IgLC = Immunoglobulin Light chain

IgE = Immunoglobulin E

ISO = International Organisation for Standardisation

IUCN = International Union for Conservation of Nature

IZVG = International Veterinary Zoo Group

MALDI-TOF = Matrix-Assisted Laser Desorption/Ionisation-Time of Flight

MC = mast cells

MMP-7 = Matrix metalloproteinases 7

NGS = next generation sequencing

NPathS = Nottingham Pathology Society

PAS = Periodic Acid Schiff

PI-IBS = post-infectious irritable bowel syndrome

PCR = Polymerase Chain Reaction

qPCR = quantitative PCR

PPE = Personal Protective Equipment

RCPath = Royal College of Pathologists

SCFA = Short-Chain Fatty Acids

SOP = Standard Operating Protocols

SR = Sirius Red

SVMS = School of Veterinary Medicine and Science

TNF = Tumour Necrosis Factor

UC = Ulcerative colitis

UK = United Kingdom

UKAS = United Kingdom Accreditation Service

UoN = University of Nottingham

WS = Warthin-Starry

WLG = Western Lowland gorilla

ZN = Ziehl-Neelsen

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Abstract

In captive gorillas, ulcerative colitis is an important cause of morbidity and mortality with no established definite aetiopathogenesis. The aim of the study was to histopathologically characterise colon lesions in captive western lowland gorillas (*Gorilla gorilla* ssp. *gorilla*) and to investigate the feasibility of applying the Nancy index, a disease activity scoring system for ulcerative colitis in humans. Colon samples from 21 animals were evaluated based on histopathological characteristics for the diagnosis of inflammatory bowel disease (IBD) in humans and divided into acute and chronic changes. The Nancy index, graded 0 to 4, was applied to all cases. Special stains including Ziehl-Neelsen, Warthin-Starry, Gram and Periodic Acid Schiff reaction were used to identify potential aetiological agents. Most common acute changes included neutrophils in the lamina propria (17/18, 94%), mucosal and submucosal oedema (12/18, 67%), and crypt abscesses (8/18, 44%). Most common chronic changes were lamina proprial lymphoplasmacytic infiltrates (17/18, 94%) and crypt dilation/distortion (6/18, 33%). Based on the Nancy index, 4/21 (19%) cases were graded as grade 4 (the highest grade), 2/21 (10%) were identified as grade 3, 11/21 (52%) grade 2, and 4/21 (19%) cases were graded 0. The changes in the colon observed in our study show comparable characteristics to the acute phase of ulcerative colitis described in humans. No unifying aetiopathogenesis could be identified. The Nancy index proved a valuable tool for the standardisation of disease activity grading and comparison for further studies in gorilla colitis.

Table of Contents

1 INTRODUCTION

1.1 WESTERN LOWLAND GORILLA (*GORILLA GORILLA* SSP. *GORILLA*)

Great apes (family Hominidae) are Old World primates found in Southeast Asia and Africa, constituted by bonobos (pygmy chimpanzees) (*Pan paniscus*), common chimpanzees (*Pan troglodytes*), gorillas (*Gorilla gorilla*) and orangutans (*Pongo abelii*, *Pongo pygmaeus*). Within this group, disease and pathology is best studied in both free living and captive common chimpanzees, with little information collected about the other above mentioned species, although published studies on free-living western lowland gorillas are increasing (Lowenstine et al., 2018).

The western lowland gorilla (WLG) (*Gorilla gorilla* ssp. *gorilla*) belongs taxonomically to the order Primates, family Chordata, class Mammalia (Mittermeier et al., 2013). The western lowland gorilla is one of the two recognised subspecies of the western gorilla (*Gorilla gorilla*), which includes the WLG and the eastern lowland gorilla (*Gorilla beringei graueri*) (Grooves, 2001). Chimpanzees (*Pan* sp.) and humans (*Homo* sp.) are the closest relatives of gorillas (genus *Gorilla*), based on phylogenetic studies (Lowenstine et al., 2018; Wall, 2013).

Since 2007, the WLG has been recognised as critically endangered (considered to be facing an extremely high risk of extinction in the wild) on the International Union for Conservation of Nature (IUCN) red list of threatened species, further classified under criterion A (a population reduction of more than 80% over three generations - one generation is approximately 22 years) in 2016 (Maisels et al., 2018).

A retrospective study of mortality in 119 captive WLG from 50 different European Zoologic collections identified diseases of the digestive system as a

major cause of death in gorillas, followed by trauma in young individuals and cardiovascular disease in aged animals (Strong et al., 2017). In apes, bacterial enterocolitis has been attributed to several species of *Salmonella*, *Yersinia*, *Campylobacter*, and *Shigella*. Amongst protozoal infections, balantiosis due to *Balantoides coli* (*B. coli*) (also referred to as *Balantidium coli* or *Neobalantidium coli*) (Mathison et al., 2020; Pomajbíková et al., 2013) is the most commonly encountered, followed by cryptosporidiosis, cyclosporidiosis and giardiasis, all of them rarely associated with clinical disease. Amoebic infections are also recorded and its clinical relevance vary from coincidental (subclinical) finding to severe disseminated clinical disease (e.g., *Entamoeba dispar* is considered an incidental enteric parasite; *Dientamoeba fragilis* is responsible for inflammatory bowel disease (IBD)-like symptoms; *Entamoeba histolytica* is the causative agent of ulcerative colitis) (Lowenstine et al., 2018).

Few case reports of ulcerative, haemorrhagic or histiocytic colitis have been published in lowland gorillas (Scott and Keymer, 1975; Van Kruiningen et al., 1991; Lankester et al., 2008; Isidoro et al., 2013; Paixão et al., 2014).

In 1975, a study was published reporting lesions of ulcerative colitis in two young gorillas and one orangutan, with lesions comparable to the disease often seen in humans. Scott and Keymer (1975) noticed that the lesions observed were indistinguishable from the active phase of UC in humans. In their study, no aetiological agents were identified (Scott and Keymer, 1975). In the early 1990s, Van Kruiningen and colleagues reported a case of bacterial histiocytic colitis in a lowland gorilla resembling Whipple's disease, a rare cause of chronic diarrhoea that can be confused with inflammatory bowel disease (IBD) in humans and other animals. In the reported case of bacterial histiocytic colitis by Van Kruiningen and

colleagues, further characterisation of the bacteria was not possible (Van Kruiningen et al., 1991).

In two case reports, infectious agents associated with haemorrhagic colitis in two gorillas included a case of *Campylobacter jejuni* with concomitant *B*. *coli* and a case of *Salmonella enterica* serotype *Infantis* (Isidoro et al., 2013; Paixão et al., 2014). *Dientamoeba fragilis* has also been described in a captive WLG with chronic diarrhoea and weight loss (Lankester et al., 2010).

Diet plays an important role in gastrointestinal health and physiology both in great apes and humans (Popovich et al., 1997). Gorillas are hindgut fermenters and thus, their diet composition in the wild is low in fat and high in dietary fibre. In the wild, WLG are folifrugivorous, meaning that their diet is composed of foliage and fruit, the latter consumed more during the wet season (July – August). The changes on food availability, from foliage-based to a more fruit-based diet affects also the gut microbiome in gorillas. A study conducted by Hicks et al. (2018) observed that the bacterial phylum *Prevotella* is more prevalent during the wet season whereas the bacterial phylum *Treponema* is prevalent in the low fruit season. The latter known to have fermentation pathways, which help in the digestion and absorption of fibres (Hicks et al., 2018).

The high fibre content of the diet provides a substrate for bacterial fermentation in the colon with short-chain fatty acid (SCFA) generation. It has been suggested that ulcerative colitis in humans is due to lack of energy provided by SCFA in low fibre diets. Butyrate, a fibre-derived SCFA, is known to be the preferred energy substrate for colonic mucosal cells and contributes to mucosal repair (Roediger, 1982). It is therefore important to consider that the high prevalence of ulcerative colitis in captive gorillas could be related to the consumption of relatively

low fibre diets when in captivity, similar to the suggested pathogenesis in humans (Popovich et al., 1997; Roediger, 1980; Roediger et al., 1982b).

Colitis represents a significant condition in captive gorillas, representing one of the major causes of death (23% of all cases) in captive gorillas in European zoological collections (Strong et al., 2017). Further characterisation and investigation of potential aetiologies and underlying risk factors is still needed.

This study focused on gastrointestinal tract (GIT) pathology with special attention on inflammation of the colon (colitis) and more specifically, ulcerative colitis in gorillas and its comparison to the disease in humans.

1.2 ULCERATIVE COLITIS IN HUMANS (*HOMO SAPIENS*)

In humans, IBD is a chronic idiopathic inflammatory disorder, predominantly observed in developed countries, further subdivided into ulcerative colitis (UC) and Crohn´s disease (CD). Histopathological criteria and diagnostic standardisation for the differentiation between both entities has been established in human medicine (Chang and Liu, 2018; Feakins, 2013; Magro et al., 2013). UC in humans affects mainly the colon and is characterised by relapsing and remitting mucosal inflammation (Ungaro et al., 2017). It most commonly affects adults, between 30-40 years of age, with no associated sexual predilection (Ungaro et al., 2017).

In humans, defects in colonic epithelial cells (colonocytes), or the mucus layer and epithelial barrier are implicated in the pathogenesis of UC (Ungaro et al., 2017). In a healthy state, the colonic epithelial barrier function is maintained by the mucus layer and epithelial cells, IgA and antimicrobial factors, specialised antigen-presenting cells (such as dendritic cells), and the coordinated activity of innate and adaptive immune cells. When this barrier is altered, disease occurs. Damage to the

mucosal barrier allows the luminal microflora to trigger a continuous and unregulated inflammatory response, which leads to enterocyte apoptosis, inhibition of mucosal healing, sustained inflammation, and ultimately dysbiosis (Ungaro et al., 2017). The hypothesised pathophysiology highlights the important role of the mucosal epithelial cells and the mucous barrier and their defects in disease progression, leading to dysbiosis and an inflammatory response causing further mucosal damage and disturbed function (Ungaro et al., 2017).

Histologically, the diagnosis of UC is made based on the observation of both acute and chronic histologic features (Chang and Liu, 2018; Stratton et al., 2017):

I. Acute features: neutrophilic inflammation; presence of crypt abscesses (dilation of crypts with presence of neutrophilic inflammation and cell debris); and presence or absence of erosion or ulceration.

II. Chronic features: alteration of the crypt architecture and increased chronic inflammatory infiltrate (lymphocytes and plasma cells) especially below the crypts.

As opposed to the histological characteristics observed in UC, CD is characterised by the formation of granulomas and a segmental, transmural colitis and/or ileitis with or without involvement of other intestinal segments (Chang and Liu, 2018). The histological hallmark in CD is the presence of epithelioid granulomas and transmural inflammation, which are rare in UC (Chang and Liu, 2018).

Paneth cells, located in normal conditions at the base of the small intestinal “crypts of Lieberkühn”, are considered to play an important role in the microbiome homeostasis and modulation, participate in the intestinal innate immune response,

and aid in the development of the intestinal epithelium (Elphick and Mahida, 2005). Paneth cells contain different proteins and peptides (e.g. α-defensins, lysozyme, tissue necrosis factor, matrix metalloproteinases 7, xanthine oxidase, immunoglobulin A, metallothionine, adipokines, serum amyloid A, lipokines, among others) contained in cytoplasmic granules which when secreted, both constitutively and in response to pathogen exposure, modulate the microbiome and mediate the inflammatory response (primarily as microbiocidal agents). Paneth cells can occasionally be found in a scattered distribution in the stomach and colon; however, their presence in this tissues is generally associated with mucosal inflammation (Lueschow SR, 2020). Paneth cell metaplasia in the colon or rectum has been described as a feature of chronic mucosal injury, and applies for both UC and CD conditions (Chang and Liu, 2018). Metaplasia is a reversible cellular adaptation in response to injury in which cells undergo transformation from one differentiated cell type into another one. In Paneth cell metaplasia, normal colonocytes undergo transformation into Paneth cells (Kumar et al., 2020).

The potential role of mast cells (MC) and eosinophils in the pathogenesis of IBD is also a recurring topic in the human literature (Stoyanova and Gulubova, 2002; Al-Haddad and Riddell, 2005; Coburn et al., 2013; Boeckxstaens, 2015; Alhmoud et al., 2020). Residential mucosal intestinal MC are activated by several types of ligands that bind to mast cell receptors. The ligands are divided into immune ligands (immunoglobulins, complement fragments and cytokines) and non-immune mediators (neurotransmitters, neuropeptides, hormones, growth factors and physicochemical stimuli). Upon binding with any of these mediators, mast cells are activated (Albert-Bayo et al., 2019).

Once intestinal mast cells are activated, they release biologically-active products (also called active mediators) which can be newly synthesised or pre-formed in the cytoplasmic granules. Newly-synthesised mediators include lipid molecules, cytokines, growth factors, neuropeptides and chemokines, whilst pre-formed mediators include biogenic amines, lysosomal enzymes, proteoglycans, cytokines, growth factors and enzymes (Albert-Bayo et al., 2019).

Activated mast cells in the intestinal tract have important pleiotropic activities during both disease and homeostasis. Intestinal mast cells are known to have several functions, including participation in the host defence against microbes, in epithelial cell secretions, in mucosal regulatory functions and in smooth muscle contraction and peristalsis (Albert-Bayo et al., 2019; Bischoff, 2009; Kumar et al., 2015). In humans, stress is known to be an important trigger of mast cell activation and is associated with symptomatic flares in IBD (Bernstein et al., 2010; Söderholm and Perdue, 2001). In some studies, perceived stress has been identified as a stimulus that triggers MC activation via eosinophil-derived CRH (corticotropin releasing hormone), IgLC (immunoglobulin light chain), IgE (immunoglobulin E) or ATP (adenosine triphosphate) (Boeckxstaens, 2015).

In a study performed on six human colon surgical specimens with chronic UC, quantitative changes in enterochromaffin cells (EC), mast cells and neuronal fibres were found, suggesting that interactions between MC and neuronal components may play a role in the pathogenesis of UC (Stoyanova and Gulubova, 2002). Quantitative changes were assessed by means of histopathology and immunocytochemical methods and they found that in tissue sections with UC there was an increased density of SP- (Substance P) and SER (Serotonin)-positive neurofibers and SER-positive endocrine cells (the EC) in close apposition with SP-

and SER-immunopositive mast cells compared with control specimens (Stoyanova and Gulubova, 2002).Therefore, mast cells are considered to play a significant role in the pathogenesis of active IBD in humans (Boeckxstaens, 2015; He, 2004; Stoyanova and Gulubova, 2002).

In dogs, increased numbers of mast cells were observed in patients with IBD and in animals with *Canine Parvovirus*-2–induced enteritis with crypt abscesses (Woldemeskel et al., 2013). There is evidence that mast cells may play a role in the pathogenesis of the intestinal diseases in animals, and in captive gorillas, stress is an important factor to consider (Cooper, 2017).

The role of eosinophils in the pathogenesis of CD and UC is still unclear. Although many studies have shown the pro-inflammatory role of eosinophils in IBD (Coburn et al., 2013), others suggested that eosinophils may play a protective (anti-inflammatory) role and found that patients with IBD have an increase in number of mucosal eosinophils which are associated with better outcomes in human patients (Alhmoud et al., 2020). Previous studies also found that eosinophils were observed in mild or quiescent disease, suggesting again a protective role of eosinophils in IBD (Al-Haddad and Riddell, 2005). Further studies are, however, required to determine the role of eosinophils in IBD in humans.

Several intestinal pathogens (including *Campylobacter* sp. *Salmonella* spp., *Shigella* spp., *Clostridium difficile* and parasites) implicated in infectious and chronic colitis in humans are commonly observed as concurrent infections in UC patients, which make the differentiation between chronic infectious colitis and ulcerative colitis with concomitant bacterial infections challenging (Lin et al., 2017). Nevertheless, no definite infectious cause has yet been identified for UC or CD (Stratton et al., 2017).

1.3 THE NANCY INDEX

Disease activity in UC in humans is defined histologically by the presence of acute inflammatory infiltrates in the colon. Assessment of disease activity is essential in UC in order to standardise histological features that correlate with clinical outcomes and to obtain reproducible results and comparable data between different studies. In humans, disease activity in UC proved to positively impact and influence prognosis, relapse of symptoms and can influence decisions of medical treatments (Christensen et al., 2017; DeRoche et al., 2014).

In humans, the Nancy histological index is the first validated grading system for the assessment of disease activity in UC (Marchal-Bressenot et al., 2016, 2017; Ponte et al., 2017). It is based on three histological criteria (ulceration, acute and chronic inflammatory infiltrate) defining five grades of disease activity (Marchal-Bressenot et al., 2017), designated as:

- Grade 0 = absence of significant histological disease
- Grade 1 = chronic inflammatory infiltrate with no acute inflammatory infiltrate (neutrophils)
- Grade 2 = mildly active disease
- Grade 3 = moderately active disease
- Grade 4 = severely active disease

The distinction between mildly, moderately and severely active disease is based on the absolute numbers of neutrophils identified on tissue sections per high power field (HPF) and on the absence / presence of ulceration. Grade 2 is determined by a mild increase (scattered, <10 cells per HPF) and grade 3 by a moderate increase

(<30 per HPF) in neutrophils. Grade 4 is determined by the presence of tissue ulceration, regardless of the numbers of chronic and acute inflammatory cells present (Marchal-Bressenot et al., 2017).

1.4 AIMS AND OBJECTIVES

The aims and objectives of this study were:

i. to characterise lesions in the colon of captive WLG by histopathological evaluation to gain insights into the aetiopathogenesis of colitis in captive gorillas;
ii. to compare the histological lesions observed in gorillas with UC in humans;
iii. and to assess the utility of the Nancy index system, used for the assessment of disease activity in ulcerative colitis in humans, to assess severity of colitis in captive gorillas.

2 MATERIALS AND METHODS

2.1 ANIMALS AND CASE SELECTION

Formalin fixed paraffin embedded tissue samples of captive WLG (*Gorilla gorilla* ssp. *gorilla*) from eleven different European zoos were retrospectively selected from the archive of the pathology service at the University of Nottingham - School of Veterinary Medicine and Science (Sutton Bonington, United Kingdom), the International Zoo Veterinary Group (IZVG) (Keighley, United Kingdom), and the German Primate Centre (Göttingen, Germany). Contributing institutions were members of the British and Irish Association of Zoos and Aquariums (BIAZA) or the European Association of Zoos and Aquariums (EAZA), or both, and subject to the

ethical codes of those organisations, in addition to the United Kingdom Secretary of State's Standards of Modern Zoo Practice as implemented in the Zoo Licensing Act 1981 (Amendment) (England and Wales) Regulations 2002, or were members of the German Veterinary Society and worked according to the current Federation of European Laboratory Animal Science Associations (FELASA) recommendations.

The criteria the author used for inclusion in the study were: 1) species (western lowland gorillas); 2) availability of formalin fixed colon samples and, where possible, stomach and small intestine; 3) a histopathological diagnosis of enterocolitis/colitis. Animals with no identified gastrointestinal lesions were selected as control. Control samples were retrieved from post-mortem archive cases where no gastrointestinal pathology was reported and formalin fixed samples from stomach, small and large intestine were available. Age, sex, clinical history, cause of death and ancillary testing results from histopathology reports were reviewed and summarised when available (Annex - Supplemental Table S1).

All cases were screened and classified based on the degree of tissue preservation as: 0) well-preserved = overall good tissue preservation; 1) mild autolysis = good tissue preservation but multifocal artefactual epithelial detachment; 2) moderate autolysis = identifiable tissue architecture and cell definition with diffuse artefactual epithelial detachment; 3) advanced autolysis = loss of tissue architecture and cellular definition with no recognisable epithelium. Cases with advanced autolysis hindering reliable histological evaluation were excluded from the study.

2.2 HISTOPATHOLOGY

The paraffin blocks were sectioned at 5 μm and processed and stained using routinely employed methods by a histology technician in the laboratory at the University of Nottingham. Sections of colon, small intestine and stomach were cut in transverse (full-thickness) sections, allowing visibility of all tissue layers. Slides were stained with haematoxylin and eosin (HE), Ziehl-Neelsen (ZN), Warthin-Starry (WS), Periodic Acid Schiff (PAS) reaction, and Gram stains following established standard operating protocols (Survana et al., 2019) for characterisation, scoring and evaluation of potential causative infectious agents. The special stain, Sirius Red (SR), was performed on colonic sections for visualisation of eosinophils (Gilbertson, 2019).

The sections of H&E stained colon were graded, by the author under supervision of a boarded certified anatomic pathologist for the first batch of samples (G1-G13) and by the author alone for the second batch (G14-G22), for the following parameters: lymphocytes, plasma cells, neutrophils, macrophages, eosinophils, and mast cells; fibrosis, oedema, and haemorrhage; crypt hyperplasia, dilation and distortion, necrosis and abscesses; Paneth cell metaplasia; mucosal ulceration. The defined parameters are detailed in Annex – Supplemental Table S2. Where available, the evaluation and grading of stomach and small intestinal samples followed the same criteria where applicable.

Mucosal eosinophil and mucosal/submucosal mast cell counts were performed on each colon section in the slides stained with SR and ZN, respectively. Although ZN is not routinely used for mast cell detection, it is known that the granules of mast cells stain with basic fuchsine (Ribatti, 2018).

Eosinophil and mast cell numbers were counted in 10 high power fields (HPF) (40x) (per 2.37mm^2), and the average was calculated. Areas were selected at high magnification (40x HPF) to identify the most eosinophil-rich (including mucosal layer) or mast cell-rich (including mucosal and submucosal layers) regions. Cells counted included only preserved cells with a visible nucleus, and intracytoplasmic fine to coarse granules. For mast cells, grade 0 was given for 0-3 cells per HPF, grade 1 (mild) for 4-6 cells per HPF, grade 2 (moderate) for 7-9 cells per HPF, and grade 3 (severe) for >9 cells per HPF. For eosinophils, based on human and veterinary literature, grade 0 was given for 1-2 cells per HPF, grade 1 (mild) for up to 20 cells per HPF, grade 2 (moderate) for 21-50 cells per HPF, and grade 3 (severe) for ≥51 cells per HPF (Alhmoud et al., 2020; Bates, 2012; Day et al., 2008; Ramirez et al., 2018).

Slides stained with ZN, WS, PAS and Gram stains were systematically evaluated for the presence of infectious agents and graded as 0 (negative) or 1 (positive). When positive, the type of infectious agent (e.g. bacteria, protozoa, ameboid, or fungal organisms) and location (e.g. luminal, superficial mucosa, lamina propria, intracryptal) were recorded. The presence of mucosal bacteria was graded as: 1 (no or rare individual bacteria in <50% of the mucosal surface), 2 (bacterial colonies in <50% of the mucosal surface), 3 (larger bacterial colonies covering >50% of the mucosal surface).

2.3 NANCY INDEX

The Nancy index was used as a standardised method of evaluating disease activity and severity and applied to all the cases in this study (Marchal-Bressenot et al., 2016). Three histologic criteria were assessed including: (1) presence of ulceration

(grade 4), (2) presence of an acute inflammatory infiltrate defined by mild (grade 2) or moderate to severe (grade 3) numbers of neutrophils in the lamina propria and epithelium; and, in the absence of acute inflammation, (3) the presence of a chronic inflammatory infiltrate defined by none or mild (grade 0) or moderate to severe (grade 1) increase of numbers of lymphocytes, plasma cells, and/or eosinophils in the lamina propria (Marchal-Bressenot et al., 2016). The diagnostic algorithm and criteria legend are shown in Figure 1 and Table 1.

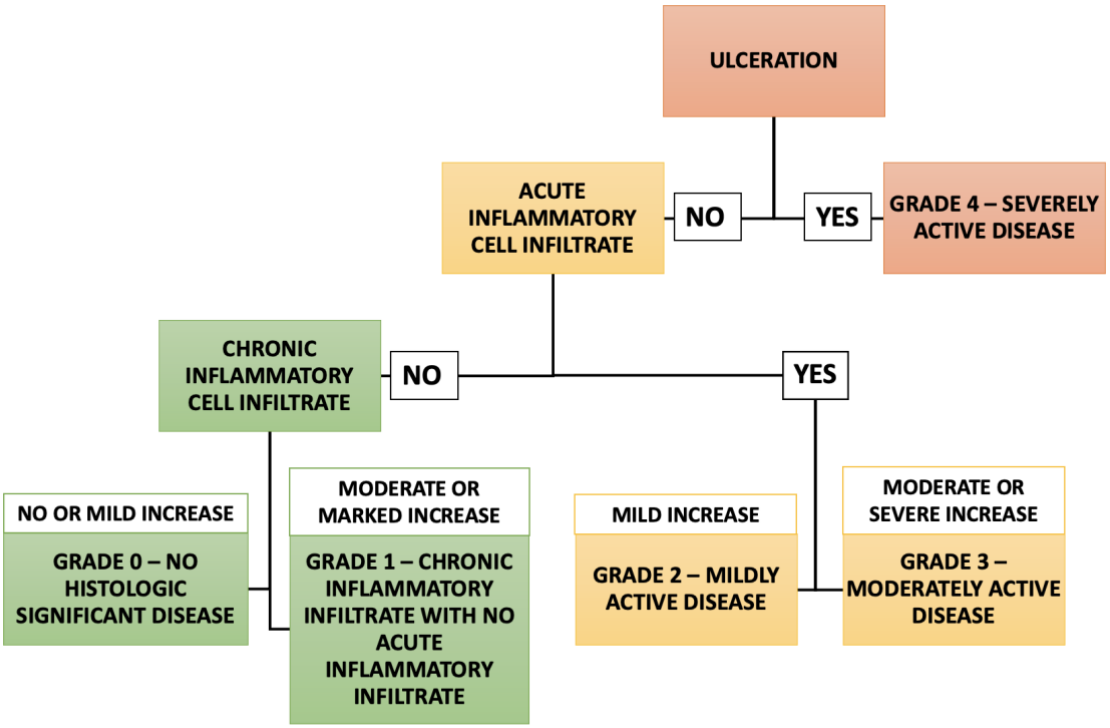


Figure 1 – Algorithm of the Nancy histological index, modified from Marchal-Bressenot A, et al. Gut 2017; 66:43-49.

Low numbers of lymphocytes and plasma cells are usually observed within the lamina propria of the small and large intestine and constitute a normal, background cell population. Neutrophils are not present in the healthy intestinal mucosa (Day et al., 2008). Grade 0 and grade 1 of the Nancy index are determined by the presence of only chronic inflammatory cells (defined as lymphocytes, plasma

cells and eosinophils) within the lamina propria. Grade 0 of the Nancy index will be given when no (0-4 cells between crypts) or a mild increase (up to 4-6 cells between crypts) is observed in the number of lymphocytes and plasma cells within the lamina propria. Grade 1 of the Nancy index is given when the increase in the chronic inflammatory cells is moderate (up to 6-7 cells between crypts) or marked (>8 cells between crypts). Disease activity is determined by the presence of acute inflammatory infiltrates (determined by the presence of neutrophils) admixed with a chronic inflammatory infiltrate. Therefore, when neutrophils are observed admixed with the chronic inflammatory infiltrate within the lamina propria, grade 2 or grade 3 are given to the sample. Grade 2 is determined by a mild (scattered, <10 per HPF) increase and grade 3 by a moderate (<30 per HPF) increase in neutrophils. Grade 4 is determined by the presence of tissue ulceration, regardless of the numbers of chronic and acute inflammatory cells present.

Table 1 – Nancy score legend, modified from Marchal-Bressenot A, et al. Gut 2017; 66:43-49.

LEGEND	DEFINITION	SCORE	
CHRONIC INFLAMMATORY INFILTRATE	defined as the quantity of lymphocytes and plasma cells	0	None
		1	Mild but unequivocal increase
		2	Moderate increase
		3	Marked
ULCERATION	defined as visible mucosal injury and regeneration and/or fibrin and neutrophils and/or tissue granulation	0	Absent
		1	Present
ACUTE INFLAMMATORY CELL INFILTRATE	defined as the presence of neutrophils	0	None
		1	Mild
		2	Moderate
		3	Severe

The HE grading scheme for the different analysed parameters (lymphocytes and plasma cells, neutrophils, ulceration) is defined in the annex, supplemental table S2.

2.4 MICROBIOLOGY

Microbiology samples were analysed using routinely employed methods by a United Kingdom Accreditation Service (UKAS) Accredited ISO (International Organisation for Standardisation) 17025 commercial veterinary laboratory or by the microbiologic laboratory of the German Primate Centre according to Standard Operating Protocols (SOP). Standard solid, and where appropriate selective liquid, media techniques were used and microbial identification was made using standard morphological and biochemical techniques, including the Analytical Profile Index (API) system. Where necessary, in eight cases (8/21, 38%), ancillary techniques such as Matrix-Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-TOF) techniques or Polymerase Chain Reaction (PCR) testing were employed as directed by a veterinary pathologist. Enteric bacterial samples were collected into sterile plain faecal sampling tubes or into charcoal or Amies transport medium swabs for transport to the laboratory, or were directly collected onto solid or liquid media during necropsy.

2.5 ETHICS

The research project was approved by the relevant Research Convenor and the Ethical Review Panel of the SVMS at the UoN (Animal Welfare and Ethical Review Body, 2021). The project described above complies with published codes

of conduct, ethical principles and guidelines of professional bodies associated with the research discipline (Applied science, Medicine and Health, Veterinary Medicine).

3 RESULTS

3.1 ANIMALS

A total of 22 cases (19 affected and 3 non-affected) were evaluated during this study (Table 2). A total of 21 cases fulfilled the criteria for inclusion of the study, including 18 affected and 3 non-affected (control) animals. Case 6 was excluded for histopathological analysis due to advanced degree of autolysis.

Table 2 – Affected and non-affected (control) Western Lowland gorillas indicating case number, common name, species name, age, sex and degree of autolysis.

CASE	***COMMON NAME***	***SPECIES NAME***	***AGE***	***SEX***	***DEGREE OF AUTOLYSIS***
1	Western lowland gorilla	*Gorilla g. gorilla*	17 y 11 m	M	Mild
2	Western lowland gorilla	*Gorilla g. gorilla*	25 y	F	Mild
3	Western lowland gorilla	*Gorilla g. gorilla*	1 y	M	Mild
4	Western lowland gorilla	*Gorilla g. gorilla*	NA	M	Mild
5	Western lowland gorilla	*Gorilla g. gorilla*	11 m	M	Mild
6	Western lowland gorilla	*Gorilla g. gorilla*	45 y	M	Advanced
7	Western lowland gorilla	*Gorilla g. gorilla*	34 y	M	Well-preserved
8	Western lowland gorilla	*Gorilla g. gorilla*	54 y	F	Moderate
9	Western lowland gorilla	*Gorilla g. gorilla*	46 y	F	Mild
10	Western lowland gorilla	*Gorilla g. gorilla*	33 y	M	Mild
11*	Western lowland gorilla	*Gorilla g. gorilla*	23 y	M	Mild
12*	Western lowland gorilla	*Gorilla g. gorilla*	19 y 2 m	F	Mild
13*	Western lowland gorilla	*Gorilla g. gorilla*	NA	F	Mild
14	Western lowland gorilla	*Gorilla g. gorilla*	NA	NA	Moderate
15	Western gorilla	*Gorilla gorilla*	38 y	M	Moderate
16	Western gorilla	*Gorilla gorilla*	1 y 3 m	M	None
17	Western lowland gorilla	*Gorilla g. gorilla*	43 y	F	Moderate

18	Western lowland gorilla	*Gorilla g. gorilla*	5 y	F	Moderate
19	Western lowland gorilla	*Gorilla g. gorilla*	56 y	F	Moderate
20	Western gorilla	*Gorilla gorilla*	32 y	M	Moderate
21	Western lowland gorilla	*Gorilla g. gorilla*	36 y	F	Mild
22	Western lowland gorilla	*Gorilla g. gorilla*	22 y	M	Moderate

NA= Not available; M= Male; F= Female; y= year; m= month
*Non-affected (control) cases.

The age of the subjects in this study ranged from 11 months to 56 years. Nine out of 22 animals (41%) were under or equal to 25 years old and 10/22 (45%) were over 25 years. In 3/22 cases (14%) (cases 4, 13 and 14), the age was not available. 12 animals were male (55%) and 9 (41%) female. In one case (case 14), information about the sex was not available.

3.2 HISTOPATHOLOGY

3.2.1 COLON

3.2.1.1 Morphological diagnoses

Histopathological diagnoses of colonic lesions in affected animals is shown in Table 3. Cases 11, 12 and 13 (controls) were histologically unremarkable.

Table 3 – Histopathological (morphological) diagnoses of colon tissue samples of affected WLG from selected cases.

CASE No.	MORPHOLOGICAL DIAGNOSES
1	Severe, multifocal, acute, **ulcerative** colitis with mild numbers of amoeba and gram negative bacteria (*Clostridia* spp., presumed)
2	Mild, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis
3	Moderate to severe, segmental, acute, **ulcerative** and neutrophilic colitis

4	Severe, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis with few ciliated trophozoites (compatible with *B. coli*)
5	Moderate to severe, multifocal, acute, **ulcerative** and neutrophilic colitis
7	Mild, diffuse, chronic-active oedematous, lymphoplasmacytic and neutrophilic colitis
8	Moderate, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis with few ciliated trophozoites (compatible with *B. coli*)
9	Moderate, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis
10	Mild, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis with very few apicomplexan schizonts (compatible with *Cryptosporidium* sp.)
14	Moderate, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis with moderate ciliated trophozoites (compatible with *B. coli*)
15	Mild, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis
16	Mild, multifocal, chronic, lymphoplasmacytic colitis
17	Mild, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis with few apicomplexan schizonts (compatible with *Cryptosporidium* sp.) and large numbers of ciliated trophozoites (compatible with *B. coli*)
18	Minimal, diffuse, acute, lymphoplasmacytic and neutrophilic colitis
19	Minimal, diffuse, acute, lymphoplasmacytic and neutrophilic colitis
20	Marked, segmental, chronic-active, lymphoplasmacytic and neutrophilic colitis and marked submucosal pneumatosis intestinalis with small numbers of ciliated trophozoites (compatible with *B. coli*)
21	Moderate, diffuse, chronic-active, **ulcerative**, lymphoplasmacytic and neutrophilic colitis
22	Mild, diffuse, chronic-active, lymphoplasmacytic and neutrophilic colitis and fibrinosuppurative peritonitis with abscess formation

B. coli = Balantoides coli

3.2.1.2 Grading

All selected cases (18 affected and 3 control cases (cases 11, 12 and 13)) were further characterised by means of histopathology (Table 4). The histologic changes of acute inflammation were variably present in the form of neutrophilic

infiltration, crypt necrosis and abscesses, oedema, haemorrhage and mucosal ulceration. The presence of neutrophils (Fig. 2, **B** and **C**) in the lamina propria (17/18, 94%), mucosal and submucosal oedema (12/18, 67%) and crypt abscesses (Fig. 2, **C**) (8/18, 44%) were the most common acute changes in these cases. Chronic changes were variably present in the form of predominantly lamina proprial and occasional submucosal lymphocytic and plasma cell infiltrates, fibrosis, crypt dilation and distortion, and Paneth cell metaplasia. Lamina proprial lymphocytic and plasma cell infiltrates were present in all the affected cases to a variable degree ranging from a resident background population (1/18, 5%) to a mild to moderate (17/18, 94%) increase. Fibrosis (4/18, 22%) and crypt dilation and distortion (6/18, 33%) were less common. Paneth cell metaplasia (1/18, 5%) was rarely observed.

Table 4. H&E histopathology grading results from histologically affected cases showing the number and percentage (in brackets) of cases per parameter evaluated (lymphocytes and plasma cells, fibrosis, crypt dilation and distortion, Paneth cell metaplasia, neutrophils, crypt necrosis, crypt abscesses, oedema, haemorrhage, and ulceration) stratified by grade (0=normal, 1=mild, 2=moderate, 3=severe). Last column indicates the total number and percentage (in brackets) of grades 1, 2 and 3.

Grade	**0**	**1**	**2**	**3**	**1-3**
Lymphocytes / Plasma cells	1 (5.6)	9 (50.0)	8 (44.4)	0 (0.0)	17 (94.4)
Fibrosis	14 (77.8)	4 (22.2)	0 (0.0)	0 (0.0)	4 (22.2)
Crypt dilation and distortion	12 (66.7)	2 (11.1)	3 (16.7)	1 (5.6)	6 (33.3)
Paneth cell metaplasia	17 (94.4)	1 (5.6)	0 (0.0)	0 (0.0)	1 (5.6)
Neutrophils	1 (5.6)	11 (61.1)	4 (22.2)	2 (11.1)	17 (94.4)
Crypt necrosis	13 (72.2)	2 (11.1)	2 (11.1)	1 (5.6)	5 (27.8)
Crypt abscesses	10 (55.6)	6 (33.3)	2 (11.1)	0 (0.0)	8 (44.4)
Oedema	6 (33.3)	7 (38.9)	5 (27.8)	0 (0.0)	12 (66.7)
Haemorrhage	11 (61.1)	4 (22.2)	1 (5.6)	2 (11.1)	7 (38.9)

Ulceration [a]	14 (77.8)	4 (22.2)	n/a	n/a	4 (22.2)

[a] Ulceration was graded as 0=absent or 1=present; therefore, grades 2 and 3 are not applicable in this parameter.
n/a = not applicable.

In the three control cases (cases 11, 12 and 13), the tissue sections of the colon were regarded as unremarkable, with an absence of neutrophils and minimal to mild lymphocytic and plasma cell populations in the lamina propria, interpreted as normal, resident (background) populations (Fig. 2, **A**).

Eosinophil and mast cell counts were performed in 19/21 (90%) colonic sections from control and affected cases using SR for eosinophil counts and ZN for mast cells. Preparing additional slides for SR and ZN stains was not possible for the remaining 2/21(10%) cases (cases 14 and 22) due to paucity of available tissue samples. Mast cells were found in all examined cases within the submucosa, often close to the muscularis mucosae, and usually with a perivascular distribution. No differences were detected on semi-quantitative evaluation of mast cells between affected and control cases. Similarly, no differences were detected when the semi-quantitative results for mast cell count were compared to the grades (0-4) of the Nancy index in the studied cases. The count of the eosinophils ranged from mild (4-6 eosinophils per HPF) to marked (51-100 eosinophils per HPF) within the lamina propria. Semi-quantitative evaluation did not reveal either an increase or a decrease in eosinophil numbers in relation to the Nancy index grading; however, the highest count of eosinophils was found in cases 1 (grade 4 of the Nancy index) (Fig. 2, **D**) and 9 (grade 3 of the Nancy index). Statistical analysis and stratification (division of the population into subsets/groups) of the data by Nancy index grade were not possible due to the variability of cases per group (with groups with no representation, e.g. Nancy grade 1) and the overall small number of cases.

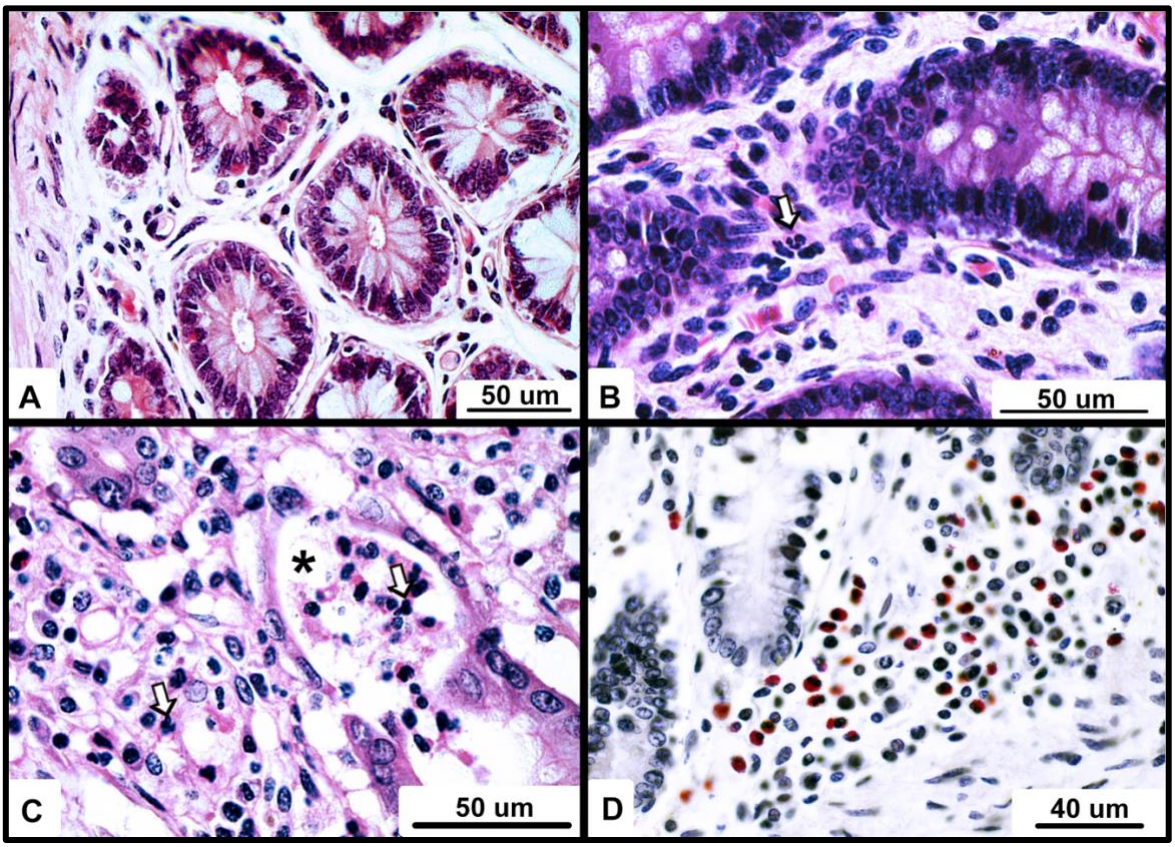


Figure 2. **Histologic changes**. **A)** Normal tissue, colon, western lowland gorilla, case 12. Note mild lymphocytic cell (background) population within the lamina propria. Haematoxylin and eosin. **B)** Mild, chronic-active lymphoplasmacytic and neutrophilic colitis, colon, western lowland gorilla, case 2. Lamina proprial mixed cell infiltrate composed of neutrophils (white arrow), eosinophils and lymphocytes. Haematoxylin and eosin. **C)** Moderate, chronic-active lymphoplasmacytic and neutrophilic colitis, colon, western lowland gorilla, case 4. Cryptal dilation (asterisk) with intraluminal cell debris and degenerated neutrophils (white arrows) (crypt abscess) and lamina proprial mixed inflammatory infiltrate (neutrophils (white arrow), lymphocytes, plasma cells and eosinophils). Haematoxylin and eosin. **D)** Severe, acute, ulcerative colitis, colon, western lowland gorilla, case 1. Large numbers of eosinophils were observed within the lamina propria. Sirius red.

3.2.1.3 Aetiological agents

Identification of infectious agents was performed by evaluation of ZN, WS, PAS and Gram stains. Case 1 exhibited mild numbers of mucosal PAS positive amoeba trophozoites (Fig. 3, **E**). Round, pale amphophilic to basophilic, 2-6 um

diameter, apicomplexan protozoan schizonts (compatible with *Cryptosporidium* sp.) (Fig. 3, **F**) were observed lining the crypt epithelium in two gorillas (2/21, 10%) (cases 10 and 17). Luminal and mucosal (lamina proprial and intracryptal) *B. coli* trophozoites and cysts were observed in six cases (5/21, 24%) (cases 4, 8, 14, 17 and 20). The largest numbers of *B. coli* trophozoites (Fig. 3, **G**) were observed in the lumen and within the autolysed mucosa of the colon exhibiting a moderate to advanced degree of autolysis.

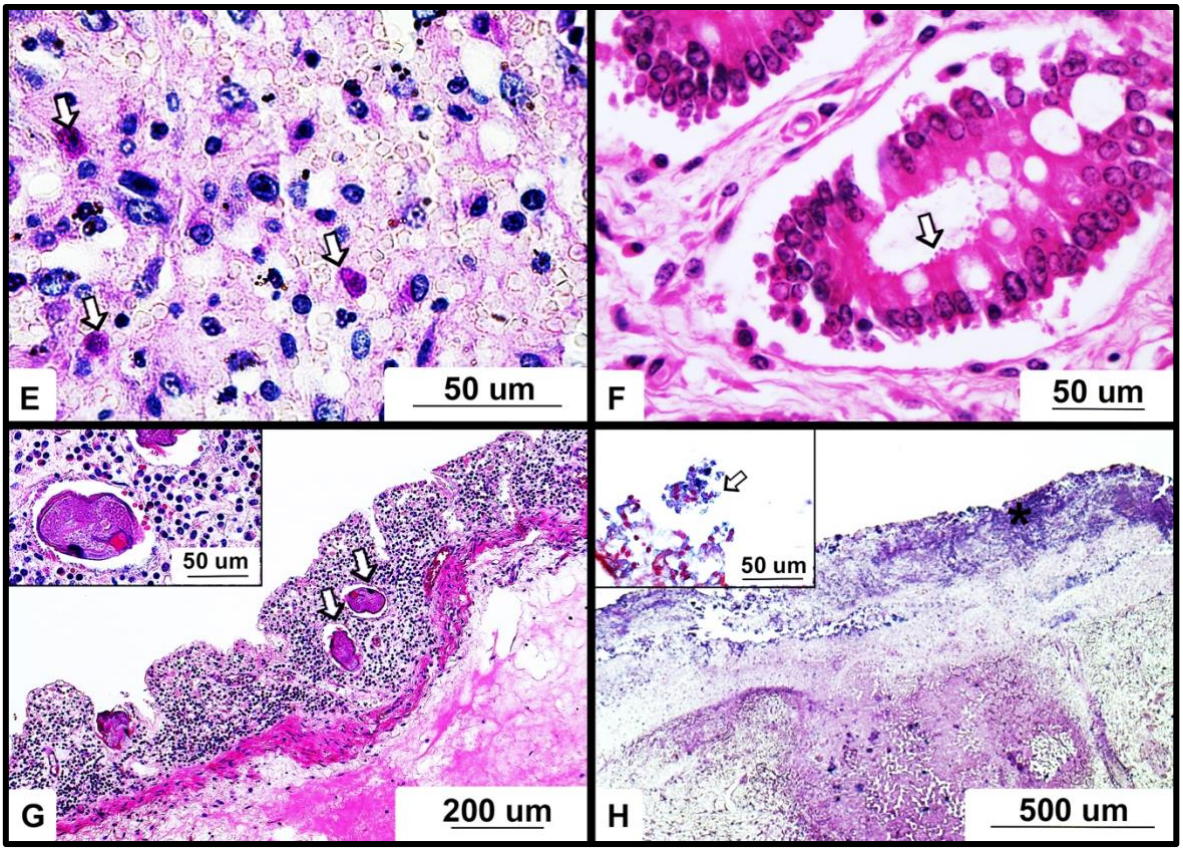


Figure 3. Infectious agents identified histologically. E) Mild numbers of mucosal PAS positive amoeba trophozoites (white arrows), colon, western lowland gorilla, case 1. PAS stain. **F)** Round, pale amphophilic to basophilic, 2-6 um diameter, apicomplexan protozoan schizonts (compatible with *Cryptosporidium* sp.) lining the crypt epithelium (white arrow), colon, western lowland gorilla, case 10. Haematoxylin an eosin. **G)** Mucosal (lamina proprial and intracryptal) *B. coli* trophozoites (white arrows), colon, western lowland gorilla, case 17. Haematoxylin an eosin. ***Inset,*** higher magnification of "G". Haematoxylin an eosin. **H)** Intraluminal and non-attaching and non-effacing mucosal Gram positive and negative mixed

bacteria (cocci, coccobacilli and bacilli) (asterisk), colon, western lowland gorilla, case 1. Gram stain.. ***Inset,*** higher magnification of "H". showing aggregates of luminal gram positive and negative bacilli and coccobacilli. Gram stain.

In all cases, a variable amount of intraluminal and non-attaching and non-effacing mucosal Gram positive and negative mixed bacteria (cocci, coccobacilli and bacilli) (Fig. 3, **H**) were observed. Acid fast (ZN positive) or argentaffin (WS positive) bacteria were not identified in any of the cases. For eight (cases 1, 10, 15, 16, 17, 18, 19 and 20) out of 21 cases, bacterial culture results from intestinal content samples were documented. *Escherichia coli* was isolated in all eight cases. Spore forming bacteria were isolated in three (cases 17, 18 and 20) cases. Spore forming bacteria include Gram positive bacteria with intracellular spores, known as endospores, commonly observed in *Bacillus* and *Clostridium* bacterial species (Basta and Annamaraju, 2021). *Campylobacter* sp. was isolated in case 20. Parasitology identified protozoal ciliates in cases 17 and 20 and *Giardia lamblia* in cases 16 and 18. All bacteriology and parasitology results are shown in table 5. Seven out of the eight cases were graded 2 (cases 10, 15, 17, 18, 19 and 20) and 4 (case 1) of the Nancy Index, indicating active disease.

Table 5. Bacteriology and parasitology results obtained from pathology reports in 8/21 examined cases. Case number (No.), bacteria isolated (Bacteriology), parasites identified (Parasitology), grade of superficial mucosal bacteria (Bac) and Nancy index grades (Nancy) are shown.

No.	Bacteriology	Parasitology	Bac	Nancy
1	non haemolytic *Escherichia coli* +	Negative	3	4
10	non haemolytic *Escherichia coli* ++ *Enterococcus* sp. ++ haemolytic *Escherichia coli* +	Negative	2	2
15	*Escherichia coli* +++	Negative	3	2

	Streptococcus sp. +++			
16	*Escherichia coli* ++ *Citrobacter freundii* +	ELISA positive for *Giardia lamblia*	2	0
17	*Escherichia coli* +++ *Streptococcus* sp. +++ *Haemolyzing Staphylococcus* sp. + Spore forming bacteria +++	Ciliates +++ (*Balantidium* sp.)	3	2
18	*Escherichia coli* +++ Spore forming bacteria +	ELISA positive for *Giardia lamblia*	1	2
19	*Escherichia coli* +	Negative	2	2
20	*Escherichia coli* +++ *Campylobacter* sp. ++ *Escherichia fergusonii* +++ Spore forming bacteria +++	Ciliates ++ (*Troglodytella abrassarti*)	3	2

+ = mild growth, ++ = moderate growth, +++ = marked growth, spore forming bacteria = include Gram positive bacteria with intracellular endospores, usually *Bacillus* (aerobic) and *Clostridium* (anaerobic) species.

3.2.2 STOMACH AND SMALL INTESTINE

The samples of stomach and small intestine were evaluated where available for detection of concomitant gastric or enteric disease. 18 out of 21 (86%) cases and 10/21 (48%) cases were examined for small intestine and stomach, respectively. In the small intestine samples, minimal to mild lymphoplasmacytic infiltration was present within the lamina propria of all animals, admixed with scattered macrophages and occasional eosinophils (interpreted as normal background change). Acute inflammatory changes (neutrophils, oedema or haemorrhage) were observed in cases 16 and 21. In all cases, large numbers of physiological Paneth cells were observed. By means of special stains, moderate numbers of Gram positive bacteria and PAS positive candida-like yeasts were

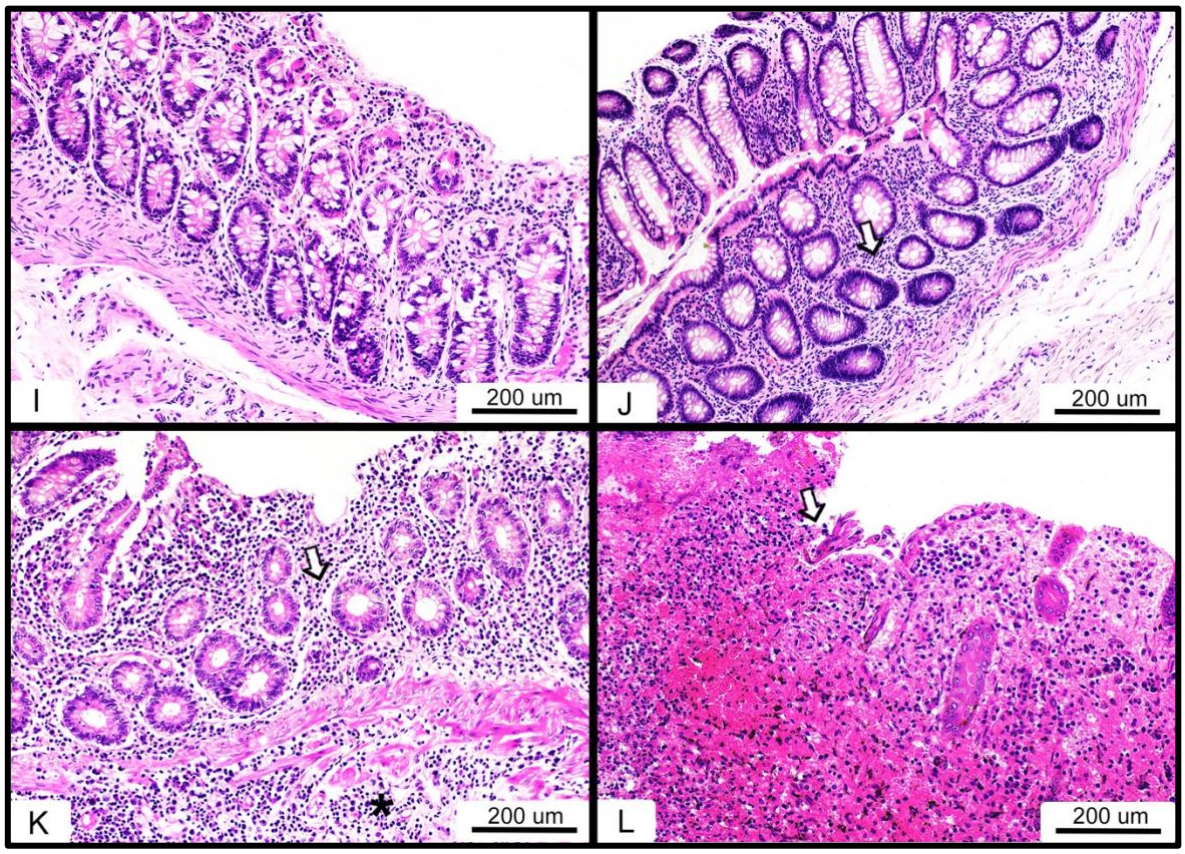
observed on the superficial mucosa in case 3. Case 16 showed moderate numbers of *Cryptosporidium* sp.

In the stomach samples, three cases (cases 1, 8 and 9) (33%) exhibited variable degrees of submucosal oedema. In case 1, a mild chronic inflammatory infiltrate (lymphocytes and plasma cells) and marked acute inflammation (neutrophils, haemorrhage, oedema and necrosis of the cryptal epithelium with mucosal ulceration) were observed accompanied by moderate numbers of Gram positive bacteria and PAS positive candida-like yeasts. Mild acute changes (mucosal oedema and haemorrhage) were observed in case 9. Finally, case 10 exhibited a moderate chronic inflammation (lymphocytes and plasma cells) affecting mainly the lamina propria.

3.3 NANCY INDEX

The Nancy index was applied to the colon sections in 21 cases, including the controls. The Nancy index is a 5-grade histological system used as a standardisation method of disease activity and severity for UC and it is obtained by histopathological examination of the tissue sections of affected colon based on a semiquantitative evaluation of acute and chronic colonic mucosal changes (Marchal-Bressenot et al., 2016).

Four out of 21 cases (19%) were scored as grade 0 (Fig. 4, **I**), 11/21 (52%) as grade 2 (Fig. 4, **J**), 2/21 (10%) as grade 3 (Fig. 4, **K**), and 4/21 (19%) as grade 4 (Fig. 4, **L**) of the Nancy index. Grade 1 was not represented. Three (cases 1, 3 and 5) out of four severely affected animals (grade 4 of the Nancy index) were under 25 years old, with ages ranging from 11 months to 17 years.



Figures 4. Nancy index. I) Normal tissue, colon, western lowland gorilla, case 12. Grade 0 of the Nancy index (No histological significant disease). Haematoxylin and eosin. **J)** Mild, chronic-active lymphoplasmacytic and neutrophilic colitis, colon, western lowland gorilla, case 2. The lamina propria is mildly expanded by increased numbers of lymphocytes, plasma cells and neutrophils (white arrow). Grade 2 (mildly active disease). Haematoxylin and eosin. **K)** Moderate, chronic-active lymphoplasmacytic and neutrophilic colitis, colon, western lowland gorilla, case 4. Grade 3 (moderately active disease). The lamina propria (white arrow) and the submucosa (asterisk, *) contain moderate numbers of lymphocytes, plasma cells and neutrophils. Haematoxylin and eosin. **L)** Severe, acute, ulcerative colitis, colon, western lowland gorilla, case 1. There is loss of mucosal epithelial lining (ulceration, white arrow). Grade 4 (severe active disease). Haematoxylin and eosin.

The “Nancy index” score system results for the WLG evaluated cases are shown in Table 6.

Table 6 – Nancy score index results of the evaluated WLG cases.

Case No.	*Acute inflammatory infiltrate*	*Chronic inflammatory infiltrate*	*Ulceration*	*Nancy score*
G1	3	2	1	**4**
G3	2	2	1	**4**
G5	2	2	1	**4**
G21	3	2	1	**4**
G4	2	2	0	**3**
G9	2	1	0	**3**
G2	1	1	0	**2**
G7	1	1	0	**2**
G8	1	2	0	**2**
G10	1	1	0	**2**
G14	1	2	0	**2**
G15	1	1	0	**2**
G17	1	1	0	**2**
G18	1	0	0	**2**
G19	1	1	0	**2**
G20	1	2	0	**2**
G22	1	1	0	**2**
G16	0	1	0	**0**
G11*	0	0	0	**0**
G12*	0	0	0	**0**
G13*	0	0	0	**0**

*Non-affected (control) cases.

Case 20, grade 2 of the Nancy index, additionally presented a segmental marked submucosal pneumatosis intestinalis (Fig. 5), characterised by the presence of gas-filled cysts and ectatic lymphatics within the submucosa of the colon, in this case. In a further case scored as grade 2 of the Nancy index (case 22) (Fig. 6), the major histological change was observed in the serosa, exhibiting

marked, subacute, multifocal fibrinosuppurative peritonitis with abscess formation affecting the serosa of different intestinal segments (ileum, colon and rectum).

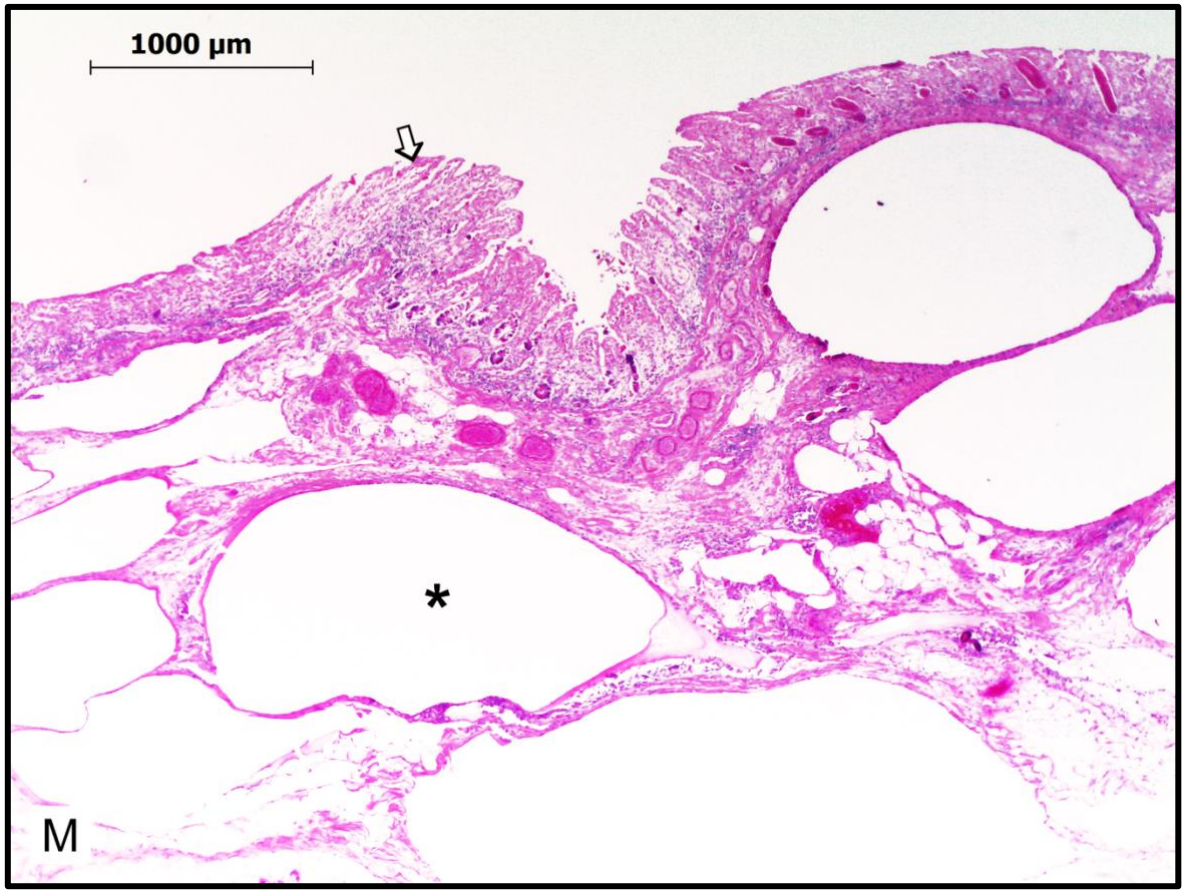


Figure 5 – M) Marked pneumatosis intestinalis, colon, western lowland gorilla, case 20 exhibiting abundant gas-filled cysts and ectatic lymphatics within the submucosa of the colon (asterisk, *). The white arrow indicates the colonic mucosal layer. Haematoxylin and eosin.

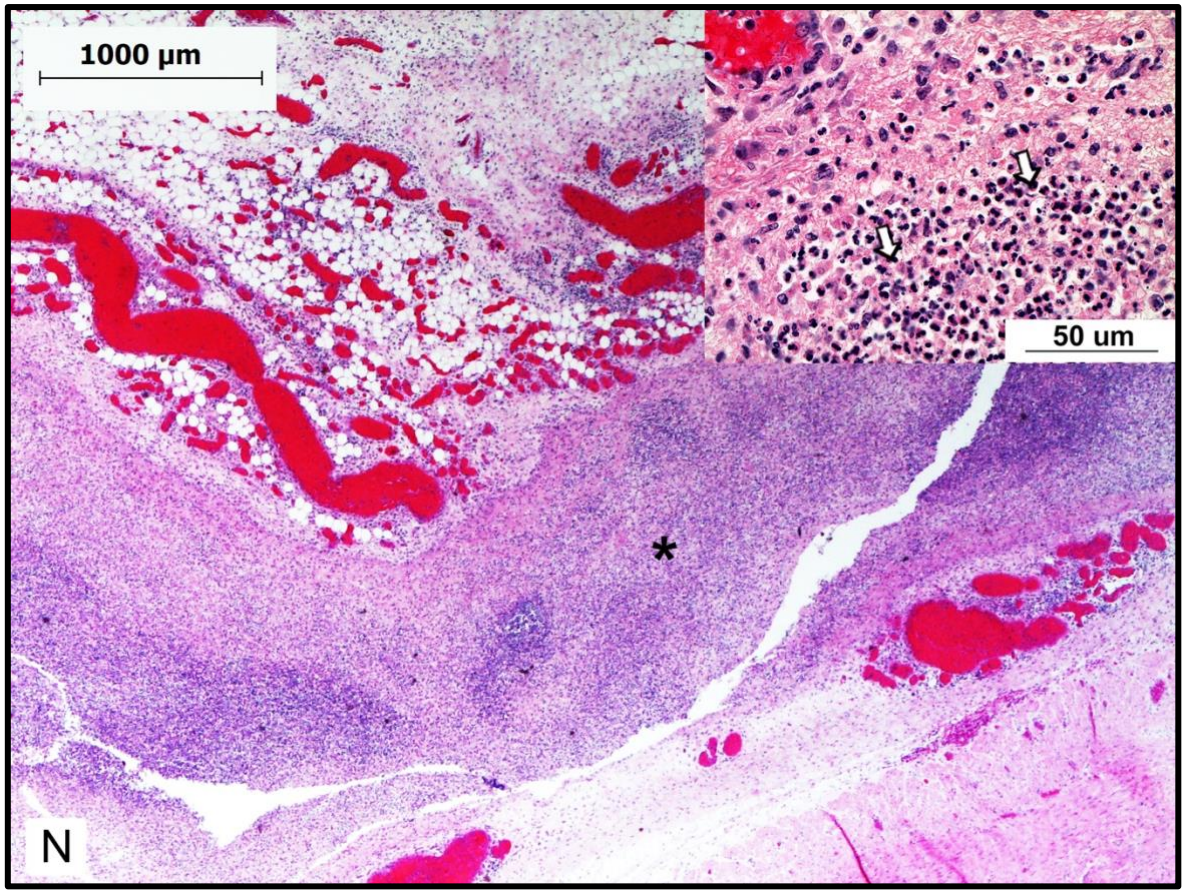


Figure 6 – N) Marked, subacute, multifocal fibrinosuppurative peritonitis with abscess formation, colon, western lowland gorilla, case 22. Large numbers of neutrophils admixed with fibrin markedly expand the serosa of the colon (asterisk). Haematoxylin and eosin. ***Inset***, higher magnification of "N" showing higher detail of the cellular infiltrate chracterised by large numbers of degenerated neutrophils (white arrows). Haematoxylin and eosin.

4 DISCUSSION

A total of 21 cases that fulfilled the criteria for inclusion were evaluated and characterised in order to: 1) gain insight into the aetiopathogenesis of colitis in captive gorillas; 2) to compare the histological lesions observed in gorillas with UC in humans; 3) to assess the utility of the Nancy index for evaluation of the severity of colitis in captive gorillas.

4.1 COMPARISON TO THE HUMAN DISEASE

UC has two incidence thresholds in humans: one in adolescents and young adults and the other in middle-aged men and women, with a peak of onset between 30-40 years (Gajendran et al., 2019; Ungaro et al., 2017). Gorillas reach sexual maturity at the age of 8 years (females) and 10 years (males) and are considered aged/elderly when they reach >35 years of age. They are considered adolescent/subadult to adult/middle age when they are above 10 years old and below 35 years old (Harcourt et al., 1980; Lowenstine et al., 2016; Margulis et al., 2007; Watts, 1991). In our study population, the age ranged from 11 months to 58 years and was subdivided into two comparative categories: under or equal to 25 years (9/21 cases, 43%) (adolescent/young adult) and over 25 years (10/21 cases, 48%) (adult/middle age).

In humans, IBD (UC and CD) is characterised by chronic and acute (active) disease changes. Chronicity is defined by the architectural distortion, the presence of diffuse band-like basal lamina lymphocytic and plasma cell infiltrates, Paneth cell metaplasia and submucosal fibrosis (Chang and Liu, 2018; DeRoche et al., 2014). In this study, chronicity was established on the basis of lymphocyte and plasma cell inflammatory infiltrates, in all cases located between and below the crypts and occasionally infiltrating the muscularis mucosa and submucosa. Architectural distortion (crypt dilation and distortion) and fibrosis were less common. Paneth cell metaplasia was only minimally present in one case. In human medicine, active disease is defined by the presence of neutrophils, crypt abscesses, and evidence of regeneration, necrosis, erosion and ulceration (Chang and Liu, 2018). The most commonly observed histological changes of acute inflammation in this case series were neutrophilic infiltration, mucosal and submucosal oedema, and crypt

abscesses. Ulceration was observed in four cases. Interestingly, the histopathological changes observed in the colon in this case series show similar characteristics to the acute phase of ulcerative colitis described in humans.

In this study, the semi-quantitative analysis of mast cells showed no differences in the number and location of mast cells between affected and control cases; however, limitations in the study included tissue preservation (advanced autolysis) and insufficient amounts of tissue to perform additional testing.

Many studies have shown the pro-inflammatory role of eosinophils in IBD, meanwhile others suggested that eosinophils may play an anti-inflammatory (protective) role (Filippone et al., 2019; Loktionov, 2019; Powell et al., 2010). In the gorilla samples, the highest numbers of eosinophils were found in two cases with grade 3 and 4 (active disease) of the Nancy index. This contrasts with findings from human studies where increased numbers of mucosal eosinophils in human IBD patients were found associated with better clinical outcomes (Alhmoud et al., 2020; Coburn et al., 2013). Previous investigations in human IBD patients also highlighted the presence of eosinophils in mild or quiescent diseases, further supporting a protective role of eosinophils in IBD (Al-Haddad and Riddell, 2005).

Nevertheless, in the present study, the presence of eosinophils may relate to intestinal parasitism given the presence of apicomplexan protozoan schizonts (compatible with *Cryptosporidium* sp.), ciliated trophozoites (compatible with *B. coli* and *Troglodytella abrassarti*) and amoebas. These results therefore need to be interpreted with caution. The limitations in relation to the eosinophil count included: 1) a small sample size in the controls versus affected animals; 2) the tissue preservation (advanced autolysis); 3) the lack of adequate reference values for

eosinophil counts in colonic samples in humans and gorillas; 4) additional testing was precluded by the paucity of tissue in the paraffin blocks.

4.2 NANCY INDEX APPLICATION IN WESTERN LOWLAND GORILLAS

The Nancy index was useful in providing a way to categorise the affected population based on the use of histological parameters in order to assess the severity and activity of the disease. The use of a simplified histopathological scoring system, like the Nancy index, can improve the consistency of interpretations among pathologists when describing histologic lesions of colonic inflammation in non-human primates. A consistent scoring system that accurately describes the disease activity will also help to determine therapeutic responsiveness (Marchal-Bressenot et al., 2016).

4.3 AETIOLOGICAL AGENTS

Common differential diagnoses of UC include: infectious enteritis (especially with intracellular bacteria), enteropathy (due to long term medication, ischaemia, or direct mucosal injury), and neoplastic disease (such as lymphoma) (Chang and Liu, 2018). Moreover, worsening of the symptoms due to secondary infections (e.g. *Clostridium difficile*, viral infections) is also observed in human patients (Chang and Liu, 2018). In this case series, mucosal and luminal amoebic trophozoites, apicomplexan protozoan schizonts (compatible with *Cryptosporidium* sp.), ciliated trophozoites and cysts (compatible with *B. coli* and *Troglodytella abrassarti*), and *Giardia lamblia* were identified in a small number of cases. Amoebic dysentery in apes can result in multifocal ulcerative typhlocolitis; however, it is important to emphasise that in non-human primates, the majority of amoeba species described

are considered non-pathogenic (commensals) (for example, *Entamoeba polecki*) and only certain species are described as pathogenic such as *Entamoeba histolytica* (Lowenstine et al., 2018; Murphy, 2015; Regan et al., 2014; Strait et al., 2012). In this case, the relatively small numbers of amoebic trophozoites suggested a secondary, rather than primary aetiology for the observed lesions. The increase in numbers of *Balantoides* protozoa in autolysed cases was assumed to be as post mortem proliferation. *Balantoides* can be identified in colon samples of clinically normal non-human primates; however, dysbiosis may pave the way for opportunistic *Balantoides* proliferation, and a contribution to the development of colitis cannot be excluded (Lankester et al., 2008; Lee et al., 1990; Lowenstine et al., 2018). *Cryptosporidium* sp. and *Giardia lamblia* are rarely associated with clinical disease in gorillas (Lowenstine et al., 2018). In this study, they were interpreted as opportunistic and were observed in animals with no or mild disease activity.

In all cases, a variable amount of superficial and/or intraluminal Gram positive and negative mixed bacteria (cocci, coccobacilli and bacilli) were observed. Intracellular bacteria were not identified in any of the cases.

In eight cases, bacterial culture results of enteric contents were available. The most commonly isolated bacterium was *Escherichia coli*, which can be a commensal microorganism of the intestinal microflora (Campbell et al., 2008). The majority of cases with isolated *Escherichia coli* presented as Grade 2 on the Nancy index; however, the lack of identification of the strain of *Escherichia coli* (commensal or pathogenic) hampers a proper evaluation of the potential for correlation between colibacillosis and disease activity in these animals. In the setting of routine post-mortem examinations, the differentiation between true-

positive culture results (contributing to clinical disease), normal bacterial microflora and post-mortem bacterial overgrowth and/or contamination continue to represent a challenge for microbiologists and pathologists (Riedel, 2014). In humans, some pathogenic strains of *Escherichia coli* have been suspected to play a role in the pathogenesis of UC (Mirsepasi-Lauridsen et al., 2016; Petersen et al., 2009). However, bacterial culture on its own does not allow an in-depth characterisation of the complex microbiome environments, hence the results of the post-mortem routine bacterial culture are not comparable to studies of dysbiosis in humans (Carding et al., 2015; Suchodolski, 2016). PCR amplification of 16S rRNA genes, next generation sequencing (NGS), quantitative PCR (qPCR), fluorescent in situ hybridization (FISH) for microbial mucosal translocation, and measurements of bacterial metabolites are required diagnostic tools used for the assessment of dysbiosis in humans and domestic animals (Duboc et al., 2013; Lloyd-Price et al., 2019; Suchodolski, 2016). Therefore, the use of the aforementioned diagnostic tests to characterise the microbiome environment of the colon in health and in disease in gorillas would be helpful to help elucidate the potential role of the microbiome in the pathogenesis of ulcerative colitis in gorillas.

In a recent study conducted in rhesus macaques (*Macaca mulatta*), lesions of idiopathic chronic diarrhoea (ICD) were studied; these are similar to the post-infectious irritable bowel syndrome (PI-IBS) observed in humans. Results suggested an association of ICD with a history of *Campylobacter* infection, with concomitant dysbiosis and hyperplasia of enterochromaffin cells (Laing et al., 2018). In humans, multiple infectious agents have been associated with the development of IBD, included *Salmonella* sp. and *Campylobacter* sp. (Adams and Bornemann, 2013; Feuerstein et al., 2019). In addition, dysbiosis occurs in humans

in the context of IBD, characterized by a reduced diversity of the microbiota (Ananthakrishnan et al., 2018). In this case series, *Campylobacter* sp. was isolated only in one case. However, the significance of this finding remains unknown. No clinical data on previous bacterial infections were available for the gorillas, but it would be informative to determine if a history of previous bacterial infection (e.g. *Campylobacter* or *Salmonella*) may contribute to the development of UC in gorillas, as seen in humans and rhesus macaques, in future larger clinical studies. Larger clinical studies would be necessary as they are more representative of the population and can produce results among variables that are statistically significantly different. A larger population could potentially be obtained by involving worldwide more zoos or extending the research to wild population.

Recent human research suggests that IBD is a multifactorial disease, likely associated with a number of risk factors including environment (e.g. diet, lifestyle, infections, geography), genetics, autoimmunity, and gut microbiota and it is unlikely that it is caused by a single primary pathogen (Ananthakrishnan et al., 2018; Gajendran et al., 2019). Lifestyle of captive gorillas differs from their wild counterparts, and environmental differences (for example diet, habitat) and stressors (introduction of new a cohabitant in a group, transfers between zoos, breeding attempts, etc.) may play a role in the development of dysbiosis and gastrointestinal disease in these animals (Meder, 2017; Smith et al., 2014; West et al., 2019).

4.4 SUMMARY AND CONCLUSION

The results of this study suggest that in WLG with ulcerative colitis there is no single infectious agent shared by the majority of affected animals studied. It is

reasonable to suspect that, similar to findings in IBD in human cases, colitis in captive gorillas is likely a multifactorial disease, in which environmental factors result in dysbiosis, which then prompts an autoimmune response (Guo et al., 2020; Porter et al., 2020). Potential factors such as genetic, environmental, immune, and microbiome factors need to be further identified and investigated in WLG.

In conclusion, the histopathological changes observed in the colon in this case series show comparable characteristics to the acute phase of ulcerative colitis described in humans. These results suggest no unifying underlying infectious agent. The Nancy index proved a useful tool for standardisation of disease activity grading of ulcerative colitis for further studies of gorilla colitis.

4.5 FUTURE PLANS AND IMPLICATIONS

Despite some limitations to the study design, this pilot study provides a useful insight into the changes observed in colitis in captive gorillas from European zoo collections, which provides a base for further larger clinical studies.

The study of the colonic (and/or gastrointestinal) microbiome in gorillas may help elucidate the role of the intestinal microbiota and its changes in the pathogenesis of colitis. This could be achieved collecting stool samples, following previously reported methods in gorilla studies and analysed for faecal glucocorticoid (FGM) metabolite, using a cortisol enzyme immunoassay (Jacobs et al., 2014).

Immunohistochemistry was also considered in this study; however, a big limitation was the lack of sufficient paraffin embedded formalin fixed tissue to perform the immunolabelling. Future studies should focus on gathering further tissues from additional animals with compatible histopathological changes and,

evaluate the inflammatory component using immunohistochemistry and/or other additional testing (e.g. *in situ* hybridisation, immunofluorescence, image analysis, etc.) aiming to better characterise and investigate the inflammatory population involved in the disease process. Immunohistochemical studies could be implemented in order to better characterise the cellular immunophenotype, investigating the cell lineage (e.g. macrophage, lymphocyte) and cluster of differentiation of the inflammatory cells (particularly lymphocytes) involved in the disease process observed in gorillas. This would give insights into the aetiopathogenesis and help elucidate the type of immune response involved.

Studies using surgical specimens (biopsy samples) and prospective data will be of value in studying the mucosal microbiota and, also, to evaluate the use of the Nancy index in clinical zoological settings. However, the latter approach is challenging, and potential limiting factors for these studies would be: the endangered status of the studied animal species, the difficult clinical management (anaesthesia risks for biopsy sample collection) and the cost of the required tests (microbiome analysis).

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6 ANNEX

Supplemental Table S1. **Gorilla data submitted by contributors**. Case number (No.), age, sex, clinical signs, cause of death, and ancillary testing (bacteriology, parasitology and immunohistochemistry (IHC)) from histopathology reports are provided for each case where available.

y= years, m= months, F= female, M= male, NA = Not available, * = control cases, + = mild growth, ++ = moderate growth, +++ = marked growth, - = negative.

Case No.	AGE	SEX	CLINICAL SIGNS	Cause of death	Bacteriology		Parasitology	IHC
					Large intestine	Small intestine	Large intestine	
G1	17 y 11 m	M	Annorexia (seven days), constipation.	Aspiration pneumonia, septic shock	Non haemolytic *E. coli* +, *Salmonella* sp. +, *Shigella* sp. +	NA	No parasites	NA
G2	25 y	F	Recent marked abdominal distension. History of chronic abscess and peritonitis.	NA	NA	NA	NA	NA
G3	1 y	M	Diarrhoea (one week).	NA	*Yersinia* sp. -, *Salmonella* sp. -	NA	NA	NA
G4	n/a	M	Severe diarrhoea, abdominal pain. Treated for dental trauma.	Cardiac fibrosis	NA	NA	NA	NA
G5	11 m	M	Progressive weight loss, weakness and hyporexia. Diarrhoea, abdominal pain, anaemia.	NA	NA	NA	NA	*Entamoeba* sp. -
G7	34 y	M	Chronic constipation, clinical evidence of colitis - mucous and blood faeces - and annorexia intermittently.	NA	NA	NA	NA	NA
G8	54 y	F	NA	Cardiac fibrosis	NA	NA	NA	NA
G9	46 y	F	Chronic dental problems, received antibiotic treatment, aspirin and metacam.	Cardiac fibrosis	NA	NA	NA	NA

G10	33 y	M	Vomiting, mild diarrhoea overnight, abdominal pain.	Cardiac fibrosis	Non haemolytic *E. coli* ++, *Enterococcus* sp. ++, haemolytic *E. coli* +	NA	NA	NA
G14	NA	NA	NA	NA	NA	NA	NA	NA
G15	38 y	M	Died after tooth extraction.	Cardiac tamponade due to aortic rupture	*Streptococcus sp.* +++, *E. coli* +++	*Staphylococcus* sp. +	No parasites	NA
G16	1 y 3 m	M	Comatose, hypoglycaemic shock.	Hypoglycaemic shock	*E. coli* ++, *Citrobacter freundii* +	NA	ELISA positive for *Giardia lamblia*	NA
G17	43 y	F	Several episodes of diarrhoea, weight loss and dysphagia in last months.	Septic/toxic shock	*E. coli* +++, *Streptococcus* sp. +++, spore forming bacteria +++, haemolysing *Staphylococcus* sp. +	*E. coli* +++, *Streptococcus* sp. +++, spore forming bacteria +++	Ciliates +++ (*Balantidium* sp.)	NA
G18	5 y	F	NA	Undetermined - Suspected septic shock	*E. Coli* +++, spore forming bacteria +	*E. coli* +++, spore forming bacteria +	ELISA positive for *Giardia lamblia*	NA
G19	56 y	F	Dysphagia. Died after tooth extraction.	Cardiac failure	*E. Coli* +	*E. Coli* +	No parasites	NA

G20	32 y	M	Constipation, vomiting, acute diarrhoea.	NA	*E. fergusonii* +++, *E. coli* +++, *Campylobacter* sp. ++, spore forming bacteria +++	*E. fergusonii* +++, *E. coli* +++, *Campylobacter* sp. +++, spore forming bacteria +++	Ciliates ++ (*Troglodytella abrassarti*)	NA
G21	36 y	F	Hyporexia, diarrhoea and severe weight loss.	Euthanasia	NA	NA	NA	NA
G22	22 y	M	1-week history of anorexia, abdominal pain and constipation. Exploratory laparatomy: marked fibrinosuppurative peritonitis and necrotic sections of bowels.	Euthanasia	NA	NA	NA	NA
G11*	23 y	M	NA	Cardiac fibrosis	NA	NA	NA	NA
G12*	19 y 2 m	F	NA	Bacterial sepsis	NA	NA	NA	NA
G13*	NA	F	NA	Bacterial sepsis and DIC	NA	NA	NA	NA

Supplemental Table S2 – **Haematoxylin and eosin (HE) grading scheme** for gastrointestinal sections of gorillas (*Gorilla gorilla* ssp. *gorilla*) for the following parameters: lymphocytes, plasma cells, neutrophils, macrophages, eosinophils, and mast cells; fibrosis, oedema, and haemorrhage; crypt hyperplasia, dilation and distortion, necrosis and abscesses; Paneth cell metaplasia; and mucosal ulceration.

Parameter/Degree	0	1 = mild	2 = moderate	3 = marked
Lymphocytes and plasma cells	0-4 cells between crypts. Crypts closely packed	Up to 4-6 cells between crypts. Crypts mildly separated	Up to 6-7 cells between crypts. Crypts are lifted and separated and the cells infiltrate the submucosa	> 8 cells between crypts. There is marked widening of intercrypt spaces and the cells infiltrate the submucosa and muscularis
Macrophages	Scattered, very few	Scattered aggregates at the top of the glands	Multifocal aggregates, unequivocal increased numbers	Marked increased numbers, diffuse
Eosinophils (Day MJ et al (2008))	1-2 per HPF	up to 20 per HPF	21-50 per HPF	51-100 per HPF
Mast cells	0-4 per HPF	4-6 per HPF	6-8 per HPF	>8 per HPF
Fibrosis	Absence	Scattered reactive fibroblasts	Unequivocal increased collagen and plump fibroblasts	Marked increased collagen and plump fibroblasts
Neutrophils	Absence	Scattered neutrophils in the lamina propria (<10 per HPF)	Moderate numbers of neutrophils and infiltration of the submucosa (<30 per HPF)	Marked numbers of neutrophils and infiltration of the submucosa and muscular layers (>30 per HPF)
Oedema	Absence	Mild widening of stroma spaces with mildly dilated lymphatic vessels	Moderate widening of stroma and moderately dilated lymphatic vessels	Marked widening of stroma and moderately dilated lymphatic vessels
Haemorrhage	Absence	Focal or multifocal scattered aggregates of extravasated erythrocytes	Multifocal	Focal extensive, multifocal to coalescing to diffuse
Crypt hyperplasia	0	1-2	2-7	>7
Crypt dilation and distortion	Absence	Minimal distortion of crypt architecture	Moderate distortion of crypt architecture and mild branching	Marked distortion and branching of crypt
Crypt Necrosis	Absence	Focal	Multifocal	Focal extensive, multifocal to coalescing to diffuse
Crypt abscesses	Absence	Rare dilated crypt with debris and degenerated neutrophils (1-2 in 10x)	Moderate numbers of dilated crypt / abscesses (3-5 in 10x)	Numerous dilated crypt / abscesses (>6 in 10x)
Paneth cells	Absence	0-5	5-10	>10
Ulceration	Absent	Present	n/a	n/a

HPF = high power field; n/a = not applicable