Immunopathological Aspects of Equine Inflammatory Bowel Disease

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Cover: Photomontage of horses and histological sections of rectal biopsies.
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Abstract

Intestinal inflammation is a major welfare issue for horses causing colic, diarrhoea and an inability to thrive. One of the diagnoses with poor prognosis is inflammatory bowel disease (eqIBD). Equine IBD comprises different entities of chronic idiopathic enteropathies, namely eosinophilic gastroenteritis, granulomatous enteritis and lymphoplasmacytic enterocolitis. An exaggerated immune reactivity towards still unidentified antigens is suggested in the disease. The aim of this thesis was therefore to elaborate methods to characterize cellular infiltrations and to determine cytokine profiles associated with various forms of eqIBD. Jejunal tissues were used to describe the immune cell populations *in situ* in healthy horses and in well characterised IBD-afflicted horses. T cells, regulatory T cells, B cells, IgM- IgG- and IgA-secreting plasma cells and MHC II-expressing cells were labelled by immunohistochemistry and quantified by image analysis. The inflammation in IBD horses was dominated by T cells and MHC II-expressing cells, whereas B cells and plasma cells were decreased compared with healthy horses. These features were most prominent in granulomatous IBD. The regulatory T cells followed the infiltration pattern of T cells, but were not significantly increased in diseased horses. Thus the histopathology in the studied forms of eqIBD shows similarities with a delayed hypersensitivity reaction. Seven reference genes were evaluated and their optimal combination determined for seven equine intestinal segments (the duodenum, mid-jejunum, ileum, caecum, right ventral and dorsal colon and rectum). Segments from healthy horses expressed IL-12, IL-17A, IL-23, TLR4, but not IFN-α. The relative expression of the three interleukins and TLR4 was analysed using quantitative PCR in rectal biopsies showing chronic or chronic active simple proctitis from horses with clinical signs of eqIBD. Horses with chronic active proctitis had increased expression of IL-17A and TLR4 compared with healthy horses, whereas horses with chronic proctitis had decreased expression of IL-12, indicating a T_H17 involvement in chronic active disease. Taken together, techniques were established to discern patterns of immune reactions and could demonstrate differences between entities when applied to eqIBD material.

Keywords: equine, intestine, immunopathology, chronic idiopathic inflammatory enteropathy, inflammatory bowel disease

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Dedication

To my family and friends who patiently and lovingly supported me throughout my studies

The road to health is paved with good intestines.

Sherry A. Rogers

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Olofsson, K.M.**, Press, C.M., Fossum, C. & Lindberg, R. Immunohistochemical characterization of immune cells in the jejunum of healthy horses and horses with inflammatory bowel disease. *In manuscript.*

II Fossum, C., Hjertner, B., **Olofsson, K.M.**, Lindberg, R., Ahooghalandari, P., Camargo, M.M., Bröjer, J., Edner, A. & Nostell, K. (2012). Expression of *tlr4*, *md2* and *cd14* in equine blood leukocytes during endotoxin infusion and in intestinal tissues from healthy horses. *Veterinary Immunology and Immunopathology*, 150(3-4), pp. 141-148.

III Hjertner, B., **Olofsson, K.M.**, Lindberg, R., Fuxler, L. & Fossum, C. (2013). Expression of reference genes and T helper 17 associated cytokine genes in the equine intestinal tract. *The Veterinary Journal*, 197(3), pp. 817-823.

IV **Olofsson, K.M.**, Hjertner, B., Fossum, C., Press, C.M. & Lindberg, R. (2015). Expression of Th17-associated cytokines and toll-like receptor 4 and their correlation to Foxp3 positive cells in rectal biopsies of horses with clinical signs of inflammatory bowel disease. *The Veterinary Journal*, 206(1), pp. 97-104

Papers II-IV are reproduced with the kind permission of the publishers.

The contribution of Karin M. Olofsson to the papers included in this thesis was as follows:

I K.M.O performed the study under supervision of Ronny Lindberg and Charles Press, and the manuscript was written together with Ronny Lindberg, Charles Press and Caroline Fossum. The IHC was performed by K.M.O and laboratory technicians in NMBU under the supervision of Charles Press.

II K.M.O performed the intestinal sampling and collection of PBMC. K.M.O. together with Lisbeth Fuxler processed the intestinal samples and cultured the PBMC, as well as contributed in the analyses and writing the manuscript, under the supervision of Caroline Fossum and Ronny Lindberg. The remaining co-author contributed to and/or performed the endotoxaemia infusion, clinical evaluation, analyses and writing the manuscript.

III K.M.O. performed the study and analysed the results together with Bernt Hjertner under the supervision of Caroline Fossum. K.M.O wrote the manuscript together with Bernt Hjertner, Caroline Fossum and Ronny Lindberg.

IV K.M.O. performed the study and analysed the results together with Bernt Hjertner under the supervision of Caroline Fossum. The IHC was performed by laboratory technicians in NMBU under the supervision of Charles Press. K.M.O. wrote the manuscript together with Bernt Hjertner, Caroline Fossum, Ronny Lindberg and Charles Press.

Abbreviations

β2M	β2 microglobulin
AEC	3-amino-9-ethylcarbazole
CASP	Chronic active simple proctitis
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CpG-ODN	Deoxycytidylate-phosphate-deoxyguanylate oligodeoxynucleotide
Cq	Quantification cycle
CSP	Chronic simple proctitis
CXCL-8	Chemokine (C-X-C motif) ligand 8, formerly IL-8
DAB	3,3'-diaminobenzidine
EDTA	Ethylenediaminetetraacetic acid
EEG	Equine eosinophilic gastroenteritis
EGE	Equine granulomatous enteritis
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H2Atype1	Histone H2A type 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
LPE	Lymphoplasmacytic enterocolitis
LPS	Lipopolysaccharide
MD-2	Lymphocyte antigen 96

MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NOD	Nucleotide-binding oligomerization domain-containing protein
PBMC	Peripheral blood mononuclear cell
PMA	Phorbol myristate acetate
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RPL32	60S ribosomal protein L32
RPMI	Roswell Park Memorial Institute
SDHA	Succinate dehydrogenase complex subunit A
TCR	T cell receptor
TFRC	Transferrin receptor 1 protein coding
T_H	T helper cells
TLR	Toll-like receptor
T_{REGS}	Regulatory T cells

1 Background

Intestinal health is a great concern in horses as gastrointestinal disorders are a major reason for medical intervention (Archer & Proudman, 2006). Depending on the duration, severity and distribution of lesions, the clinical presentation may include different symptoms such as colic, diarrhoea and/or altered nutritional status. One of the chronic intestinal inflammations with poor prognosis is referred to as equine inflammatory bowel disease (IBD). The term IBD includes sporadically occurring chronic idiopathic enteropathies. Histologically distinguishable entities of IBD are equine eosinophilic gastroenteritis (EEG), equine granulomatous enteritis (EGE) and lympho-plasmacytic enterocolitis (LPE).

The disease complex IBD has been recognised in humans since the early 1900s and in recent decades it has also been diagnosed in cats and dogs (Jergens *et al.*, 1992) as well as cattle (Fushimi *et al.*, 2015; Cebra *et al.*, 1998). In horses, these enteropathies have been described since the 1970s and the clinical aspects, as well as histological features, are well characterized (Schumacher *et al.*, 2000). The pathogenesis of IBD is proposed to involve an imbalanced immune reaction towards the commensal intestinal flora or other antigens in the intestinal lumen. However, little is known about the immunological processes in the equine disease development. The present thesis sought to develop methods to study equine intestinal immune responses, to discern pathological events in the development of equine IBD and to distinguish differences between the histological types of the disease.

1.1 Clinical presentation of equine IBD

Equine IBD can affect horses of both sexes, including geldings. Documented cases of IBD have ranged in age from 7 months to 26 years, although afflicted horses are often young adults, mainly 2-12 years. Young horses, aged 0-5 years, are at higher risk for developing idiopathic focal eosinophilic enteritis

(IFEE) compared with other age groups (Archer *et al.*, 2014; Kemper *et al.*, 2000). Afflicted horses have been reported for various breeds; American Paint horse, American Paso Fino (La Perle *et al.*, 1998; Schumacher *et al.*, 1990), Arabian, Appaloosa (Scott *et al.*, 1999), crossbreds, Hanoverian (Southwood *et al.*, 2000; Clark, 1988), Hunter (Edwards *et al.*, 2000), Icelandic horse (Paper IV), Irish draft (Archer *et al.*, 2006), miniature horse (Wong & Crisman, 2004), Morgan (Scott *et al.*, 1999), National show horse (Scott *et al.*, 1999), Quarter horse, Standardbred, Tennessee walking horse (Schumacher *et al.*, 1990), Thoroughbred, Warmblood and ponies. Neither Kemper *et al.* (2000) nor Archer *et al*. (2006) detected a breed predisposition in their material, but Standardbreds have been implicated as overrepresented (Schumacher *et al.*, 2000).

Varying clinical manifestations are seen in horses suffering from equine IBD. Commonly reported clinical findings are colic, weight loss and/or diarrhoea, while anaemia, subcutaneous oedema and dermatitis are less frequent. Rare presentations include rectal prolapse (Gibson *et al.*, 2001) and high fever (Bosseler *et al.*, 2012).

Colic is a wide term and includes a vast spectrum of aetiologies. If a horse has a colic episode there is a high risk of recurrence, even if the horse is not IBD-afflicted (Scantlebury *et al.*, 2011; Cohen & Peloso, 1996). In IBD horses, colic is thought to be due to a reduced mobility of the intestine, ileus and subsequently intestinal distention (Scott *et al.*, 1999) and can occur without any of the other characteristic clinical signs of IBD (Bassage *et al.*, 1997). According to Archer (2009), partial obstruction of the intestinal lumen is one of the most common causes of recurrent colic and includes strictures as a consequence of IBD lesions. Horses with IBD can also present with acute endotoxaemia as a consequence of the intestinal inflammation (Grulke *et al.*, 2008).

Malabsorption resulting in hypoalbuminaemia, hypoproteinaemia and weight loss may be caused by reduced absorption through the intestinal mucosa, reduced transportation of nutrients, and/or maldigestion (Mair *et al.*, 2006; Roberts, 1983). The severity of the albumin and protein losses is correlated to poor prognosis (Metcalfe *et al.*, 2013). Infectious diseases are the main cause of malabsorption in foals, whereas the most common cause in adult horses is infiltrative diseases (Mair *et al.*, 2006). In IBD the intestine is inflamed, occasionally flattened with ulcerations and lymphangiectasis. This leads to reduction in plasma protein by loss of intestinal fluids into the intestinal lumen, as well as reduced absorptive properties of the intestinal epithelium (Roberts, 1983; Meuten *et al.*, 1978).

The symptoms of IBD can appear recurrently, intermittently or even seasonally (Archer *et al.*, 2014; Schumacher *et al.*, 2000; Southwood *et al.*, 2000). The duration of disease varies from an acute onset to a lengthy chronic illness. The acute manifestation occurs almost exclusively in cases of eosinophilic IBD (Archer *et al.*, 2006; Edwards *et al.*, 2000; Schumacher *et al.*, 2000), whereas other clinical parameters are mainly non-exclusive for the different entities. It is noteworthy that other diseases of the intestinal tract can display the same clinical signs as IBD, making diagnosis challenging.

1.2 Diagnosing equine IBD

There is no laboratory test that specifically can diagnose IBD. Instead multiple tools are used to identify and exclude other diseases. In the clinic, a presumptive diagnosis of equine IBD can be made when all other plausible diagnoses have been excluded. The relevant differential diagnoses for IBD vary depending on the presenting clinical signs (Table 1). Some of the differential diagnoses are regarded as highly unlikely based on the prevalence in an area and the susceptibility of the horse. Several diagnoses can be excluded during clinical examinations, whereas others are excluded in specific analysis, such as microbiology testing and radiological examinations.

1.2.1 Clinical pathology

The use of oral D-xylose or glucose absorption test for screening of small intestinal dysfunction may show normal, reduced or delayed uptake by the mucosa for the given sugars (Mair *et al.*, 1991). A disturbed uptake is not diagnostic for IBD, but indicative of infiltrative disease (Roberts, 1983). In horses with eosinophilic IBD a partial malabsorption or a delayed uptake can be seen, whereas horses with granulomatous IBD or lymphoma can display a severe malabsorption (Mair *et al.*, 1991; Lindberg *et al.*, 1985). Using haematology and biochemistry, some but not all IBD-afflicted horses display deviations from normal. Anaemia, hypoproteinaemia, hypoalbuminaemia and elevated liver enzymes have been noted in all the various IBD entities (Schumacher *et al.*, 2000). Concerning white blood cell counts, neutrophilia can be found in both EGE and EEG, and neutropenia can be present in EGE (Schumacher *et al.*, 2000; Gibson & Alders, 1987). The change in neutrophil count is presumed to reflect secondary events, such as endotoxaemia. However, blood eosinophilia is only described in the eosinophilic form of IBD (Gibson & Alders, 1987; Platt, 1986; Lindberg *et al.*, 1985), but has also been found in conjunction with intestinal lymphoma (Duckett & Matthews, 1997). Furthermore, Lindberg *et al.* (1985) reported varying immunoglobulin levels in

Table 1. *Differential diagnoses for the main clinical symptoms of equine IBD*

Chronic/recurrent colic	Hypoproteinaemia/weight loss	Chronic diarrhoea
Alimentary lymphoma	Alimentary lymphoma	Alimentary lymphoma
Crib-biter/windsucker[1,2]	Amyloidosis	Disruption of intestinal flora
Congestive heart failure*	Congestive heart failure	Enteric bacterial infection
Dental abnormalities[1]	Dental abnormalities	*Brachyspira* spp.[8, 9]
Displacement of the colon	Enteric bacterial infection	*Salmonellosis*
Enteric bacterial infection	*Lawsonia intracellularis*	Enteric parasitic infection
Lawsonia intracellularis	*Mycobacterium avium* spp.	*Cyathostominosis*
Enteric parasitic infection	*Rhodococcus equi*	*Giardia* spp.[10]
Cyathostominosis	Enteric fungal infection	*Strongylus vulgaris*
Strongylus vulgaris	*Aspergillus fumigatus*	Hyperlipidaemia
Enterolithiasis	*Histoplasma capsulatum*	Impaction
Gas colic, intermittent	Enteric parasitic infection	Intestinal muscular hypertrophy[6]
Gastrointestinal ulceration	*Cyathostominosis*	Liver disease
Impaction	*Parascaris equorum*	NSAID toxicity/right dorsal colitis
Intestinal adhesions	External bleedings or burns	Peritonitis
Intestinal diverticulum	Gastrointestinal ulceration	Sand ingestion/accumulation
Intestinal fibrosis[3]	Grass sickness[5]	
Intestinal muscular hypertrophy[4]	Immunodeficiency	
Intussusception	Intestinal muscular hypertrophy[4, 6]	
Liver disease	Kidney disease	
Miscellaneous neoplasia	Liver disease	
Neural dysfunction/ grass sickness	Lymphatic obstruction	
NSAID toxicity/right dorsal colitis	Malnutrition	
Obstruction, intra- or extraluminal	Miscellaneous neoplasia	
Peritonitis	NSAID toxicity/right dorsal colitis	
Sand ingestion/accumulation	Pancreatic insufficiency	
Urogenital disease	Pleuritis/peritonitis	
	Pulmonary disease[7]	

Modified after: Archer (2009), Kalck (2009), Lecoq & Lavoie (2009), Mair *et al.* (2006), *Mair & Hillyer (1997), Love *et al.* (1992) and Roberts (1983)

1. Scantlebury *et al*. (2011)
2. Escalona *et al.* (2014)
3. Schultheiss et al. (1995)
4. Chaffin *et al*. (1992)
5. Wylie & Proudman (2009)
6. Cordes & Dewes (1971)
7. Dickinson & Lori (2002)
8. Hampson *et al*. (2006)
9. Shibahara *et al.* (2005)
10. Kirkpatrick & Skand (1985).

blood from IBD horses. Reduced IgG(T) was detected in cases of both EEG and EGE horses, whereas IgG was only reduced in EGE cases and IgM only in EEG cases. However, the values for IgM did not reach the cut off value for IgM deficiency as defined by Perkins *et al.* (2003).
The clinical relevance and functionality of immunoglobulin discrepancies in the serum of IBD horses compared with healthy horses are not known, butcould result from a disturbed humoral immune response as described for human IBD patients (Rai *et al.*, 2015) or a loss through the inflamed intestine (Lindberg *et al.*, 1985). Taken together, clinical pathology can indicate the presence of IBD in horses and be used to establish a plausible diagnosis. The diagnosis then needs to be verified and classified by histological evaluation of intestinal tissue.

1.2.2 Intestinal biopsy

Various approaches to collect equine intestinal biopsy material for histopathology examination are possible (Kalck, 2009). Endoscopy can be used to both visualize the mucosa and sample it, but collected tissue samples are small and several sampling sites are needed. In gastroscopy, the stomach, the duodenum and, in miniature breeds, the jejunum can be sampled. Colonoscopy can be used to sample the small colon and the rectum. To collect better sized rectal sample, rectal biopsy can be applied (Kaikkonen *et al.*, 2014; Lindberg *et al.*, 1996). No visualization of mucosa is then possible, but mucosa and submucosa are included in the samples, facilitating histopathological diagnosis. Evaluation of the significance of rectal biopsies revealed that one third to half of them were diagnostic of the specific type of inflammation in the gut, revealed at necropsies of diseased horses (Barr, 2006; Lindberg *et al.*, 1996). Furthermore, full-thickness intestinal biopsy or material from intestinal resections can be made at exploratory surgery. Necropsy is the most exhaustive method for diagnosing IBD, as all intestinal segments can be evaluated, grossly and histologically, facilitating exclusion of differential diagnoses.

1.3 Equine eosinophilic gastroenteritis (EEG)

Idiopathic inflammatory enteropathy in horses, characterised by infiltration of eosinophils, was first named as chronic eosinophilic gastroenteritis (Pass & Bolton, 1982) and later as eosinophilic granulomatosis (Lindberg *et al.*, 1985). Gross lesions can be seen distributed intermittently or segmentally in any part of the gastrointestinal tract (Schumacher *et al.*, 2000). The afflicted tissue may display macroscopic mucosal ulcerations, serosal plaques and circumferential band-formed areas of inflammation. Circumferential mural bands have been

suggested to be pathognomonic of EEG (Scott *et al.*, 1999) and are composed of inflammatory cells and fibrosis. Depending on the distribution of the gross lesions in the intestine, numerous other terms have been used, i.e. eosinophilic enterocolitis (Gibson & Alders, 1987), idiopathic focal eosinophilic enteritis (IFEE) (Southwood *et al.*, 2000), segmental eosinophilic colitis (Edwards *et al.*, 2000) or diffuse eosinophilic enteritis (Mäkinen *et al.*, 2008). In accordance with Schumacher *et al*. (2000), intestinal eosinophilic equine IBD will subsequently be referred to as equine eosinophilic gastroenteritis / enterocolitis (EEG). IFEE has been suggested to be a sub-entity of EEG (Archer *et al.*, 2006; Proudman & Kipar, 2006) as the described horses have the characteristic mural bands and a more acute clinical presentation than other EEG-afflicted horses. The mural bands may cause constriction of the intestinal lumen resulting in luminal obstruction, proximal distention of the intestine and acute clinical presentation (Archer *et al.*, 2006; Perez Olmos *et al.*, 2006; Scott *et al.*, 1999).

In EEG, intestinal tissue eosinophilia is histologically accompanied by lymphocytes, macrophages, plasma cells and mast cells (Lindberg *et al.*, 1996; Pass & Bolton, 1982), which are present in the mucosa, submucosa and may be transmural. The lymphocytic infiltrates in EEG lesions have been shown to be dominated by T cells (Bosseler *et al.*, 2012; Mäkinen *et al.*, 2008). Other features of the entity are microscopic ulcerations of the mucosa, partial blunting of villi, fibrosis and occasional goblet cell hyperplasia (Lindberg *et al.*, 1996; Lindberg *et al.*, 1985). Eosinophilic granulomas with a central core of degenerate eosinophils, surrounded by epithelioid and giant cells often occur.

Tissue eosinophilia and eosinophilic granulomas outside the intestinal tract is not uncommon. Eosinophilic infiltrates in the pancreas, liver, mesenteric lymph nodes, skin and lung are all described (La Perle *et al.*, 1998; Hillyer & Mair, 1992; Sanford, 1989; Lindberg *et al.*, 1985; Nimmo Wilkie *et al.*, 1985; Pass & Bolton, 1982). In one described case, severe lung involvement resulted in secondary cardiac failure (Pucheu-Haston & Del Piero, 2013). The terms hyper-eosinophilic syndrome (Schumacher *et al.*, 1991) and multisystemic eosinophilic epitheliotropic disease (MEED) (Nimmo Wilkie *et al.*, 1985) have been used to encompass these additional features. MEED is the more accepted term. Notably, the term can also include horses that display eosinophilic lesions in several organs without lesions in the intestinal tract (Nimmo Wilkie *et al.*, 1985). Two horse afflicted by chronic idiopathic enteritis and tissue basophilia have been described (Gibson & Alders, 1987; Pass *et al.*, 1984). The observed inflammation shared features with EEG including infiltration of eosinophils, lymphocytes, plasma cells and macrophages, in addition to the

observed basophils. In the review of equine IBD by Schumacher *et al.* (2000), both these horses were included in the MEED classification.

1.3.1 Eosinophilic gastrointestinal diseases in other species

In humans, the condition idiopathic eosinophilic gastrointestinal disorder (EGID) is characterized by accumulation of eosinophilia in any segment of the intestine, associated with abdominal pain and failure to thrive (Rothenberg, 2004). The disease is suggested to be caused by environmental factors, such as food antigens, in genetically susceptible patients (Jawairia *et al.*, 2012; Zuo & Rothenberg, 2007). Eotaxin, IL-4, IL-5 and IL-13 are increased in several of the disorders included in EGID and delayed T_H2-type immune responses are considered involved. Even if the disease shares many features, it is not considered an entity in the human IBD complex. EGID may precede IBD in some patients (Mutalib *et al.*, 2015), suggesting a possible link between the pathogenesis of the two diseases.

Cattle also have been noted to suffer from idiopathic eosinophilic enteritis (Fushimi *et al.*, 2015; Cebra *et al.*, 1998). Antibiotic or antihelmintic treatment was not effective, whereas glucocorticoid treatment induced remission. Among small companion animals, both cats and dogs can be affected by eosinophilic enteritis (Sattasathuchana & Steiner, 2014; Jergens *et al.*, 1992). A cat held in a closed facility developed eosinophilic IBD, which may suggest a non-infectious aetiology (Griffin & Meunier, 1990). In dogs, oxidative stress and dysbiosis were thought to be the underlying cause for developing disease (Minamoto *et al.*, 2015).

1.4 Equine granulomatous enteritis (EGE)

Granulomatous diseases can occur in various equine organs, such as the alimentary tract, respiratory tract, skin, or spread systemically (Costa, 2016). If an antigen is not eliminated and/or a continuous inflammatory stimulus occurs, macrophages can cluster together at the site to form granulomas. When macrophages cluster, T cells also accumulate at the site and recruit further effector cells, such as eosinophils (Co *et al.*, 2004).

Equine granulomatous enteritis (EGE) was first described in the 1970s (Merritt *et al.*, 1976; Cimprich, 1974). The inflammation is characterised by infiltration of macrophages, lymphocytes, epithelioid and giant cells that form granulomas or diffuse granulomatous infiltrates. The granulomas are non-necrotizing and are occasionally delineated by fibroblasts. Lesions are found in the mucosa, submucosa or as transmural infiltrates (Lindberg, 1984; Cimprich, 1974). Villous atrophy, crypt hyperplasia, lymphangiectasis, oedema, mucosal

erosion and/or ulceration are common features. When the EGE-afflicted intestinal mucosa was observed using electron microscopy, shortened and widened villi, some fused, occurring as ridges, or a complete lack of villous projections was revealed (Lindberg & Karlsson, 1985). Granulomatous lesions are, apart from the intestine, common in mesenteric lymph nodes and may also be seen in the liver, spleen, pancreas, kidney, endometrium, synovial membranes and skin (Lindberg, 1984; Cimprich, 1974).

Gross lesions are more often diffuse in EGE than the lesions observed in EEG (Schumacher *et al.*, 2000). The lesions are mainly present in the small intestine, but occasionally also in the large intestine (Lindberg, 1984; Cimprich, 1974). The mucosa is often grossly altered, displaying thick intestinal folds, sometimes with a corrugated to cobblestone appearance or a flattened surface (Lindberg, 1984; Meuten *et al.*, 1978). At necropsy, apparent dissemination to regional lymph nodes and a general thickening of the intestinal wall are described (Lindberg, 1984; Roberts & Kelly, 1980; Meuten *et al.*, 1978).

1.4.1 Granulomatous gastrointestinal diseases in other species

In humans, idiopathic granulomatous colitis is referred to as Crohn's disease and is one of the two major entities in human IBD. It shares features with EGE and also Johne's disease in cattle (Greenstein, 2003). Johne's disease is caused by *Mycobacterium avium* subspecies *paratuberculosis*, but it is heavily debated whether mycobacteria may be a causative agent in Crohn's disease. Instead, Crohn's disease is thought to be driven by an exaggerated T_H1 and T_H17 response, which overwhelms the anti-inflammatory effects of T_{REGS} (Brand, 2009).

Young dogs of the Boxer or French bulldog breed may present with granulomatous colitis / histiocytic ulcerative colitis (Churcher & Watson, 1997). Microscopically, periodic acid Schiff (PAS)-positive macrophages are characteristic and infiltration of T cells, IgG plasma cells, MHC II-expressing cells and granulocytes appear (German *et al.*, 2000). This granulomatous disease is thought to be either driven or exaggerated by invasive *Escherichia coli*, and antibiotic treatment can induce remission (Mansfield *et al.*, 2009). In cats, a granulomatous form of IBD exists, but is rare (Day & Hall, 2008). *Mycobacterium paratuberculosis* has been detected by PCR in intestinal material from both cats and dogs (KuKanich *et al.*, 2013). However, positive PCR results did not correlate to alimentary illness and animals diagnosed with IBD were negative for the bacterium.

1.5 Lymphoplasmacytic enterocolitis (LPE)

Lymphoplasmacytic enterocolitis (LPE) is rarely documented in horses (Schumacher *et al.*, 2000). In LPE, the lamina propria is infiltrated by lymphocytes and plasma cells, which occasionally also are noted in the submucosa. Thickened villi (MacAllister, 1990) and villous blunting (Kemper *et al.*, 2000) or atrophy (Clark, 1988) are reported features. Lesions are in general in the small intestine and occasionally also in the large intestine (Kemper *et al.*, 2000). Grossly, a thickened intestinal wall can be present, as a result of submucosal oedema and sometimes muscle hypertrophy (Kemper *et al.*, 2000; MacAllister, 1990). The mucosa may be oedematous, segmentally to diffusely congested, and multifocally ulcerated. Mesenteric lymph nodes may be oedematous (MacAllister, 1990) or displaying lymphoid hyperplasia (Clark, 1988).

1.5.1 Lymphoplasmacytic gastrointestinal diseases in other species

In humans, no specific lymphoplasmacytic entity is referred to in the IBD complex. However in ulcerative colitis, which is part of human IBD, lymphocytes and plasma cells increase in the basal lamina propria. The characteristic lesions involve architectural alterations of the mucosa and infiltration of neutrophils. Previously, the disease was thought to be T_H2 driven, but is now called an atypical T_H2 response where T_H17 play a role in disease development (Roda *et al.*, 2011; Xavier & Podolsky, 2007). In human microscopic colitis, primarily lymphocytes infiltrate the lamina propria and epithelium for no known reason, causing diarrhoea (Mellander *et al.*, 2016). The disorder is generally not considered an entity of human IBD, although some investigators have suggested its inclusion (Jegadeesan *et al.*, 2013) and some patients show both microscopic colitis and IBD (Mellander *et al.*, 2016).

For cats and dogs, the lymphoplasmacytic IBD is the dominant entity in the disease complex (Jergens *et al.*, 1992). In the lymphocytic infiltration of dogs, T_H1, but not T_H17, are suggested to contribute to the lesions, and T_{REGS} are significantly lowered (Maeda *et al.*, 2015; Schmitz *et al.*, 2012; Ridyard *et al.*, 2002). For cats, the number of T cells was not significantly different in healthy cats compared with cats affected by IBD, but lamina propria CD8 positive cells decreased in IBD-afflicted cats and MHC II-expressing cells increased (Waly *et al.*, 2004).

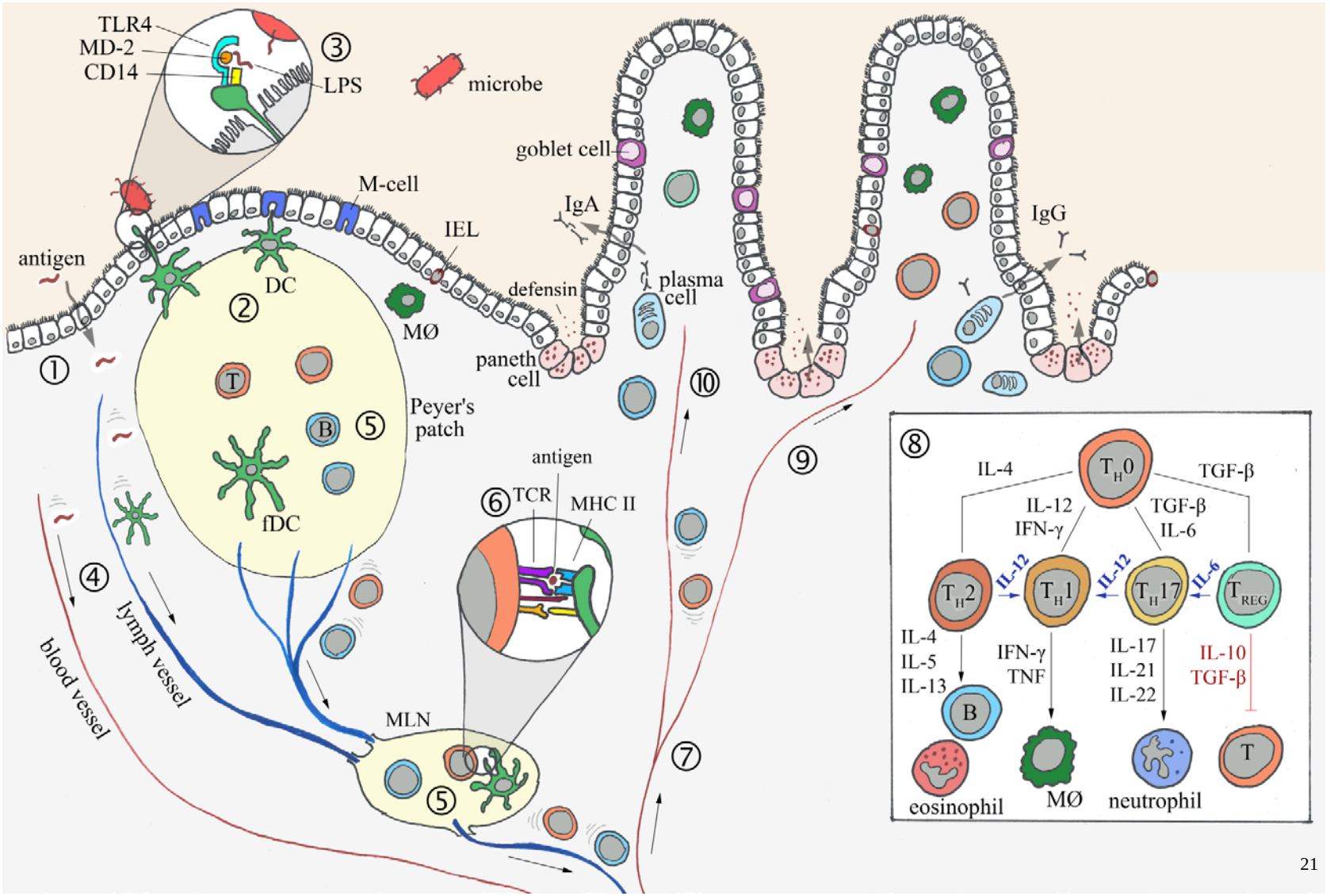
1.6 General aspects of equine mucosal immunology

For a deeper understanding of the mechanism underlying the various IBD lesions, further knowledge of equine intestinal immune system is needed. Equine mucosal immunology has mainly been studied in the respiratory tract, while less is known of the intestine. The gut-associated lymphoid tissue (GALT) is present in both the small and large intestine of the horse as lymphoid follicles, Peyer's patches, diffuse lymphoid infiltrates of the lamina propria and mesenteric lymph nodes. Lymphoid and myeloid cells, as well as eosinophils, are found throughout the gastrointestinal tract of healthy individuals, whereas neutrophils are scarce and basophils are rarely seen. Mast cells are present, but not routinely stained or immunolabelled for confirmation. A general view of the intestinal immune system with emphasis on the parameters considered in the present thesis is summarised in Fig. 1.

Figure 1.

A simplified illustration of intestinal immune reactions when encountering an antigen. ①When an antigen breaches the mucosal epithelial barrier resident innate immune cells excrete cytokines and chemokines, for further recruitment of innate immune cells. Depending on the type of antigen, neutrophils and eosinophils are attracted, ultimately releasing their antimicrobial molecules by degranulation.② Antigen can be recognised by dendritic cells in the subepithelial dome, being delivered to the lamina propria by transcytosis through M-cells or by sampling from the intestinal lumen. ③ Antigens are recognized by innate immune cells through pattern recognition receptors that bind conserved ligands expressed by pathogens, such as lipopolysaccharide (LPS) on Gram-negative bacteria. Binding of LPS to TLR4 and the co-receptors CD14 and MD-2 triggers an intracellular signalling cascade resulting in gene activation with cytokine and chemokine production. ④ Dendritic cells present antigen to cells of the Peyer's patch or migrate to a mesenteric lymph node. Lymphatic vessels transport antigen to the mesenteric lymph nodes either as free antigen or as material engulfed in dendritic cells. ⑤ In the GALT (Peyer's patch or lymph node) antigen fragments are presented to naïve T cells on MHC II by dendritic cells. ⑥ Interaction between MHC II and the T cell receptor stimulates up-regulation of costimulatory molecules and activation of T cells under influence of cytokines produced by the antigen presenting cell. Activated T cells bind to B cells presenting the same antigen. T cell-produced cytokines drive B cell proliferation and immunoglobulin class-switching, and the binding sustains the growth of the T cells. Activated T cells and B cells leave the GALT via lymphatic vessels and enter the systemic blood circulation. ⑦ From the blood circulation, activated lymphocytes home to the gut via high endothelial venules with B cells differentiating to plasma cells and T cells affecting immune cells in the lamina propria. ⑧ The type of T helper cell response is influenced by the overall cytokine milieu and promotes an inflammatory reaction or immune tolerance. ⑨ At inflammation, the T cells exert their effects excreting cytokines and chemokines, accumulating innate cells ultimately forming granulomas. ⑩ If the antigen encountered is recognised as harmless, homeostasis prevails. In homeostasis, induced regulatory T cells produce IL-10 and TGF-β favouring IgA production.

For simplicity, the mucus layers are not illustrated. Abbreviations in the figure: B = B cell; DC = dendritic cell; fDC = follicular dendritic cell; IEL = intraepithelial lymphocytes; Ig = immunoglobulin; MØ = macrophage; MLN = mesenteric lymph node; T = T cell; TCR = T cell receptor; T_H = T helper cells; T_H0 = naïve T cell


TLR4
MD-2
CD14
③
LPS
microbe
goblet cell
M-cell
IgA
IEL
IgG
antigen
DC
②
defensin
plasma cell
MØ
paneth cell
①
T
B
⑤
Peyer's patch
⑩
⑨
antigen
⑥ TCR
MHC II
fDC
④
lymph vessel
blood vessel
MLN
⑤
⑦
⑧
IL-4
T_H0
TGF-β
IL-12
IFN-γ
TGF-β
IL-6
T_H2
IL-12
T_H1
IL-12
T_H17
IL-6
T_{REG}
IL-4
IL-5
IL-13
IFN-γ
TNF
IL-17
IL-21
IL-22
IL-10
TGF-β
B
T
eosinophil
MØ
neutrophil

1.6.1 Pattern recognition receptors and cytokines

Pattern recognition receptors (PRRs) are molecules expressed by innate immune cell and recognise conserved motifs on antigens (pathogen-associated molecular patterns; PAMPs) or structures on dying cells (damage-associated molecular patterns; DAMPs). The Toll-like receptor group (TLRs) recognises extracellular or endosomal antigens and there are 12 known TLRs in human (Akira *et al.*, 2006). The first TLR described in humans is now known as TLR4 (Medzhitov *et al.*, 1997). TLR4 is expressed on macrophages as well as dendritic cells and recognises lipopolysaccharide (LPS) from bacteria, but also structures from fungi, parasites and virus (Akira *et al.*, 2006). In the TLR4 receptor complex, CD14 binds LPS and subsequently associates with the TLR4 and MD-2 dimer (Guha & Mackman, 2001). In the healthy human intestinal epithelium, TLR4 expression is barely detected, but the expression is increased in IBD (Cario & Podolsky, 2000).

In horses, TLR4 is expressed in healthy lung tissue and is increased after infusion with LPS (Singh Suri *et al.*, 2006) and in respiratory airway obstruction (Berndt *et al.*, 2007). The receptor is expressed in the alveolar macrophages, endothelium and bronchial epithelium in lungs and in macrophages of lung vessels (Singh Suri *et al.*, 2006). TLR4 can also be induced in lamellar tissue of the horse's hoof after induction of hyperinsulinaemia (de Laat *et al.*, 2014). In the endometrium, TLR4 is expressed constitutively, and is not affected by insemination (Nash *et al.*, 2010). Due to its increased expression in human IBD and the known expression in horses, TLR4 was included in the gene expression analysis of equine IBD horses, in the present thesis.

Cytoplasmic receptors including the NOD-like receptors (NLRs) complement the TLRs to detect intracellular antigen. One NLRs extensively studied in human IBD is the NOD2 receptor that recognises muramyl dipeptide, a degradation production from most bacteria (Girardin *et al.*, 2003). NOD2 is highly conserved between species and in horses the gene is located on chromosome 3 (Boyle *et al.*, 2013). All TLRs and NOD2 signal via the transcription factor nuclear factor-kappaB (NF-κB) (Hu *et al.*, 2010; Kawai & Akira, 2007). The activation of NF-κB leads to its translocation from the cytoplasm into the nucleus, where it activates the cell and, amongst other effects, increases the transcription of genes encoding pro-inflammatory cytokines.

Cytokines are small proteins that function as signal substances between different cells and can be organised into categories; lymphokines, monokines, chemokines, interferons and interleukins. Cytokines can be seen as

immunomodulating agents that regulate both innate and adaptive immune responses and "communicate" with cells of other organ systems. In the only previous study of cytokine expression in equine IBD, expression of the pro-inflammatory cytokine TNFα was increased in IBD-afflicted horses, whereas expression of IL-2, IL-4, IL-5, IL-10 and TGF-β was the same as in healthy horses. In accordance, TNFα is increased in human IBD and chosen as a target for immunotherapy (Ahluwalia, 2012). Other cytokines of interest in human IBD are IL-17A and IL-23, possibly important for tissue destruction in the disease (Neurath, 2014). These T_H17-associated cytokines are also proposed, but not proven, to be involved in the development of feline and canine IBD (Allenspach, 2011). The present thesis focuses on the expression of the cytokines IFN-α, IL-12, IL-17 and IL-23.

IFN-α and IFN-γ

Interferons (IFN) are commonly divided into type I IFNs (including IFN-α, -β, -ε, -ω, -κ, -ν, -δ, -τ and -ζ), type II IFNs (IFN-γ) and type III IFNs (IFN-λ1, -λ2, -λ3). In horses, an additional type I IFN has been describe, IFN-μ, which is inducible in equine PBMC (Detournay *et al.*, 2013). Type I IFNs are most known for their interference with viral infections. IFN-α production increases at viral infection, but can also be increased in autoimmune diseases (Ning *et al.*, 2011). In horses, IFN-α is e.g. induced in equine tracheal cells after equine herpes virus and in blood after equine influenza virus infection (Quinlivan *et al.*, 2007).

Type II IFN, i.e. IFN-γ, is associated with T helper cell (T_H) 1 responses. Human IBD patients have increased levels of IFN-γ in their blood (Singh *et al.*, 2016) and IFN-γ positive cells are increased in the lamina propria of one entity of human IBD (Crohn's disease) (Camoglio *et al.*, 1998). In horses, IFN-γ is e.g. increased in blood after a *Toxoplasma gondii* infection and in bronchoalveolar lavage fluid in horses with inflammatory airway disease (IAD) (Do Carmo *et al.*, 2015; Richard *et al.*, 2014). At a pulmonary *Rhodoccoccus equi* infection in horses IFN-γ is upregulated, and a local T_H1 immune response in the lungs is suggested to clear the pathogen (Hines *et al.*, 2003).

IL-12 and IL-23

IL-12 and IL-23 are heterodimeric cytokines and share one subunit (p40) whereas the other subunit is unique (IL-12; p35 and IL-23; p19). Both IL-12 and IL-23 are regarded as pro-inflammatory cytokines (Vignali & Kuchroo, 2012) and can be secreted by macrophages and dendritic cells. IL-12 directs naïve T cells to a T_H1 response, whereas IL-23 stabilizes the T_H17 subset (Wu *et al.*, 2013). Previously, IL-12 was thought to have a pivotal role in the

progression of chronic intestinal inflammations and other immune-mediated inflammations as the diseases were ameliorated by antibodies against IL-12p40 (Neurath *et al.*, 1995). Later, after the discovery of IL-23, some of the effects of neutralising the p40 subunit were concluded to be due to regulation of IL-23. In IBD, it has been suggested that IL-12 regulates the systemic inflammation, whilst IL-23 regulates the intestinal inflammation (Uhlig *et al.*, 2006). In horses, IL-23 has been detected in chronically inflamed eyes using immunohistochemistry (Regan *et al.*, 2012). Additionally, IL-23 is expressed in IAD (Hughes *et al.*, 2011) and IL-23 rather than IL-12 is expressed in response to *Rhodococcus equi* infection (Nerren *et al.*, 2009).

IL-17A

There are six members in the IL-17 family (IL-17A-F) where IL-17A and the closely related IL-17F are the most studied. IL-17A is not exclusively produced by T_H17 cells, but this T cell subset is the high producer (Jin & Dong, 2013). Other cells that experimentally or *in vivo* can secrete IL-17A are γδ T cells and invariant natural killer T cells (Cua & Tato, 2010). In a study of the kinetics of IL-17A in disease, it was concluded that in an acute disease (*Candidia albicans* infection), as well as in a chronic disease (EAE), IL-17A was first produced by γδ T cells and the production was gradually overtaken by T_H17 cells (Hirota *et al.*, 2011). Interestingly, in the chronic disease, but not the acute disease, IL-17A production decreased in favour of a T_H1 phenotype and these cells were "ex-IL-17A"-producing cells.

In horses, IL-17A has been sequenced and shows 84% identity with human IL-17A (Tompkins *et al.*, 2010). IL-17A is increased in horses suffering from chronic respiratory airway obstruction (RAO) when exposed to triggering environmental factors (Ainsworth *et al.*, 2007; Debrue *et al.*, 2005), and in chronically inflamed eyes (Regan *et al.*, 2012), but not in osteoarthritis (Kamm *et al.*, 2010).

1.6.2 T cell subsets

Cytokines produced at initiation of an immune response profile the ensuing T cell response. Traditionally, αβ T cells are divided into cytotoxic T cells and T helper cells and regulatory T cells. The general view is that intracellular bacteria trigger IL-12 production in dendritic cells leading to a T_H1 response with IFN-γ production and enhanced phagocytosis by macrophages. Helminths trigger production of IL-4 inducing T_H2 responses with IL-4, IL-5 and IL-13 production, as well as recruitment of eosinophils. T_H2 cytokines stimulate class-switching and differentiation of B cells to plasma cells in the lamina propria. Extracellular bacteria trigger TGF-β and IL-6 production in dendritic

cells, thereby inducing a T_H17 response with production of IL-17A, IL-17F and IL-22. TGF-β can also induce T_{REGS}, for instance when encountering antigen in the presence of retinoic acid (Xiao *et al.*, 2008). As previously mentioned, the T_H17 phenotype is stabilized by IL-23 and the cells can directly and indirectly attract neutrophils to the site of inflammation (Pelletier *et al.*, 2010; Fossiez *et al.*, 1996). Previously it was thought that once a T_H cell had gained a phenotype it was set, however more recent studies show that T_H cells demonstrate plasticity between subtypes (Ueno *et al.*, 2015). IL-12 can drive both T_H2 and T_H17 cells towards a T_H1 phenotype, whereas IL-6 can drive T_{REGS} to a T_H17 phenotype. γδ T cells share features with innate cells, being more abundant in tissue than in circulation and being important in the first line of defence against infections in the intestine (Ismail *et al.*, 2011). These cells recognise other types of antigens than those recognized by αβ T cells and are not completely dependent of MHC presentation of the antigens (Chien & Konigshofer, 2007). Interestingly, γδ T cells in the intestinal mucosa increase in Crohn's disease, and are suggested to contribute to the pathogenesis (Kanazawa *et al.*, 2001) whereas this is not the case in canine IBD (German *et al.*, 2001).

In horses, subpopulations of T cells are detected by aid of monoclonal antibodies directed to equine CD2, CD3, CD4, CD5, CD8 using flow-cytometry or immunolabeling of frozen tissue sections (Platt *et al.*, 2010; McClure *et al.*, 2001; Tschetter *et al.*, 1998). Anti-CD3 is routinely used to label T cells in formalin-fixed sections and immunohistochemical labelling of T_{REGS} has been described (Steinbach *et al.*, 2009). A protocol for discrimination of equine T_H1, T_H2 and T_{REGS} in flow-cytometry by using IL-10, IL-4 and IFN-γ production has also been elaborated (Wagner *et al.*, 2010). Equine T_{REGS} seem to be heterogeneous as they can express either or both the cytokines IL-10 and IFN-γ (Robbin *et al.*, 2011). T_{REGS} were detected by an anti-FoxP3 antibody, complemented by the labelling of CD4 and CD25 (Hamza *et al.*, 2011). T_H17 cells have been identified by detection of IL-17 and IL-23 in formalin-fixed inflamed uvea (Regan *et al.*, 2012). Labelling of CD8 γδ T cells has been performed on several tissue including intestine and IEL (Tschetter *et al.*, 1998). As T cells drive the inflammation in IBD in several species and T_{REGS} are supposed to dampen this reaction both these cell types were included in the present analysis of equine IBD. Additionally, T_H1 and T_H17 cells are involved in the development of human IBD and therefore the expression of IL-12p40, IL-17A and IL-23p19 was studied in the present material.

1.6.3 B cells and immunoglobulins

The B cell carries immunoglobulin as their B cell receptor, which can be secreted as antibodies by plasma cells. Dependent on the heavy chain, various subclasses of immunoglobulins are formed, namely IgA, IgD, IgE, IgG or IgM. IgA is the dominant immunoglobulin at mucosal surfaces and plays a key role in their immune defence (Lewis *et al.*, 2010). The serum levels of IgA are low, and in horses they have been described to decrease during intense training (Souza *et al.*, 2010). Even if IgD has been found in horses, its function remains unknown. In humans, IgD can be excreted into the respiratory tract lumen, binding bacteria or be bound to granulocytes in the blood, probably functioning as a pattern recognition receptor (Chen & Cerutti, 2011; Edholm *et al.*, 2011). IgE levels are low compared with other Ig subclasses and vary between horses (Wagner, 2009). IgE mediates type I hypersensitivity in skin allergy of horses (Wagner *et al.*, 2006) and increases in insect bite hypersensitivity and at parasite infection (Schaffartzik *et al.*, 2012; Wagner, 2009). Equine IgG can be further subdivided into subclasses; IgG1 (formerly IgGa), IgG2 (IgGa) IgG3 (IgG(T)), IgG4 (IgGb), IgG5 (IgG(T)), IgG6 (IgGc) and IgG7 (IgG(T)) (Walther *et al.*, 2015; Wagner *et al.*, 2004). IgG is induced in secondary responses, and is important in the response against many pathogens. It is also suggested that IgG(T) can bind to mast cells and contribute to skin allergy in horses (Wagner *et al.*, 2006). IgM is the first immunoglobulin to appear in phylogenetic development. It is produced initially by foetuses and is responsible for primary immune responses. IgM deficiency has been documented in horses, primarily foals or horses with neoplastic disease (Thomas *et al.*, 2005; Perkins *et al.*, 2003). The role of B cells in equine IBD is not clear and therefore their distribution in the jejunum was determined by immunohistochemistry and morphometric analysis in the present thesis.

1.7 Suggested aetiologies of IBD

The aetiology of IBD is unknown. Findings in human patients with IBD indicate an immune-mediated disease, which has been evaluated in mouse models of IBD and successively also suggested for veterinary species. The immune system in the intestine needs to be both reactive towards pathogens and tolerant towards the beneficial, commensal flora (Goldszmid & Trinchieri, 2012). If the balance between responsiveness and non-responsiveness is faulty, the intestine is at risk of severe disease from ignoring pathogens or invoking a deleterious immune reaction towards harmless antigens. This balance is thought to be disrupted in IBD, resulting in an exaggerated, inappropriate immune response (Xavier & Podolsky, 2007). Possible causes and contributing

factors to the exaggerated immune response have been proposed, but the aetiology is still obscure. In humans, a higher risk of developing IBD was seen in a population consuming drinking water with high iron content (Aamodt *et al.*, 2008). Furthermore, a high prevalence of IBD is found in westernised countries, with high living standards (Cosnes *et al.*, 2011). Whether the risk is associated with antimicrobial usage, pollution, diet changes or other factors is not fully understood. Two of the most compelling pieces of evidence against an infectious agent as the sole cause of IBD are the sporadic appearance of the disease and the positive treatment outcome achieved with immunosuppressive drugs. However, it is still possible that microorganisms in conjunction with environmental factors and a genetic predisposition can play major roles in the pathogenesis of IBD (Hansen *et al.*, 2010; Podolsky, 2002).

In the search for an infectious agent in equine IBD, transmission studies were performed using equine IBD-affected tissue. However, in inoculated animals (rabbits, guinea pigs and one healthy young horse) no lesions could be detected (Pass & Bolton, 1982; Merritt *et al.*, 1977). Additionally, no particular environmental factor, such as management routine, feeding regimes or medication has successfully been linked to the disease (Lindberg *et al.*, 1985). However, certain geographical areas have been associated with increased risk for eosinophilic equine IBD (Archer *et al.*, 2014). IBD has also been observed more frequently in horses during late spring and summer (Southwood *et al.*, 2000; Nimmo Wilkie *et al.*, 1985) or fall (Archer *et al.*, 2014), suggesting a seasonal factor in the pathogenesis. These environmental factors could be linked to toxins (Woods *et al.*, 1992), diet, allergic substances or microorganisms such as parasites. One case of equine IBD (EGE) has been linked to the ingestion of hairy vetch (Anderson & Divers, 1983).

1.7.1 Hypersensitivity reaction

Two types of hypersensitivity reactions (type I and type IV) have been suggested to be involved in the development of equine IBD. Hypersensitivity is an inappropriate, exaggerated immune reactivity and is classified into four major categories with additional sub-categories (Uzzaman & Cho, 2012). In an immediate (type I) hypersensitivity reaction, mast cells interact with an antigen on their Fc-IgE receptor and cross-linking induces release of pre-formed mediators, such as histamine and prostaglandin D_2. The release of mediators causes acute reactions and attracts immune cells to the site and newly synthesised cytokines can prolong the inflammatory response. A delayed (type IV) hypersensitivity is mediated by persistent activation of T cells. Different effector cells are recruited dependent of the T_H subset that initiates the reaction, i.e. T_H1 and macrophages are seen in type IVa hypersensitivity, T_H2 and

eosinophils in type IVb, cytotoxic T cells in type IVc and T cells and neutrophils in type IVd (Pichler, 2003). T_H17 cells are also thought to contribute to delayed hypersensitivity, mainly to the type IVd reaction (Iwakura *et al.*, 2008). A link between type I and type IVb is suggested as T_H2 cells induce IgE production (Uzzaman & Cho, 2012).

Several authors have suggested hypersensitivity reactions as a plausible mechanism in the development of equine IBD (Sanford, 1989; Lindberg *et al*, 1985; Lindberg, 1984; Roberts, 1983; Pass & Bolton, 1982). Aluminium was suggested to have invoked an immediate hypersensitive inflammation in a cluster of IBD horses (Fogarty *et al.*, 1998). The mechanism was thought to be through alteration of the barrier function, activating dendritic cells and macrophages and increasing cytokine expression, including IL-17A and CXCL-8 (Pineton de Chambrun *et al.*, 2014; Lerner, 2007). However, this report has been disputed as to whether the disease was IBD as lesions were non-classical and debris from parasites as well as iron-containing cells could have given false positive readings for aluminium (Collery *et al.*, 1999). Mast cells are infrequently seen in eosinophilic equine IBD, and therefore a role for an immediate hypersensitivity is not agreed upon (Mäkinen *et al.*, 2008; Lindberg *et al.*, 1985). However recurrent flares of an immediate reaction in combination with delayed responses have been suggested for EEG. In eosinophilic equine IBD, vasculitis is a common feature (Schumacher *et al.*, 2000) and could be driven by immune complex deposition in small vessels in the inflamed intestine (Lindberg, 1985), hence possibly a type III hypersensitivity.

The granuloma formation in human IBD is sometimes referred to as a delayed hypersensitivity reaction (Co *et al.*, 2004) and both canine and feline IBD has been suggested to be mediated by hyper-reactive innate immune cells (Allenspach, 2011). IBD can be challenging to distinguish from dietary hypersensitivity in both humans and small companion animals (Vojdani & Perlmutter, 2013; Mandigers & German, 2010). Little is known of food hypersensitivity in horses. Celiac disease is a cell-mediated hypersensitivity reaction to gluten (Vojdani *et al.*, 2008) that could possibly affect horses. A few cases of equine IBD have had anti-gluten antibodies, although this was also recognised in healthy controls fed a high-gluten diet (van der Kolk *et al.*, 2012). An indirect effect of diet can be a change in the intestinal microbiota, which creates a dysbiosis and provokes an inflammation.

1.7.2 Microbiological agents

In humans, a dysbiosis of the intestinal microbiota has been suggested to drive IBD (Hold *et al.*, 2014). The same theory has been suggested for colitis in

horses (Costa *et al.*, 2012) and for IBD in dogs (Allenspach *et al.*, 2010). Alternatively, the dysbiosis can be a consequence of intestinal inflammation. Therefore an altered microflora as a primary cause of IBD has been debated in both humans (Sartor & Mazmanian, 2012) and horses (Lindberg, 1984). Even if the dysbiosis is not causative, the microbiota can still serve as a factor in the pathogenesis, by containing pathogens or by a defective host immune response towards commensal bacteria.

Mycobacterial infections have been a suggested cause or contributor to the development of IBD in horses and humans (Naser *et al.*, 2014; Schumacher *et al.*, 2000). Horses are susceptible to *Mycobacterium avium* subspecies *paratuberculosis* experimentally (Larsen *et al.*, 1972), but the bacterium has not been isolated from clinical cases. Infection with *Mycobacterium avium* subspecies *avium* has been recorded in equine intestines (Cline *et al.*, 1991; Buergelt *et al.*, 1988; Platt, 1986; Merritt *et al.*, 1975; Cimprich, 1974) resulting in lesion indistinguishable from granulomatous equine IBD. However, a true causal relationship has not been confirmed in Swedish EGE cases, as no intracellular bacteria was found in Ziehl Neelson nor auramine-rhodamine or other microbial stains (Lindberg, 1985), concurring with findings by Cimprich (1974). Culturing mycobacteria is difficult and time consuming. Therefore the exclusion of a mycobacterial infection is difficult. In human, some genes are however associated with an increased susceptibility for IBD and also an increased susceptibility for mycobacterial infections (Jostins *et al.*, 2012). Even if it is not a conclusive finding, this dual genetic susceptibility could indicate an underlying genetic immune disturbance that increases the risk of a mycobacterial infection causing IBD.

Parasites are not a rare finding in equine intestines, even if continuous deworming is commonly performed (Höglund *et al.*, 1997). Cyathostomes are regarded to be the most important parasites in equids today (Stratford *et al.*, 2011; Love *et al.*, 1999). The parasite can be present in dewormed horses and give clinical disease when migrating *en masse* into the lumen of the intestine, resulting in protein losses even in adult horses (Love *et al.*, 1999). *Anoplocephala perfoliata* has also been suggested to be important, especially in horses presenting with signs of colic, and the infection is possibly underdiagnosed (Back *et al.*, 2013). Parasites have been suggested to be part of the induction of equine IBD (Cohen *et al.*, 1992; Church *et al.*, 1986; Platt, 1986; Breider *et al.*, 1985; Jasko & Roth, 1984) and can attract immune cells, such as eosinophils as well as other cell types, when invading the tissue (Rodríguez-Bertos *et al.*, 1999; Pearson *et al.*, 1993). In both *Anoplocephala* infection (Rodríguez-Bertos *et al.*, 1999) and eosinophilic equine IBD (Lindberg, 1985), lesions can be found at the ileocaecal junction. However, in

the several reports on eosinophilic equine IBD, parasites or remnants of parasites were rare findings (Mäkinen *et al.*, 2008; Archer *et al.*, 2006; Southwood *et al.*, 2000; Lindberg, 1985; Pass & Bolton, 1982). The lack of intralesional parasites could nevertheless be due to the eradication of the initiating parasites. In contrast to parasites as a cause of disease, a protective role for helminths has been suggested in experimental IBD (Ruyssers *et al.*, 2008). The helminths are thought to skew the immune response towards a T_H2 and T_{REG} response rather than a T_H1 driven response, which ameliorates the severity of disease.

Less explored microbial agents in the IBD context are fungi and viruses. Fungi have been demonstrated in eosinophilic lesion in horses with chronic enteritis (Morton *et al.*, 1991), but were absent in other studies of eosinophilic intestinal lesions (Mäkinen *et al.*, 2008; Gibson & Alders, 1987). Virus has been suggested as a possible aetiology where inflammatory lesions are noted in several organs including the intestine, even if virus have not been isolated in affected horses (Nimmo Wilkie *et al.*, 1985). In humans, measles virus and Epstein-Barr virus have been suggested to induce vascular changes and/or IBD lesions (Gehlert *et al.*, 2004; Uhlmann *et al.*, 2002; Wakefield *et al.*, 1993), but viruses are also present in healthy individuals and for measles, an aetiological role has been strongly challenged (Bustin, 2008; Davis & Bohlke, 2001).

1.7.3 Genetic associations

Even if contradictory opinions exist on breed predilection (Archer *et al.*, 2006; Kemper *et al.*, 2000; Platt, 1986), related horses have developed IBD (Sweeney *et al.*, 1986; Lindberg *et al.*, 1985). This finding suggests that heritability contributes to the disease development. For the human IBD entity Crohn's disease, candidate gens associated with intracellular bacterial processing and Paneth cells function have been found, whereas the entity ulcerative colitis has been linked to barrier dysfunctions (Lees *et al.*, 2011). In addition in both these entities, genes associated with the T_H17 and T_{REG} pathways, as well as PAMP recognition (*NOD2*) and genes associated with barrier function (*MUC19*) are found to be important (Khor *et al.*, 2011). TLR4 polymorphism is associated with human IBD, at least in Caucasians (Cheng *et al.*, 2015), as is the polymorphism in MHC II (Goyette *et al.*, 2015). These finding demonstrate the importance of both innate and adaptive immune reactivity in the development of IBD in genetically susceptible individuals.

2 Hypothesis and aims

Equine IBD is a disorder driven by exaggerated immune reactions induced by intestinal lumen antigens and environmental factors. The disorder is manifested as an imbalance in the cytokine milieu and the interplay between T cell subsets.

The general aim of the present thesis was to improve the understanding of equine mucosal immunology with specific emphasis on the intestine and the development of chronic idiopathic enteropathy in the horse.

The specific aims were to:

- Establish / optimise methods for gene expression analysis and for immune cell type identification in multiple intestinal segments.
- Determine the composition of immune cell populations in chronically inflamed intestinal tissue of different histological entities of equine IBD.
- Explore the usability of rectal biopsies for cytokine profiling.
- Evaluate cytokine gene expression in healthy and IBD afflicted tissues.

3 Comments on material and methods

This section includes an overview of the material and methods used in the present thesis. For further details, please see the individual papers (Paper I-IV).

3.1 Sampling considerations

3.1.1 Horses

Different groups of horses were used for sampling, depending on the material required. Healthy Standardbred horses at the Department of Clinical Sciences were used for either induction of endotoxaemia or collection of equine PBMC for *in vitro* stimulation (Paper II). Standardbred horses, stabled at a closed facility, were biopsied for control rectal biopsy material (Paper IV). All horses were considered normal, by daily monitoring and regular clinical evaluation by staff. Horses of various breed were sampled at an abattoir for full-thickness intestinal control samples (Paper I-III). Horses sampled at the abattoir had little known history, apart from the declaration of health given at slaughter. Even if episodes of gastrointestinal malaise could possibly have occurred, no signs of disease were noted at sampling, as evaluated by gross examination of carcasses and histological examination of the intestines. Hence, the material served as a good representation of the non-diseased equine population and was a suitable control material.

Equine IBD is a rare condition, thus sampling sufficient and appropriate material is challenging. The diseased intestinal material came from clinical cases admitted to the University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala or the Regional Animal Hospital, Strömsholm, Sweden. The material was either rectal biopsies sampled from horses of various breed (Paper III, IV) or archived material from Standardbred horses, sampled at necropsy (Paper I). For Paper I, the jejunum was chosen for analysis as IBD lesions are commonly found in the small intestine

(Schumacher *et al.*, 2000), and the jejunum was frequently afflicted by lesions in the archived material. The clinical cases used in the present thesis had clinical signs suggestive of IBD, displayed chronic enteropathy of unknown aetiology, and were all sporadic cases.

The archived material used in Paper I came from thoroughly examined clinical cases, with characteristic IBD lesions, sampled in the 1970s to 1980s. Horses suffering from severe chronic enteropathy had smaller chance for treatment at that time as prognosis was regarded as poor (Lindberg, 1984; Roberts & Kelly, 1980). This resulted in more cases for necropsy and confirmation of equine IBD. However, archived formalin-fixed material is not useable for transcript analysis, as RNA is destroyed and fresh material was therefore needed. Material for gene expression analysis was sampled from clinical cases in the 2010s. In recent time, horses are more readily treated, preferably with antihelmintics and corticosteroids and prognosis is considered fair to moderate (Kaikkonen *et al.*, 2014). The diagnosis of the material for gene expression was therefore based on the anamnesis, clinical findings and rectal biopsy histology, as none of the horses was necropsied.

Horses used in the present thesis were of all genders. Age has been shown to influence mucosal immunity in several species, especially in the young animal (Cesta, 2006). In equine neonates and foals, T_H1 responses dominate over T_H2 responses whereas the proportion of T_H1 cells and T_{REGS} are the same in foals as in adult horses (Wagner *et al.*, 2010). Increased age has been noted to decrease the number of T_{REGS} in blood (Robbin *et al.*, 2011). In geriatric horses over the age of 20 years, conflicting results have been published for immune cell populations, but IFN-γ expression increased with age (Hansen *et al.*, 2015). Equine IBD rarely affects animals younger than 1.5 years, and this age group was therefore excluded from the studies. The age of the sampled horses, for both the healthy and diseased horses, varied between 1.5 –24 years, which corresponds to the age span for published cases of equine IBD. The oldest horse was however excluded due to deviating reference genes (see 4.2 *Appropriate reference genes*), and the upper age range was thereby 20 years.

3.1.2 Blood sampling

Collection of PBMC from healthy horses was done through venous puncture and sampling blood in heparin tubes (Paper II). Heparin prevent clotting and have less effect on cells than EDTA-coated tubes (Brunialti *et al.*, 2002; Son *et al.*, 1996).

Jugular whole blood from horses with induced endotoxaemia was sampled through a percutaneous cathether into PAXgene tubes (Paper II). Using PAXgene tubes minimizes RNA degradation in blood after collection (Chai *et*

al., 2005). Horses were sampled before infusion of lipopolysaccharide, at the end of infusion and up until 48 hours after infusion.

3.1.3 Tissue sampling

The material used needed to be as fresh as possible, as intestinal material deteriorates quickly post-mortem (Lee *et al.*, 2005; Scheifele *et al.*, 1987) whereas GALT is less rapidly affected (Solarino *et al.*, 2009). The drawback in using rectal biopsy to diagnose equine IBD is that only approximately 35-50% of biopsies show inflammation (Barr, 2006; Lindberg *et al.*, 1996). On the other hand, the method is much less invasive than exploratory laparotomy and it is possible to perform on more cases and renders more tissue for evaluation than endoscopy. Therefore, rectal biopsy of both healthy and diseased horses was performed and samples were placed immediately after sampling in RNA*later* or formalin (Paper III, IV). The intention was to collect full-thickness samples at necropsy from horses that had received a preliminary diagnosis from rectal biopsies. However, clinically suspected cases of IBD identified for inclusion in the thesis work did not develop severe disease requiring euthanasia, or were unable to be transported to a necropsy facility or responded to treatment. Hence, archived diseased material was used for studies of full-thickness material (Paper I).

Full-thickness abattoir control material was collected within 45 minutes of killing. All control material was collected in the 2010s and preserved in formalin and RNA*later* (Paper I-III). The archived necropsy material was sampled in the 1970s and 1980s from clinically well-defined horses and sampling was done directly following euthanasia (Paper I) (Lindberg, 1985). Tissue from several organs and intestinal segments was fixed in formalin and archived in paraffin blocks.

All tissues sampled in RNA*later* were intended for transcript analysis (Paper II-IV). After collection, samples were kept in the solution for 24h at 4°C, thereafter at -80°C, to avoid mRNA degradation. Tissues sampled in formalin were left in the solution for 24h at room temperature. Thereafter, samples were processed routinely by step-wise dehydration in increasing alcohol concentration followed by paraffin embedding and sectioning.

3.2 Immunohistochemistry

All material used for IHC was formalin-fixed and indirect IHC was applied to locate immune cells *in situ* (Paper I, IV). An unlabelled primary antibody followed by a secondary labelled antibody and, when needed, signal amplification were used (Table 2). Formalin-fixed material requires heat-

treatment to recover the immunoreactivity of the epitope that was lost during fixation. This loss of immunoreactivity is suggested to occur due to either intermolecular cross-linkage (Metz *et al.*, 2004) or changes to the protein structures (Fowler *et al.*, 2011). Sections were placed in Tris-EDTA or citrate buffer (Table 2), and either microwaved at 92°C or autoclaved at 121°C for heat-induced epitope retrieval, as suggested by the manufacturer of the primary antibody or in-house evaluation. Another effect of the antigen-retrieval is a reduction in non-specific background staining (Shi *et al.*, 2011). H_20_2 in methanol (Streefkerk, 1972) reduced the background as well by eliminating endogenous peroxidase. To reduce the risk of background staining further, application of appropriate serum (serum from the animal species in which the secondary antibody was produced) was applied and in sections labelled for IgG an avidin-biotin blocking kit was applied to bind endogenous biotin (Paper I). Despite this, sections had background staining with the IgG protocol, in the submucosa, crypt abscesses and the necrotic centres of eosinophilic granuloma. Specific labelling was noted in areas that were not of interest to quantitate, namely IgG (vascular lumen), IgA (intestinal epithelium), IgM (apical portion of enterocytes) and MHC II (intestinal epithelium and vascular endothelium). None of these additionally labelled cells hindered the analyses in the present work as these areas were excluded from the quantitative evaluation in Paper I.

3.2.1 Primary antibodies

Labelling of T cells (Paper I, IV), T_{REGS} (Paper I, IV), B cells (Paper I), Ig-producing cells (Paper I) and antigen-presenting cells (Paper I) was performed on rectal and/or jejunal tissue. For detection of all T cells, the CD3 co-receptor complex is a useful epitope as it is associated with the TCR on all mature T cells (Owen *et al.*, 2012). CD3ε is one of the polypeptides in the co-receptor and antisera raised against it in other species cross-react with the equine epitope (Collins Kelley *et al.*, 1997). The CD3 antibodies labelled appropriate areas of the control material in Paper I and IV (Fig. 2, 3, 6A). To label T_{REGS}, an antibody raised against the transcription factor FoxP3 was used (Paper I and IV). FoxP3 is present in T cells and promotes a regulatory T cell profile (Hori *et al.*, 2003) or makes activated T cells hypo-responsive (Wang *et al.*, 2007). The clone FJK-16s, targeted at murine FoxP3, cross-reacts with the equine counterpart (Steinbach *et al.*, 2009). In the equine lymph node T_{REGS} were noted in the T cell-rich areas, but rarely in the lymphoid nodules (Fig. 2, 3).
In B cells, the transmembrane heterodimer CD79 is almost exclusively expressed by B cells, forming a complex with the B cell receptor. CD79 is present in a somewhat wider range of B cells than CD20. CD79-expression is

Table 2. *Specifications for the immunohistochemical indirect labelling*

	Primary Antibody					Secondary Antibody			
Target	Species	Type	Dilution (buffer)	Company	Clone	Species	Type	Dilution	Company
CD3 (Paper I)	Rabbit anti-human	Polyclonal	1:400 (1% BSA in TBS)	Dako	-	Goat anti-rabbit	EnVision$^+$	-	Dako
CD3ε (Paper IV)	Mouse anti-human	Monoclonal	1:400 (1% BSA in TBS)	Dako	F7.2.38	Goat anti-mouse	EnVision$^+$	-	Dako
CD20	Rabbit anti-human	Polyclonal	1:100 (1% BSA in TBS)	Thermo Fisher	-	Goat anti-rabbit	EnVision$^+$	-	Dako
FoxP3	Rat anti-mouse	Monoclonal	1:1 000 (1:6 biotin in TNB)	eBioscience	FJK-16s	Mouse anti-rat	Biotinylated	1:200	Vector
IgA	Goat anti-horse	Polyclonal	1:400 (PBS)	Bethyl	-	Rabbit anti-goat	Biotinylated	1:50	Bethyl
IgG, Fc specific	Goat anti-horse	Polyclonal	1:1 500 (PBS)	Sigma-Aldrich	-	Rabbit ant-goat	Biotinylated	1:50	Vector
IgM	Goat anti-horse	Polyclonal	1: 6 000 (PBS)	Bethyl	-	Rabbit anti-goat	Biotinylated	1:50	Bethyl
MHC II	Mouse anti-human	Monoclonal	1:20 (1% BSA in TBS)	Dako	TAL.1B5	Goat anti-mouse	EnVision$^+$	-	Dako

BSA: Bovine serum albumin
PBS: Phosphate-buffered saline
TBS: Tris-buffered saline
TNB: Tris NaCl blocking buffer (PerkinElmer).

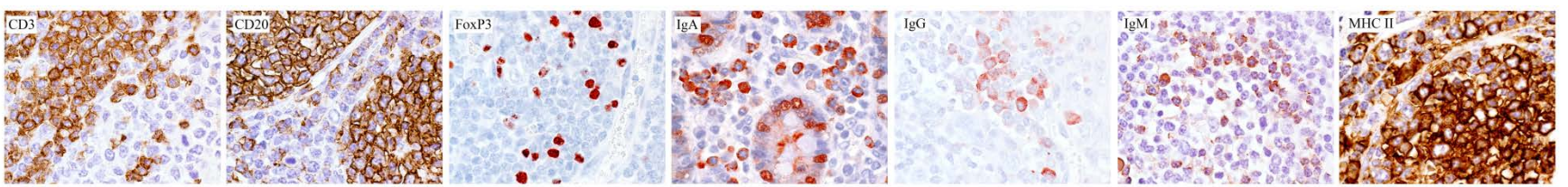


Figure 2. Sections labelled with each primary antibody and photographed at x100, in tonsil (all except IgA) or intestinal lamina propria (IgA).

present in pro-B cells (Koyama *et al.*, 1997) and CD20 is present in all B cells stages from pre-B cells (Uchida *et al.*, 2004; Tedder & Engel, 1994). Neither CD79 nor CD20 is expressed in normal plasma cells, and both antibodies labelled the appropriate areas of lymphoid tissue (Fig. 4, 6C). Anti-CD79 is commonly used in IHC on equine material, however a major disadvantage is the specific labelling of smooth muscle cells (Ramos-Vara, 2014). In a pilot study for Paper I, anti-CD79 was used, but muscle cell labelling obscured immune cell labelling in intestinal villi, making image analysis unreliable. The B-cells markers CD19 and CD21 were not considered as they can also be present in plasma cells (Bataille *et al.*, 2006; Huang *et al.*, 1995), which was an unwanted feature in the present work as the intention was to discriminate B cells from plasma cells. CD20 has previously been used in studies on equine B cells (Kalsow *et al.*, 1999). According to the literature, CD20 labels equine B cells efficiently (Valli *et al.*, 2016; K alsow *et al.*, 1998). Therefore, CD20 was chosen for the following analysis of B cells (Paper I) (Fig. 2, 4, 6C).

To label plasma cells, anti-horse Ig antibodies were used for the subtypes IgA, IgG and IgM (Paper I) (Fig. 2, 5). Labelling of immunoglobulins has been long used in formalin-fixed material (Taylor & Burns, 1974), even so several equine IgG-specific antibodies needed to be evaluated to find an optimal protocol in the present work. Previous publications with the anti-IgA (Taouji *et al.*, 2002) and anti-IgM (Pate *et al.*, 2012) antibodies used on formalin-fixed equine material found these antibodies to give adequate labelling, as did our in-house evaluation. In equine lymph node and tonsil, IgA positive cells were most prominent in sub-epithelial areas of the tonsil, IgM most prominent in the centre of germinal centres and IgG in the cortex (Fig. 5).

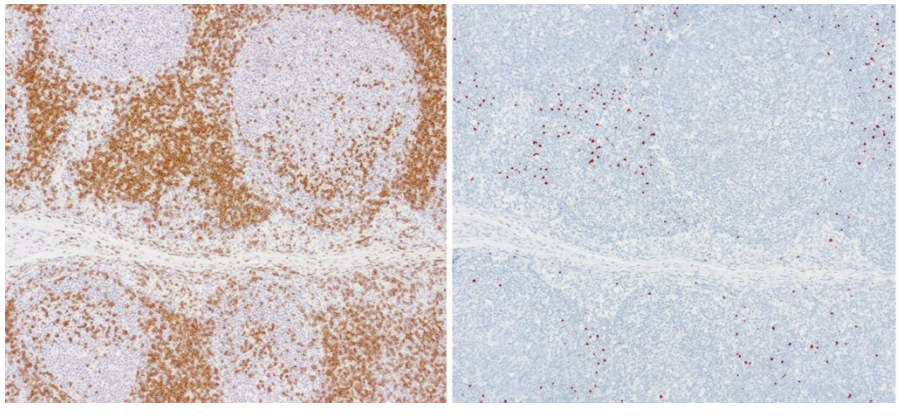


Figure 3. Consecutive section of an equine lymph node labelled for T cells (CD3; left) and T_{REGS} (FoxP3; right). Magnification x20.

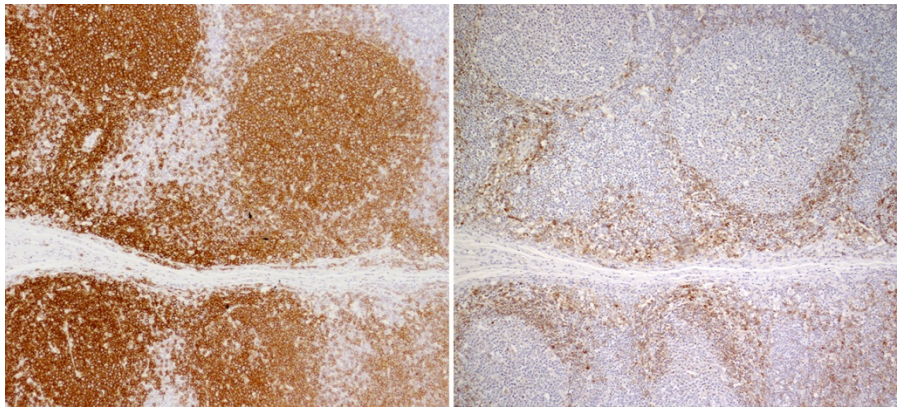


Figure 4. Consecutive sections of an equine lymph node labelled for B cells using either anti-CD20 (left) or anti-CD79 (right). Anti-CD20 labelled cells equally intensely in both the germinal centre and the mantle zone, whereas anti-CD79 labelled the germinal centre B cells less intensely than the mantle zone subset. This is accordance with the literature for human B cells (Aoun & Pirruccello, 2007). Magnification x20.

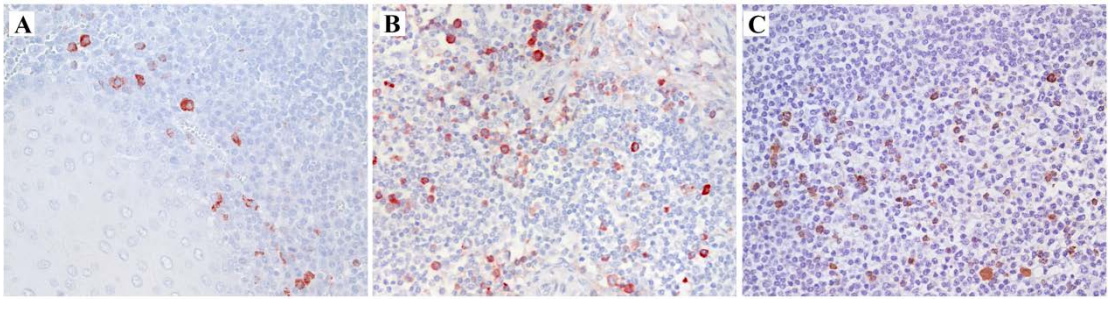


Figure 5. Lymph node immunolabelled for (A) IgA, (B) IgG and (C) IgM. Some background staining is seen in the connective tissue of sections labelled with IgG.

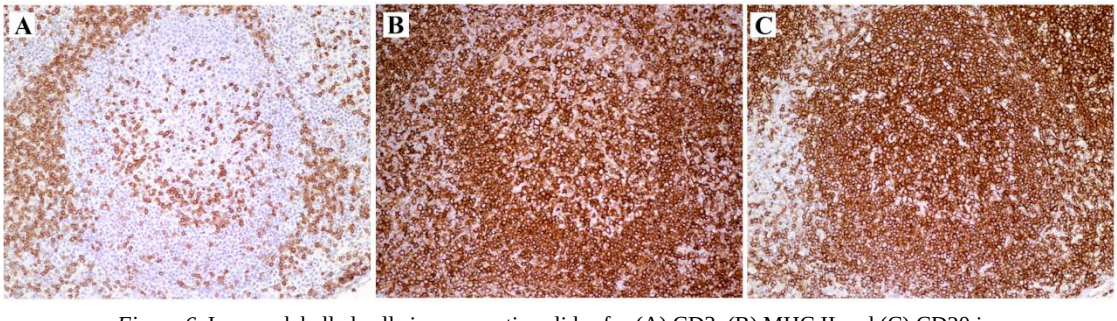


Figure 6. Immunolabelled cells in consecutive slides for (A) CD3, (B) MHC II and (C) CD20 in a lymph follicle of a mesenteric lymph node, demonstrating the co-localisation of labelled cells. For MHC II and CD20 labelled cells overlap extensively, whereas co-localisation of MHC II and CD3 is less marked and CD20 and CD3 rarely appear in the same areas of the lymph node. Magnification x20.

Sections were labelled using an anti-MHC II antibody (Fig. 2, 6B). MHC II is mainly expressed by dendritic cells, macrophages and B-cells, but constitutive expression has also been described in equine T cells in peripheral blood (Lunn *et al.*, 1993; Crepaldi *et al.*, 1986). The antibody clone TAL.1B5 used in the present thesis is targeted at the alpha chain of a human MHC II, namely human leukocyte antigen (HLA)-DR (Thomas *et al.*, 1988). This particular clone has been used in previous publications for detection of equine antigen-presenting cells (Bruynsteen *et al.*, 2013; Mäkinen *et al.*, 2008; Kalsow *et al.*, 1999), but as activated T cells and B cells could express MHC II the correct terminology for labelled cells should be MHC II-expressing cells. Apart from labelling macrophage-like cells in the lymph node, tonsil and lamina propria, MHC II-labelling was noted in endothelium of mostly small capillaries and in epithelium. This is in accordance with labelling of the same MHC II-type in human tissues (Daar *et al.*, 1984).

3.2.2 Amplification of labelling

The use of a secondary antibody amplifies the labelling of the primary antibody. Different approaches for detection of the primary antibodies were used; labelling by biotin conjugated secondary antibody followed by an avidin-biotin complex (ABC; Fig. 7A), or a secondary antibody and enzyme-conjugated dextran polymer (EnVision^{+}; Dako; Fig. 7B). The labelling for FoxP3 needed further amplification than the use of a secondary antibody alone. Hence, sections were labelled with primary antibody, streptavidin and biotin followed by a tyramide signal amplification (LSAB and TSA; Fig. 7C). In the FoxP3 protocol, biotinylated tyramide was applied, enabling HRP to oxidise the biotinylated tyramine to a free radical. The tyramine could thereby react to the primary and secondary antibody complex and deposit multiple biotin sites on the complex. A second application of streptavidin and HRP was applied, allowing the complex to bind to the many biotin sites. The labelling is in this way amplified up to 30-fold compared with non-TSA methods (Kricka, 1993).

For all the applied protocols, either AEC or DAB was used as chromogen to detect the labelling by forming an insoluble precipitate via enzymatic reaction with HRP.

3.2.3 Descriptive analysis and morphometry

By the use of bright-field microscopy all included intestinal tissue sections were described histologically. Where the analysed tissues were preserved in RNA*later*, corresponding tissue fixed in formalin was evaluated by light microscopy. To obtain objective quantitative data on the different cell populations, morphometry was applied. For the morphometric analysis, images

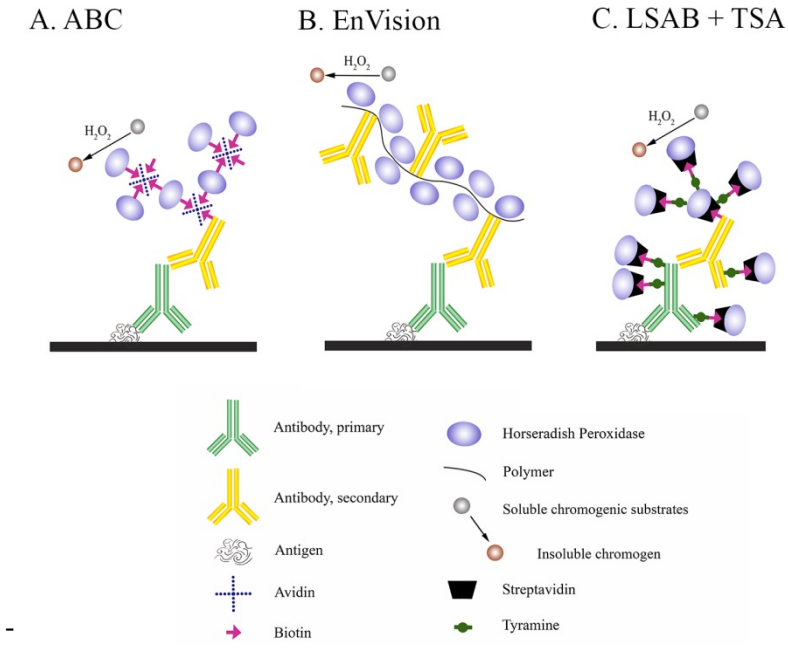


Figure 7. The schematic outlay of (A) the avidin-biotin complex assay (ABC), (B) the secondary antibody and enzyme-conjugated polymer assay (EnVision$^+$) and (C) the labelled streptavidin and biotin (LSAB) and tyramide signal amplification assay (TSA).

of tissue were digitalized at either magnification x20 (Paper I) or x40 (Paper IV) in a standardized, randomized order. All sections were blinded to the operator to avoid biased selection of investigated areas. Sections pertaining to a series were all digitalized in one session to avoid alterations in microscope settings resulting in contrived differences between images to be compared. In Paper IV, individual cells were counted in an unbiased counting frame (Gundersen, 1977) in ten different images, captured in the same level of the tissue, from each horse. All cells within the mucosa were accounted for. In Paper I, an image analysis approach was used. First, the distributions of immunolabelled cells were described in all compartments of the intestinal wall of both healthy and diseased horses followed by morphometry. Immune cells normally populate the lamina propria and expand during the development of equine IBD. Therefore the mucosa was chosen as the tissue compartment for morphometric analysis. As lesions occasionally were graded as mild and were multifocal in the EEG cases, large areas for analysis were needed. This made manual counting of cells infeasible, due to the number of cells included. Additionally, difficulties to quantitate MHC II-expressing cells have previously been described as well, due to the fact that individual labelled cells are hard to discern (Elwood *et al.*, 1997). Therefore the area labelled for each antibody was assessed by image analysis in the NIS Elements software (Fig. 8).

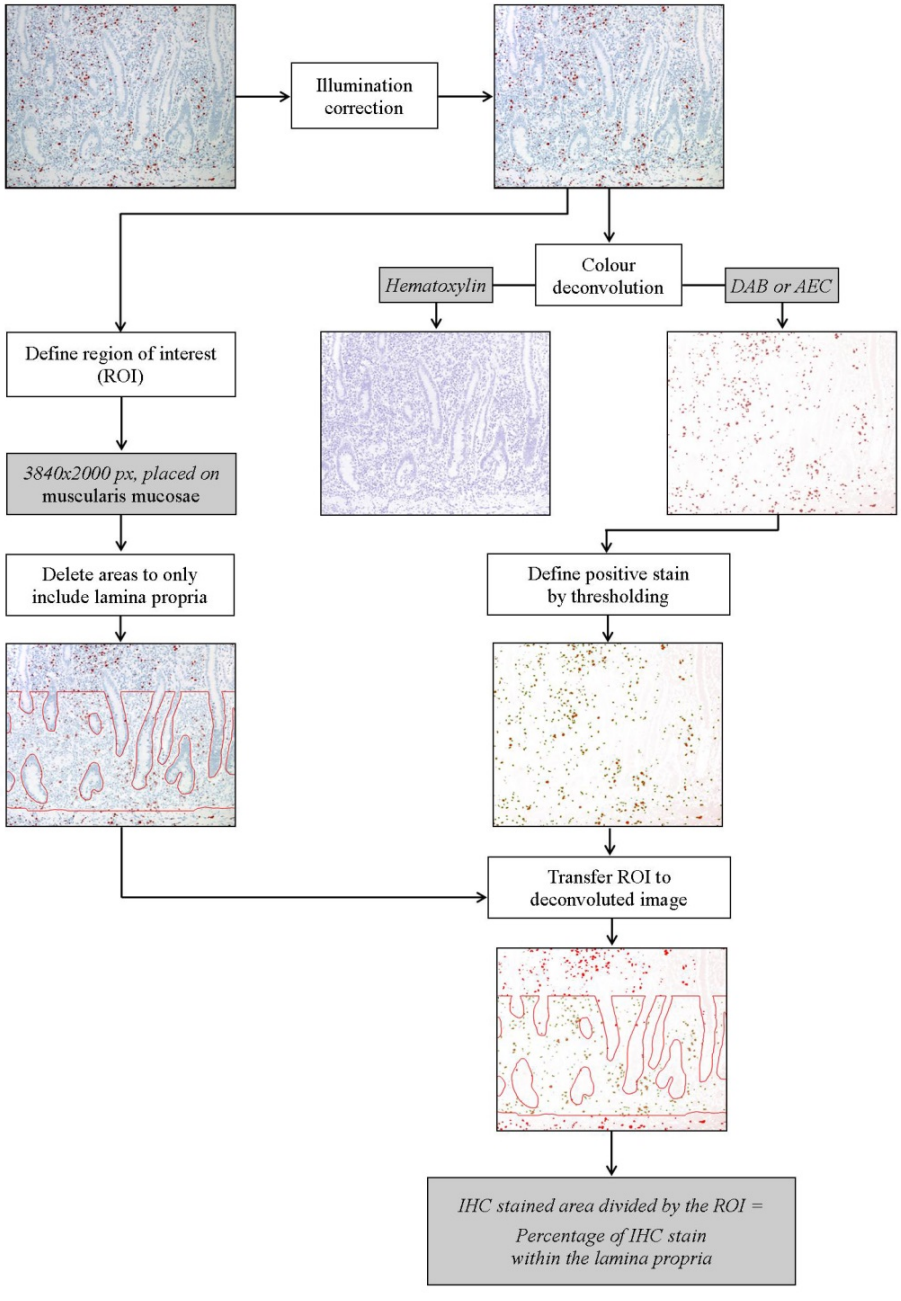


Figure 8. Schematic view of the image analysis; from immunolabelled image via definition of area to analyse (region of interest: ROI), colour deconvolution and applying threshold for detection of immunolabelling, to the percentage of immunolabelling within the analysed area.

To calculate labelled area, all images were white balanced and uneven illumination corrected (Landini, 2014). Thereafter, deconvolution for haematoxylin and DAB or AEC was performed, i.e. the image was split into sub-images where the resulting images only contained one of the stains. Labelling was defined in images only containing the DAB or AEC stain by thresholding in the red, green and blue channels. The threshold values were established for each antibody and applied to all pertaining sections, with only minor adjustments for intensity. Only the IHC labelling in the lamina propria was to be measured, therefore a region of interest (ROI) was created delineating the lamina propria. Within the ROI, artifacts and non-relevant structures were excluded. The area of the IHC labelling was divided by the ROI, resulting in a percentage of labelling in the total area. The average percentage for each labelling and for each horse was used to compare the cell infiltration between groups.

3.3 Evaluation of gene expression

3.3.1 Induction of equine PBMC

To develop appropriate methods for transcript analysis in equine material, *in vitro* activated blood cells expressing the genes of interest were used as reference material (Paper II, III). Dependent on the inducer used, blood cells increase their expression of certain genes. Ionomycin in combination with PMA stimulates production of cytokines by activating a phosphorylating cascade and producing a calcium influx (Morgan & Jacob, 1994; Chatila *et al.*, 1989). Foremost the cytokines IFN-γ, IL-2, TNFα, RANTES and TGFβ are induced after ionomycin and PMA stimulation (Ai *et al.*, 2013; Rostaing *et al.*, 1999). CpG-ODN is present more frequently in bacterial than in vertebrate genomes and acts as a PAMP (Krieg *et al.*, 1995). The CpG motif is recognized by TLR9 and stimulates cytokine responses (Bauer *et al.*, 2001), such as increased IFN-α and IFN-β expression (Wattrang *et al.*, 2005; Krug *et al.*, 2001).

The TLR4-MD-2-CD14-receptor complex recognises LPS and activates cytokine production (Guha & Mackman, 2001). In humans, LPS also stimulates the up-regulation of TLR4, both after *in vivo* and *in vitro* stimulation (Wittebole *et al.*, 2005; Muzio *et al.*, 2000). Inducing endotoxaemia in horses is known to alter the gene expression in the blood, with up-regulation of pro-inflammatory cytokines (Nieto *et al.*, 2009). The method in Paper II was used to evaluate the expression of the TLR4-complex in *in vivo* samples. An *in vivo* equine model of endotoxaemia was used to study the expression of TLR4, MD-2 and CD14 in blood cells at different time points after LPS infusion.

Healthy horses were via continuous intravenous infusion given 83.3 ng LPS per kg, per hour, for a total of 6 hours to induce endotoxaemia (Paper II). All infused horses showed clinical signs of endotoxaemia and blood was sampled for subsequent RNA isolation (according to 3.1.2 *Blood sampling*).

To obtain equine PBMC for *in vitro* stimulation, blood from healthy horses was pooled and layered onto Ficoll-Paque in a centrifuge tube (Paper II). After centrifugation, the band containing the PBMC was collected and washed. The cells were re-suspended in cell culture medium (RPMI 1640 medium, HEPES buffer, l-glutamine, penicillin, streptomycin, 2-mercaptoenthanol and 5% foetal calf serum), adjusted in cell concentration and seeded in 25 cm^2 plastic flasks. Stimulation using CpG-ODN 2216 (3 µL/mL) or PMA (50 ng/ml) and ionomycin (1 µL/ml) was done for 6 or 4 hours, respectively, at 37 °C, 6.5% CO_2. After stimulation, cells were harvested in TRIzol and stored at -80 °C until RNA isolation.

3.3.2 RNA isolation

RNA from blood sampled in PAXgene tubes was isolated using the PAXgene RNA Kit, i.e. a silica spin column-based nucleic acid purification method. In between wash-steps, a DNase solution was applied to the column and eliminated remaining contaminating DNA. For RNA extraction from cultured PBMC and intestinal tissues, an acid guanidinium thiocyanate-phenol-chloroform extraction method was used according to Wikström *et al.* (2011): Homogenisation and disruption of samples were performed in TRIzol followed by further shearing of DNA using a needle and syringe. After addition of chloroform and phase separation by centrifugation, the upper aqueous phase containing RNA was collected and purified by a column based method (E.Z.N.A. Total RNA Kit).

For intestinal samples used in Paper II and III, homogenization was facilitated using a motorized pestle (Andersson *et al.*, 2011). The pestle left the muscle layers intact, thus samples included RNA mainly from the mucosa and to a minor extent the submucosa and serosa.

The amount of total RNA retrieved using the pestle varied and to improve the consistency in RNA yield a BulletBlender was used for homogenization in Paper IV. The BulletBlender homogenizes the tissue by beads added to the sample and application of external motorized force. The entire sample was homogenized if sufficient numbers of beads were added and the agitation done at high speed (see Paper IV, supplementary table S1). However, if too many beads were used the tube was damaged and this led to chemical contamination of samples or rupture of the tube. The addition of more beads to the sample-containing tube rendered higher RNA concentration according to

spectrophotometry, but this could in part be a false high value due to the damaged tube giving off chemicals that absorb UV light at the same wavelength as RNA (Dotti & Bonin, 2011). An optimal number of beads was based on the amount of RNA extracted and the purity of samples.

3.3.3 RNA quality control

To ensure suitable sampling and extraction methods, the RNA was evaluated for the presence of intact 18S and 28S ribosomal RNA, as an indicator of minimal RNA degradation. RNA from *in vivo* induced blood leukocytes was optically evaluated after fragment separation by electrophoresis on agarose gel stained with ethidium bromide (Paper II). For the tissue and *in vitro* induced samples, two approaches for RNA quality control were used; spectrophotometry with absorbance at 230, 260 and 280nm wavelength ($A_{260}/A_{230,}$ A_{260}/A_{280}; NanoDrop) and separation of RNA molecules by capillary electrophoresis resulting in an electropherogram (Experion) (Paper II-IV). Spectrophotometry cannot distinguish RNA from contaminating DNA, therefore inaccurate values can be generated (Imbeaud *et al.*, 2005). The Experion software uses a complex algorithm taking into account both RNA integrity and degradation in the electropherogram, and is thereby a more suitable method for RNA quality control (Bustin *et al.*, 2009). The outcome of the NanoDrop was hence primarily used to confirm purity of sample, excluding contamination from the extraction process and residual proteins visible in the 230nm and 260nm wavelength, and for approximation of RNA quality and concentration before the use of Experion.

3.3.4 DNase treatment and synthesis of cDNA

RNA of good quality and sufficient concentration was synthesised to cDNA. 1µg of RNA was always used in the reaction for tissue derived samples and *in vitro* induced PBMC. In the *in vivo* induced material, the amount of RNA used to synthesise cDNA varied between horses. Therefore within each horse, the amount of RNA in each cDNA reaction was adjusted to the sample with the lowest RNA concentration. Hence, samples from one individual were always comparable between time-points and, as reference genes were used for normalisation, the fold change in gene expression was comparable between horses.

To reduce the effect of possible contamination of congenital DNA all RNA samples were treated with DNase. Thereafter each sample was split into two, one part converted to cDNA by reverse transcriptase and one part serving as a non-reverse transcribed control sample.

3.3.5 Real-time qRT-PCR

SYBR Green-based assays were used for all real time qRT-PCR reactions. Primers were designed or selected from the literature and all assays optimized under in-house conditions (Table 3). To get an idea of how well the assay might perform, amplicons were analysed for secondary structures using the web-based mfold software (Zuker, 2003). When designing the primers, sequences spanning an intron were preferred to differentiate between amplicons from cDNA and from contaminating genomic DNA. If not spanning an intron (as for H2Atype 1, IFN-α, SDHA, TFRC) or if the mRNA sequence has pseudogenes in the genome, it is of upmost importance that the level of genomic DNA is kept to a minimum. For this reason, non-reversed transcribed controls for every sample were always evaluated using either the TFRC or GAPDH assay. These control samples always indicated no or neglectable levels of contaminating DNA. False positive results due to nonspecific fluorescence are an issue in SYBR Green-based assays as the fluorescent dye intercalates any double stranded DNA, such as primer dimers and non-specific amplicons. To estimate primer dimer formation, the primers were analysed using the software Oligo7 (Rychlik, 2007) and all melt curves produced after the PCR reaction were checked for one assay specific peak.

3.3.6 Reference genes and normalisation of gene expression

Differences in RNA quality, the effectiveness of the cDNA synthesis and the amount of cDNA added to the PCR reaction affect the results of all genes analysed in a sample. To correct for variations in efficiency of the real-time qRT-PCR and quantification between samples, an invariant endogenous control is needed. Amplification of reference genes and target genes in the same sample provides an internal control if reference genes are selected with care (Bustin *et al.*, 2009). A reference gene is only useful if it is stably expressed in all included materials, otherwise variation is introduced and results in questionable data (Dheda *et al.*, 2005). The selection of reference genes is therefore pivotal.

For the *in vitro* and tissue samples, the evaluation was achieved using the commercial software qBase^{PLUS} (Paper II-IV). The software compares the stability of reference genes on the assumption that the expression ratio for two reference genes is identical in all samples regardless of the samples analysed. If this assumption is not true and the ratio differs between samples, either one or both of the reference genes are unsuitable for normalisation due to variation in expression. A pair-wise comparison of ratios results in an M-value for each analysed reference gene in qBase^{PLUS} (Hellemans *et al.*, 2007; Vandesompele *et al.*, 2002). The M-value indicates each reference gene's stability in the

analysed material. Additionally, after normalisation the reference gene should be expressed equally in all samples. This equality of expression is calculated as coefficients of variation (CV). In consecutive steps, the least suitable gene is excluded until a threshold of stability is reached, yielding a set of recommended genes for normalisation. The expression of up to seven commonly used reference genes (β2M, GAPDH, H2Atype1, HPRT, RPL32, SDHA, TFRC) was evaluated in equine PBMC and several intestinal segments from both healthy and diseased horses (Paper III, IV).

In Paper II, the expression of HRPT and SDHA was used for normalisation of *in vivo* stimulated PBMC. The relative change in expression of target genes was calculated using the $2^{-\Delta\Delta Cq}$ normalization method (Livak & Schmittgen, 2001). The returned value is a fold change between a sample and a control, in this case the time-point 0. ΔΔCq is calculated according to the following equation:

$$\Delta\Delta Cq = (Cq_{goi;\,Sample} - Cq_{ref;\,Sample}) - (Cq_{goi;\,Control} - Cq_{ref;\,Control})$$

goi = gene of interest; ref = reference gene

In the *in vitro* stimulated cells and in all intestinal samples (Paper II-IV), expression of the gene of interest was normalised using the qBase^{PLUS} software and a suitable set of reference genes. The following equation is used by the software:

$$NRQ = \frac{E_{goi}^{\Delta Cq,\ goi}}{\sqrt[f]{\prod_0^f E_{ref_0}^{\Delta Cq,ref_0}}}$$

goi = gene of interest; ref = reference gene

The equation is based on the former described equation, but takes the efficiency (E) of the PCR reaction into account. This eliminates the assumptions that need to be made using the Livak and Schmittgen-method. Additionally, the use of several reference genes is possible in the qBase-equation compared to the former equation using a single reference gene's expression. The normalised gene expression was presented as a fold change in all papers included in this thesis.

Table 3. *Primer sequences and optimized real time qRT-PCR assay conditions*

Gene	Primer sequence	Primer location	Target sequence	Size (bp)	Annealing temperature (°C)	Primer concentration (nM)	E (%)	r^2	Melting point (°C)
β2M	S: CGGGCTACTCTCCCTGACT	60-80	NM_001082502.3	72	55	300	95	0.999	82
	A: GGGTGACGTGAGTAAACCTGAAC	133-109							
CD14	S: GTTGTCAGTCAGTGCAGCTC	68-87	NM_001081927.1	166	60	400	96	0.996	83.5
	A: GAAGATGCTCCAGGAGAAGA	233-214							
GAPDH	S: ATCTGACCTGCCGCCTGGAG	779-798	NM_001163856.1	68	64	400	102	0.999	79
	A: CGATGCCTGCTTCACCACCTTC	846-825							
H2A type 1	S: ATATTCAGGCCGTGCTGCT	380-398	XM_005603621	105	57	400	100	0.994	81
	A: TTTGGGTTTCAAAGCGTTTC	484-466							
HPRT	S: AATTATGGACAGGACTGAACGG	104-125	AY372182.1	121	57	300	95	0.996	80.5
	A: ATAATCCAGCAGGTCAGCAAAG	224-203							
IFN-α	S: CCAGTTCCGGAAGCCTCAAG	207-226	NM_001099441.1	67	55	300	102	0.996	79
	A: GAAGAGGTGGAAGATCTGTTGGAT	273-250							
IL-12p40	S: TGCTGTTCACAAGCTCAAGTATGA	642-665	NM_001082516.1	76	59	300	101	0.998	84
	A: GGGTGGGTCTGGTTTGATGA	717-698							
IL-17A	S: CCAGAAGGGCCTCAGATTACCACA	173-196	NM_001143792.1	139	56	300	95	0.996	84
	A: ACCTTCCCTTCGGCATTGACACAG	311-288							
IL-23p19	S: AGTGCGAGGATGGCTGTGAT	291-310	NM_001082522.2	124	59	400	97	0.992	82
	A: GGCTCCCCTGTGAAAATGTCT	414-394							
MD-2	S: TAACATGAAGTCCCCGATTT	152-171	NM_001081898.1	256	57	400	105	0.997	81
	A: TCCCCTGAAGGAGAATGATA	407-388							
RPL32	S: AGCCATCTACTCGGCGTCA	81-99	XM_001492042.5	144	59	400	100	0.995	81.5
	A: TCCAATGCCTCTGGGTTTC	224-206							
SDHA	S: GAGGAATGGTCTGGAATACTG	1478-1498	DQ402987.1	91	57	300	98	0.994	80
	A: GCCTCTGCTCCATAAATCG	1568-1550							
TFRC	S: TGGCTACTTGGGCTATTGTAAACG	255-278	NM_001081913.1	90	63	300	97	0.990	78
	A: GGTGGTTCTGTTCCCTCTATCTCC	344-321							
TLR4	S: GGAACTGGACCTGAGCTTTA	289-308	NM_001099769.2	101	60	400	95	0.995	80.5
	A: CAATTTCACACCTGGACAAA	389-370							

Present target and primer locations may deviate from those in the published papers, but sizes remain the same.

E: Efficiency, from serial dilutions of reference cDNA; A: Antisense (3'-5'); S: Sense (5'-3')

4 Results

4.1 Cellular contribution to chronic intestinal inflammation (Paper I, IV)

Eosinophils are the characteristic cellular component for EEG, presenting as tissue eosinophilia and eosinophilic granulomas. In the jejunal tissue used (Paper I), mild infiltrates of eosinophils were noted in healthy horses, and were primarily scattered in the submucosa and in the basal areas of lamina propria, in accordance with Rötting *et al*. (2008). In diseased horses, EEG horses displayed moderate to marked tissue eosinophilia (Table 2 in Paper I) whereas EGE horses showed sparse to no eosinophils. Due to this apparent difference in infiltration, eosinophils were not enumerated in more detail, but included in the descriptive analysis. In the rectal tissue (Paper IV), all horses regardless of disease status displayed normal infiltrates of eosinophils.

T cells dominated the lymphocyte population in jejunal tissue and were numerous in the apical lamina propria of healthy horses (Paper I). Diseased horses displayed abundant T cells at the sites of intense cell infiltration, which were distributed throughout the tissue. The expansion of T cells was prominent and correlated to less B cells in the examined area (Paper I). T cells and T_{REGS} also correlated in the jejunum (Paper I) if all horses were included in the analysis, but not if only diseased horses were compared (Fig. 9, Paper I). In healthy horses, T_{REGS} resided diffusely in the jejunal lamina propria, while in the diseased horses this T cell subpopulation accumulated in areas of intense cell infiltrates and in streaks outside granulomas. In the rectal tissue obtained from healthy horses and horses with simple proctitis (Paper IV), T cells constituted a large part of the immune cell population. Clustering of T cells and T_{REGS} was noted in the diseased horses and the regulatory cells were somewhat more numerous in horses with an active component in their inflammation (CASP). No correlation was found between the numbers of T cells and T_{REGS} in the rectum (Fig. 9, Paper IV).

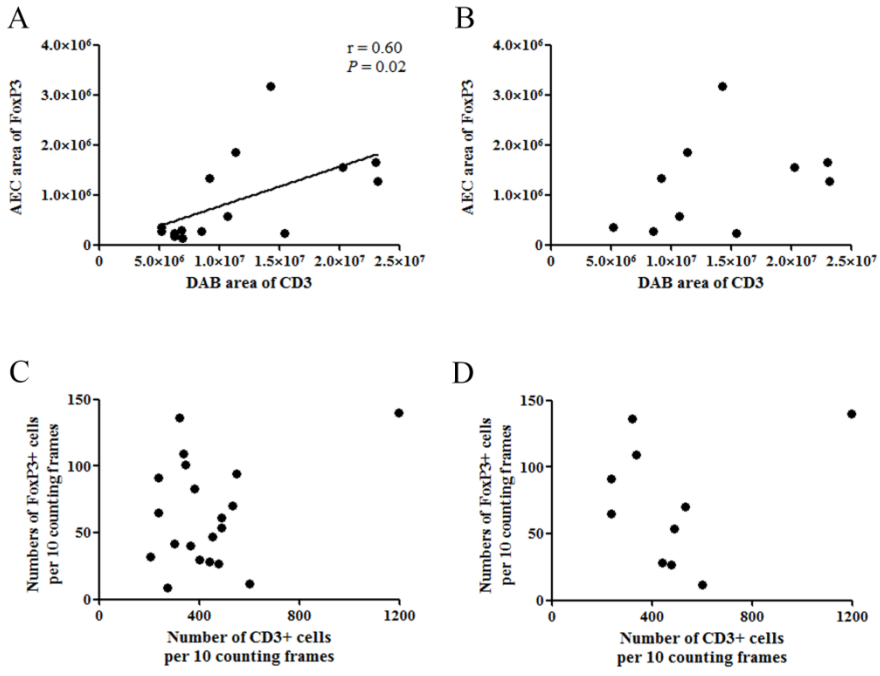


Figure 9. The correlation in infiltration between T cells and T_{REGS} in (A) the jejunum of both healthy and diseased horses, (B) the jejunum of diseased horses, (C) the rectum of both healthy and diseased horses and (D) the rectum of diseased horses. (A) and (B) are quantitated by image analysis and (C) and (D) by cell counting in an unbiased counting frame.

In both the jejunal and rectal epithelium, almost all IEL showed labelling for T cells (Paper I, IV), only a few were labelled by FoxP3 or by MHC II and none displayed B cell characteristic (Paper I). In the healthy equine ileum, approximately 50% of the IEL are CD4+ T cells and 50% CD8+ T cells (Tschetter *et al.*, 1998). In the present material, the numbers of IEL was not significantly different in jejunum obtained from healthy and diseased horses, but was markedly increased in a few of the diseased horses (Paper I). Additional findings in the jejunal epithelium were reduction in Paneth cells seen along with villus atrophy and crypt abscesses in the granulomatous cases. Two EEG horses had crypt abscesses, one also displaying goblet cell hyperplasia (Paper I) (Fig. 10). In the rectal tissue, one horse diagnosed with CASP displayed loss of acid mucin in the goblet cells (see supplementary Fig. S2 in Paper IV).

MHC II-expressing cells were together with T cells the dominant cell type in the jejunal lamina propria and submucosa, and the infiltration of the two cell types was correlated (Paper I). In healthy horses, most MHC II positive cells were situated in the villi lamina propria, just below the epithelium with

occasional cells within the epithelium. In horses with granulomatous or eosinophilic inflammation, MHC II+ cells accumulated in lymphoid aggregates. Most MHC II+ cells were according to their morphology macrophages. As several cell types can be induced to express MHC II, some of these cells expressing MHC II may not be professional antigen presenting cells (Kambayashi & Laufer, 2014; Daar *et al.*, 1984). When comparing the infiltrates of B cells and MHC II+ cells, both by light microscopy and by morphometry, MHC II-expressing cells far exceeded the B cells. Additionally, MHC II-expressing cells were a dominant presence in lymphoid aggregates in the diseased horses, whereas B cells were mostly found in the periphery of these lesions. In healthy horses, B cells were found in the basal areas of the jejunal lamina propria, and only accounted for a smaller fraction of the immune cells. For EEG afflicted tissues, B cells were rare in the areas displaying most intense immune cell infiltration. Instead B cells were present in areas of more normal histology and then in numbers comparable with the healthy horses. For EGE-afflicted tissues, B cells were generally fewer compared with healthy horses and their distribution was in clusters rather than diffusely spread. Areas with nodules of B cells (Fig. 11) could be noted in diseased horses and may reflect lymphoid neogenesis (Aloisi & Pujol-Borrell, 2006).

Plasma cells secreting IgA, IgM and IgG were present, in decreasing order, in the healthy jejunal lamina propria (Paper I). IgA and IgM cells were located at the junction between villous and mid-area lamina propria, whereas the few IgG plasma cells were present in the basal areas of lamina propria. In diseased horses, IgA labelled cells were detected in the periphery of intense cell infiltrations, and occasionally forming streaks of labelled cells. IgG plasma cells were also found in minor streaks in one EEG-afflicted horse, whereas in the EGE-afflicted horse these plasma cells were scarce and diffusely spread. IgM plasma cells followed the distribution of IgA cells in the EEG group with less positive cells than in healthy horses, whereas IgM cells were rare in the tissue samples from the EGE group. A strong positive correlation was seen between decreased areas of B cells and IgG+ cells (Paper I). IgM+ cells were negatively correlated to both T cells and MHC II+ cells. In the epithelium, IgA+ enterocytes showed varying intensity of labelling, with the least intense staining in the bottom of crypts and in the villi tips. The level of IgA labelling in the epithelium was comparable between healthy and EEG horses and both these groups showed more labelling than in the EGE horses (Fig. 12).

As T cells and MHC II-expressing cells dominated the lesions in both EEG and EGE, a delayed type hypersensitivity reaction is proposed for both entities. The granuloma formation by macrophages in both groups suggests either an

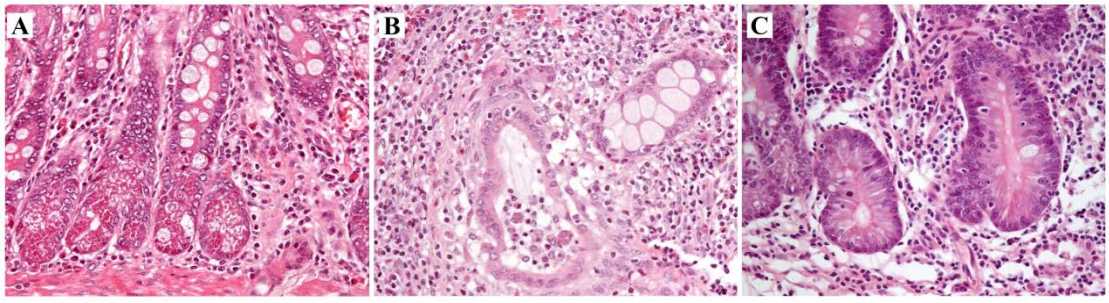


Figure 10. The epithelium of (A) a healthy horse, (B) an EEG-afflicted horse with crypt abscess and goblet hyperplasia and (C) an EGE-afflicted horse with degranulation or loss of Paneth cells. Magnification x40.

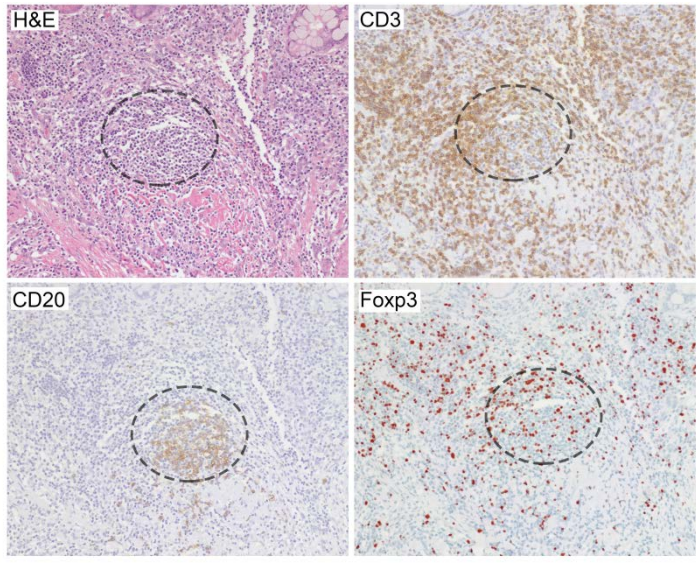


Figure 11. Immunohistochemical staining in consecutive sections in eosinophilic gastroenteritis Jejunal tissue stained (H&E) and immunolabelled (CD3: T cells, CD20: B cells, FoxP3: T_{REGS}). The dotted circle outlines the B cell dense area. Magnification x40.

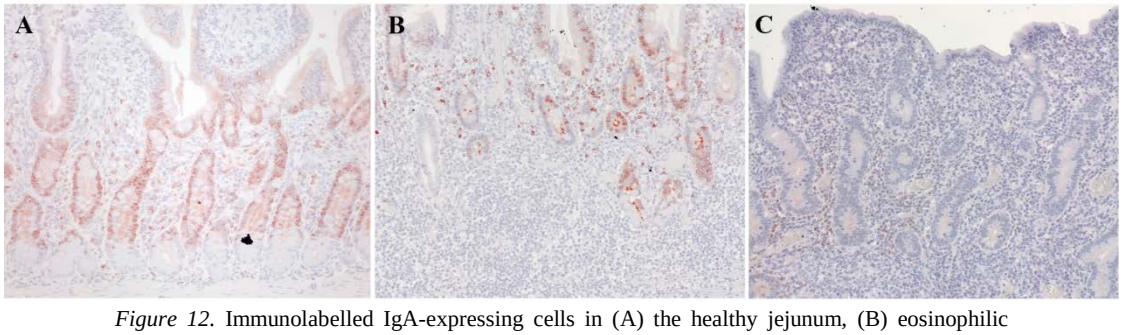


Figure 12. Immunolabelled IgA-expressing cells in (A) the healthy jejunum, (B) eosinophilic enteritis and (C) granulomatous enteritis. Magnification x20.

inability of the effector pathways to eradicate the triggering antigen or an inability of the regulatory pathways to terminate an immune response when the antigen has been eliminated. Whether the quantity and function of T_{REGS} in the diseased horses were adequate is not known, but their presence in IBD lesions suggests that an attempt to limit the inflammation has occurred, even if not successful. The rarity of B cells and plasma cells, especially in EGE horses, indicates a lesser role for this lymphocyte lineage in disease development.

Furthermore, it was possible to demonstrate and quantitate presence of T cells as well as T_{REGS} in the rectal biopsies. To quantify the T cell-associated gene expression in rectal biopsies, appropriate reference genes were evaluated.

4.2 Appropriate reference genes (Paper II, III, IV)

For studies on equine PBMC the genes GAPDH, HPRT and SDHA were evaluated as potential reference genes. Independent of sampling methods and *in vivo* or *in vitro* stimulation, SDHA and HPRT were identified, using the qBase software, as the two most optimal reference genes, and hence were used for normalisation (Paper II). In contrast, the combination of GAPDH and HPRT were more suitable for normalization in healthy equine ileum, right dorsal colon and rectum (Paper II).

To further study gene expression in equine intestinal material, the sampling sites were extended to incorporate seven distinct segments (the duodenum, mid-jejunum, ileum, caecum, right ventral and dorsal colon and rectum) and seven putative reference genes (β2M, GAPDH, H2Atype1, HPRT, RPL32, SDHA and TFRC) were evaluated (Paper III). No combination of the evaluated reference genes was stable enough for normalization across all analysed segments from healthy horses. However, sets of three or four reference genes suitable for segment-specific normalization were identified for the duodenum, mid-jejunum, right and ventral dorsal colons and rectum. The ileum and caecum showed considerably lower reference gene stability. When focusing on rectal material from healthy horses and horses with IBD two or three genes were identified as useful candidates for normalization of gene expression, namely H2Atype1 and TFRC, or SDHA, GAPDH and RPL32, respectively. Combined data from both groups identified RPL32, GAPDH, HPRT and SDHA for normalisation across the groups. The most stably expressed of these genes were GAPDH and RPL32, therefore they were used in Paper IV. qBase was again used to control for stability in GAPDH and RPL32 in the rectal samples in Paper IV. Both GAPDH and RPL32 were again stably expressed in both healthy and diseased material and suitable for normalisation.

4.3 Gene expression in equine PBMC (Paper II, III)

Apart from the reference genes, seven target genes (MD-2, CD14, TLR4, IFN-α, IL-12p40, IL-23p19 and IL-17A) were evaluated in stimulated and unstimulated blood cells (Paper II, III). In LPS *in vivo* stimulated samples, the expression of CD14 was overall unaffected (Paper II). TLR4 expression reached its peak in relative expression between 6 to 12 hours after the start of infusion, and showed up to a 20-fold increase. The expression levels of TLR4 remained elevated in all horses except one that was excluded due to severe signs of endotoxaemia. For two of the horses, the expression levels of TLR4 were similar to the levels of MD-2, whereas in one horse, MD-2 levels exceeded TLR4 and in the fourth horse MD-2 was expressed at lower levels than TLR4.

Induction of PBMC with CpG-ODN had no effect on the expression of CD14, IL-17A or IL-23p19 (Paper II, III). The expression of MD-2 and TLR4 increased two and half times, IL-12p40 increased over 100 times, and IFN-α increased more than 300 times after CpG-ODN stimulation compared with unstimulated cells. These findings are in agreement with TLR9 activation by CpG-ODN, driving a T_H1 cytokine profile. Stimulation of cells with PMA and ionomycin had no effect on the expression of CD14 or IL-23p19, but reduced the relative expression of IFN-α, IL-12p40, MD-2 and TLR4. In contrast, the expression of IL-17A increased 300-fold, thus indicating a T_H17 profile. It was concluded that CpG-ODN stimulated cells were useful as positive control material in the MD-2, IL-12p40, IFN-α and TLR4 assays and PMA and ionomycin stimulated cells in the IL-17A assay. Additionally, the assays were considered effective in detecting the expression of the targeted genes in biological material. The increased TLR4 and MD-2 expression as well as clinical signs of endotoxaemia corresponded in time. This validated the described method for gene expression analysis also for samples induced *in vivo*.

4.4 Gene expression in healthy intestine (Paper II, III, IV)

The seven genes of interest evaluated in PBMC were also evaluated in healthy intestinal material. CD14, MD-2 and TLR4 were evaluated in full-thickness samples of the ileum, right dorsal colon and rectum and showed no significant differences in expression between horses or segments (Paper II). When TLR4 was evaluated in rectal biopsy material, variations in expression were within a 2-fold change for all healthy horses but one (Paper IV). IFN-α, IL-12p40, IL-17A and IL-23p19 were evaluated in seven segments of the equine intestine. IFN-α was not detectable in any of the segments. The expression of IL-17A in

the ileum and IL-23p19 in the caecum and right dorsal colon varied somewhat, whereas the other cytokines and sites showed expression within a 2-fold range (Paper III). The expression of IL-23p19 never varied more than twofold in healthy rectal biopsies. Likewise, all horses except two in the case of IL-12p40, or one in the case of IL-17 were within a 2-fold range. Overall, all cytokines but IFN-α were readily detected and segments and individuals in the healthy subjects showed little variation in target gene expression. Therefore, IL-12p40, IL-17, IL-23p19 and TLR4 were chosen for further analyses.

4.5 Gene expression in chronically inflamed intestine (Paper IV)

The gene expression of IL-12p40, IL-17A, IL-23p19 and TLR4 was evaluated in rectal tissue displaying lesions consistent with idiopathic chronic enteropathy. The expression varied for some of the targeted genes, but was not related to either the sex or age of the horse (Fig. 13). The expression of the targeted genes in the diseased horses did not differ from the healthy subset when compared as one group including both horses with and without an acute inflammation. However, when the diseased horses were divided into their respective histological diagnosis, differences were noted. Horses with chronic simple proctitis (CSP) had a lower expression of IL-12p40 than healthy horses and horses with chronic active simple proctitis (CASP). The expression of IL-17A was significantly higher in the CASP group compared with healthy or CSP horses. In all horses, the expression of IL-17A correlated positively with the number of T_{REGS} infiltrating the tissue. No significant differences in expression of IL23p19 were found. Nevertheless, a correlation between IL-23p19 expression and the number of T_{REGS} was seen. CSP horses showed no difference in expression of TLR4 compared with the other groups, whereas the CASP horses had a significantly higher expression of TLR4 than the healthy horses.

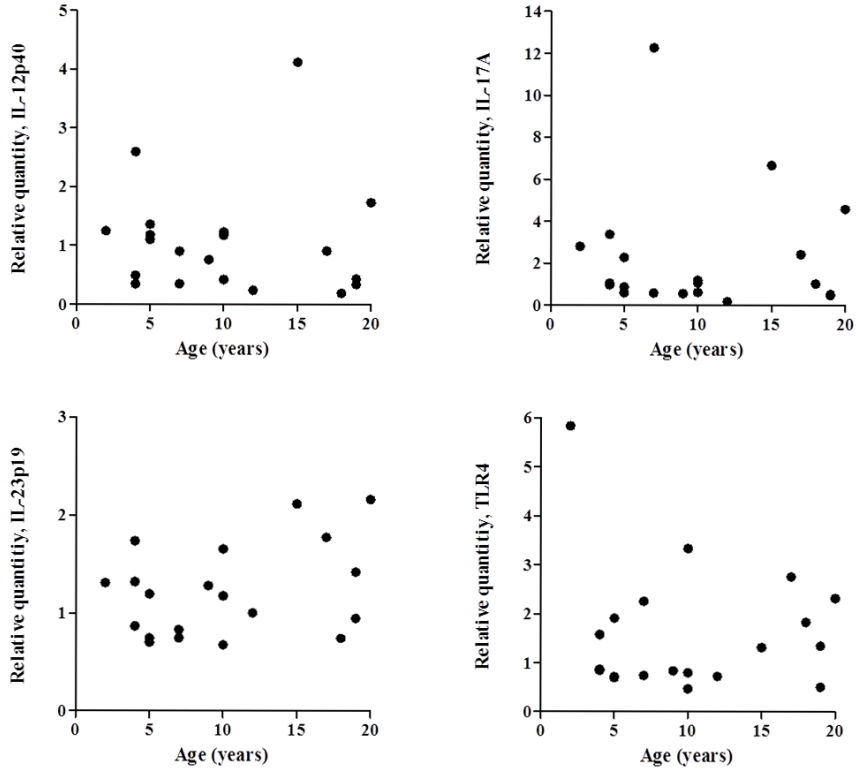


Figure 13. The expression of IL-12p40, IL-17A, IL-23p19 and TLR4 in rectal biopsies from both healthy and diseased horses. No correlation for age and gene expression was found for any of the tar geted genes.

5 Discussion and concluding remarks

Equine IBD is diagnosed by combining clinical and histological findings. In the standing horse, the only easy accessible tissue for histological analysis is rectum, from which biopsies can be collected. Using routine staining (H&E, Ab-PAS) this tissue is diagnostic in approximately half of the cases. This thesis therefore aimed to expand technologies for examination of rectal material and to elaborate methods that could be applied in the diagnosis of intestinal inflammatory conditions in the horse. For that purpose, rectal biopsies displaying simple chronic enteropathy were used for gene expression analysis (Paper IV), as specific IBD lesions were found in too few cases to be included. However, simple proctitis is not pathognomonic of IBD (Lindberg *et al.*, 1996). Additionally, not all horses afflicted by IBD have changes in their rectum. The use of rectal biopsies from the 2010s reflects the current clinical situation, where rectal material is the main tissue available for the diagnosis of IBD. Extended histological examinations were therefore carried out on archived material (Paper I). This material, albeit limited in its suitability in some analyses, was a good source of well-diagnosed cases with characteristic lesions and severe disease status. The further analysis of formalin-fixed archived material was however limited to immunohistochemistry. The number of reagents specific for all equine materials is still limited, and more research is needed to create immunological reagents for the detailed investigation of disease processes in the horse. The present thesis therefore focused on elaboration of methods such as immunohistochemistry and real time qRT-PCR to study equine intestinal immune reactions.

The advantage of using IHC is its ability to label cells *in situ*. Precautions were made to label all sections in a series in the same run and photograph them in one session to minimize inter-run differences. Notably, all archived samples were re-bedded before sectioning and diseased material displayed labelling of each antibody. Therefore, the length of time that the material had been archived was considered to have had little impact on the results. For the IHC

labelling of FoxP3, the antibody labelled the correct population, but several attempts to increase the detection were needed as the chromogen staining was faint. The TSA amplification as described by Junginger *et al.* (2012) for detection of FoxP3 cells in canine lamina propria was applied and performed well on equine intestinal tissue. For IgG, an antibody from Jackson ImmunoResearch (anti-horse IgG, Fc specific; 108-005-008) labelled plasma cells, but also gave an intense, obscuring background, whereas an antibody from Rockland (anti-horse IgG, Fc specific, 608-1103) labelled non-plasma cells in addition to IgG-producing cells. The anti-horse IgG antibody from Sigma-Aaldrich (Fc specific, SAB3700143) that was eventually used labelled plasma cells and only produced minor background. However, the cells labelled in IBD-diseased horses were few. It is therefore possible that the antibody, even though it was a polyclonal antibody, might not detect all subclasses of equine IgG. To better delineate the understanding of the IgG involvement in equine IBD, monoclonal antibodies directed to all subclasses of equine IgG are needed to be used on diseased tissues.

The quantitative analysis of the IHC labelling was restricted to the lamina propria as it is thought to be the effector site for immune cells in the development of IBD (Xavier & Podolsky, 2007). The lamina propria also constituted the main area of rectal biopsies. In Paper IV, labelled cells in the rectal mucosa were manually counted in a pre-defined area. To count cells in this way gives an estimate of the cell density that is comparable to other studies, but requires a randomized selection of areas analysed and is not the method of choice in stereology (Gundersen *et al.*, 1988). Instead, when applying image analysis (Paper I) the area labelled by an antibody was divided by the total area of the analysed lamina propria. The resulting percentage of labelling is thus comparable between groups. The image analysis provides an estimate of area rather than cell density. Consequently, the results take into account the alteration in immune infiltrate, and make group differences more reliable. The image analysis method used is applicable to any immunolabelled or stained section. Importantly, the image analysis is most valuable when done in conjunction with a descriptive analysis and should be seen as a quantitative complement to the evaluation by an experienced pathologist (Gurcan *et al.*, 2009).

For gene expression analysis, a rigorous selection of reference genes is necessary for all types of tissue, species and condition. Appropriate reference genes ensure a correct normalisation of the data set in both healthy and diseased material (Vandesompele *et al.*, 2002). The method used in Paper III was time-consuming, but necessary to gain reliable results. The real time qRT-

PCR protocols for the various cytokine genes were optimised using RNA prepared from equine PBMC exposed to PMA/ionomycin or CpG-ODN. However, none of these inducers increased the expression of IL-23p19. According to Liu *et al*. (2009), the use of *Rhodococcus equi* induced IL-23p19, but not IL-12p40, in cultures of PBMC obtained from foals. This approach could possibly be used to produce improved positive controls for the IL-23p19 assay. The reference gene expression analysis established in the present thesis is the most comprehensive available for equine intestinal segments and will be valuable in any future analysis of equine intestinal materials. Likewise, the protocols for RNA isolation and purification from the rectal biopsies will aid in recovery of high quality RNA from limited amounts of tissues. Furthermore, primer design and optimised conditions for real time qRT-PCR analysis of equine IL-17A and equine IL-23p19 expands the possibilities to explore inflammatory conditions in any equine organ of interest, as long as appropriate reference genes for the material are used.

Both descriptive and quantitative analysis of the healthy full-thickness material of the jejunum (Paper I) identified a predominance of T cells in the lamina propria, which is in agreement with previous publications (Divers *et al.*, 2006; Packer *et al.*, 2005). The distribution of T cells in the healthy mucosa resembled that described for the canine small intestine mucosa (German *et al.*, 1999). In diseased horses, a T cell dominance of the lymphocytic infiltrate has been described for both EEG lesions and LPE in horses (Mäkinen *et al.*, 2008; Divers *et al.*, 2006). However, in non-lesion sites of EEG, MHC II-expressing cells were the most numerous (Mäkinen *et al.*, 2008). In the present material, no differentiation was made between lesion and non-lesion sites in the image analysis (Paper I), but MHC II positive cells were markedly increased in all diseased horses. This increase was significant in EGE when quantified. Taken together, the results strongly indicate a role for MHC II-expressing cells and T cells in the development of equine IBD. An important role for these cells has also been suggested for equine recurrent uveitis (ERU), which is an idiopathic, immune-mediated disease of the eye (Romeike *et al.*, 1998). MHC II-expressing cells and T cells are increased diffusely in the inflamed uvea, suggesting a role for macrophages with aberrant immune responses in the disease development (Romeike *et al.*, 1998). T cells and MHC II-expressing cells are also central in human IBD (Neurath, 2014; Zenewicz *et al.*, 2009; Coombes & Powrie, 2008). In accordance, dendritic cells are suggested to be pathogenetic in canine IBD with increased numbers of dendritic cells and MHC II-expression in both lymphoplasmacytic and eosinophilic IBD (Junginger *et al.*, 2014). It would therefore be of much interest to study further

MHC II-expressing cells in equine intestinal disorders, as these cells clearly are involved in disease development in several species afflicted with IBD.

Numerous contributing factors have been described for IBD in several species including genetic, microbial and environmental factors. The chronic lesions in both EEG and EGE show great similarities to delayed hypersensitivity reactions, but in the former entity massive infiltrations of eosinophils are noted. A possible difference between EEG and EGE could be diverging initiating antigen, driving different types of immune responses. Alternatively, it could simply reflect a genetic predisposition for that type of immune reaction or reflect environmental influences.

Chronic eosinophilic inflammation is commonly caused by parasites, allergens and viruses attracting eosinophils to sites of inflammation through chemokines such as eotaxins and cytokines, including IL-4, IL-5 and IL-13. Transient eosinophilia has been diagnosed in the lungs of otherwise healthy horses, lacking an increase in IL-4 and IL-5 (Riihimäki *et al.*, 2008). This finding demonstrates a partial redundancy of T_H2 cytokines in the recruitment of eosinophils to equine mucosal surfaces. Eosinophils are also recruited in insect bite hypersensitivity of horses (Schaffartzik *et al.*, 2012). The reaction is induced by type I hypersensitivity and possibly by type IV reaction (Anderson *et al.*, 1991). The insect bite hypersensitivity is a prominent allergic reaction to *Culicoides* antigens and is characteristically diagnosed in Icelandic horses born in an environment free of the particular insect, that later move to an area where the insect is present. This clinical history supports the theory that an early encountering with an antigen is important to induce tolerance rather than inflammation. T_{REGS} are suggested to be important in determining whether a horse will develop *Culicoides* hypersensitivity or not. When exposed to the insect's saliva, allergic horses had lower numbers of T_{REGS} and expression of regulatory cytokines compared with healthy horses (Hamza *et al.*, 2012; Heimann *et al.*, 2011). Additionally, T_{REGS} of the allergic horses *in vitro* were significantly less able to supress the production of pro-inflammatory cytokines than T_{REGS} from healthy horses (Hamza *et al.*, 2013). These findings suggest an imbalance between the effector T cells and the T_{REG} responses in the diseased animals.

In the present material, no intralesional microorganisms were detected in any of the included diseased horses, whereas in some healthy individuals intestinal ciliate protozoa were present, which are considered to be normal inhabitants of the equine gut (Uzal *et al.*, 2016). However, a microbial aetiology to equine IBD is still possible as the initiating insult can have been eradicated at the time of sampling or undetected by the methods used (Stratford *et al.*, 2011).

In a pilot study (Wahlund, 2011), the expression of type I interferons was quantitated in equine rectal biopsies from clinically suspected IBD cases. Type I interferons were not readily detected in any of the horses, with the exception of IFN-κ, which did not differ significantly between healthy and diseased horses. To further the understanding of IFNs in IBD, studies on characteristic lesions in fresh material would be of interest and could if detected indirect indicate a viral infection in the pathogenesis.

Microbial antigens are sensed by receptors on innate immune cells. Dysfunctional TLRs and their contribution to human IBD were reviewed and it was concluded that atypical TLR signalling has an important role in disease development (Cario, 2010). In both human and canine IBD-afflicted intestinal tissues, TLR4 is up-regulated on recruited dendritic cells (Allenspach *et al.*, 2010; Burgener *et al.*, 2008; Hart *et al.*, 2005) and these cells respond more vigorously to LPS with pro-inflammatory cytokine production (Baumgart *et al.*, 2009; Hart *et al.*, 2005). A genetic association and increase in susceptibility to human and canine IBD have been found for TLR alleles, including polymorphism in TLR4 (Cheng *et al.*, 2015; Kathrani *et al.*, 2010; Franchimont *et al.*, 2004). The gene polymorphism possibly makes some hosts more susceptible to bacterial infections or over-reactive towards non-harmful antigens. In the present material, expression of TLR4 could be induced by exposure of equine PBMC to CpG-ODN *in vitro*, and *in vivo* after endotoxin infusion (Paper II). In the intestinal tissue, TLR4 expression varied between individuals, but was significantly increased in chronic active proctitis (CASP; Paper IV). It is possible that in the chronic active group the lamina propria cells were exposed to LPS and therefore increased their TLR4 expression.

Theoretically, signalling via TLR4 would generate inflammatory mediators attracting neutrophils. In horses with chronic recurrent airway obstruction (RAO), bronchial epithelial cells have increased expression of TLR4 and neutrophils, but not eosinophils, infiltrate the lung. Hence in both IBD and RAO, increased TLR4 is seen together with recruitment of neutrophils. These disease conditions are thought to be a reaction towards environmental antigens, such as dusty, mouldy hay. Even healthy horses respond to provocation with this type of hay (Kleiber *et al.*, 2005), but RAO horses are suggested to have an exaggerated, sustained immune response. The increased expression of TLR4 could indicate an enhanced recognition of LPS with recruitment of granulocytes for both IBD and RAO.

RAO has been thought to be a type I hypersensitivity with T_H2 cells stimulating IgE production (Lavoie *et al.*, 2001). More recent data indicate that type IV reactions might be a major contributor to the pathology (Tahon *et al.*, 2009). Additionally, in RAO horses, increased expression of both T_H1 and T_H2

cytokines has been noted. IL-17A is also increased in RAO (Debrue *et al.*, 2005), which could contribute to the neutrophilia observed. However, one study showed that the neutrophilia preceded the increase in IL-17, suggesting another role for the cytokine in the disease (Ainsworth *et al.*, 2006). In the closely linked disease, inflammatory airway disease (IAD), several types of inflammation of the respiratory tract are included; neutrophilic, eosinophilic and mastocytic. IAD is either an environmental disease, induced by poor housing, or an early onset of RAO (Leclère & Lavoie, 2016). In IAD, cytokine levels vary with the type of cell infiltrates. IFN-γ, IL-4 and IL-12 are increased in mastocytic inflammation, IL-1β and IL-17 in neutrophilic inflammation (Beekman *et al.*, 2012; Lavoie *et al.*, 2011), whereas IL-5 and IL-10 as well as CXL-8 are increased in all types (Beekman *et al.*, 2012). Taken together, plasticity in the T_H subset appears crucial in the development of the hypersensitivity reaction in the lung, whereas the role for T_{REGS} in the diseases is still unknown.

A similar question remains for equine IBD, whether the chronic reaction is due to a continuous stimulation of an antigen or to a faulty regulation of the inflammation. T_{REGS} are suggested to be defective in human IBD (Hardenberg *et al.*, 2011). Interestingly, T_{REGS} and IL-10 are reduced in canine IBD compared with healthy dogs and dogs with lymphoma (Maeda *et al.*, 2015; Junginger *et al.*, 2012). T_{REGS} in the equine intestine have not been evaluated previously, but have been suggested to be impaired in horses with insect bite hypersensitivity (Hamza *et al.*, 2012). In the present material, T_{REGS} were noted in all horses examined (Paper I, IV), but their number did not correlate with the infiltrating T cells in diseased horses. This variation in proportion of T cells and T_{REGS} might be due to sampling at different time points in the disease progression. Further work on T_{REGS} function in equine IBD is needed to see whether their cytokine production is altered. Cytokines of special interest are IL-10 and TGF-β as these cytokines are suggested to mediate the regulation in horses (Hamza *et al.*, 2011). If, as in dogs, a discrepancy between lymphoma and IBD can be made by evaluating the infiltrating T_{REGS} this would be helpful in distinguishing between equine intestinal lymphoma and LPE, which can be difficult for well differentiated lymphoma today.

In the present material, the distribution of B cells and plasma cells in the healthy jejunal lamina propria (Paper I) corresponded well with the distribution of these cell types described by Packer *et al.* (2005). For IBD-afflicted horses, previous studies have found few B cells as well as less plasma cell than T cells (Mäkinen *et al.*, 2008; Divers *et al.*, 2006). It is noteworthy that in one of the studies no comparisons with normal tissue were made and in the other study

the disease LPE was evaluated. Therefore, the present thesis is the first to discriminate between healthy tissue and EEG and EGE lesions. A reduction in the B cell linage for diseased horses was shown, which was most significant in the EGE group (Paper I). In the diseased horses, B cells were occasionally seen in foci in the centre of lymphoid aggregates. The same phenomenon was described in equine eyes afflicted by ERU (Regan *et al.*, 2012; Romeike *et al.*, 1998). In the B cell dense areas of IBD horses, MHC II-expressing cell and T cell, including T_{REGS}, were co-localised with the B cells, but few immunoglobulin-producing cells were detected. A possible explanation for these B cell foci is lymphoid neogenesis, which is found in chronic inflammation of human tissues, and represents an attempt to maintain immune responses towards a persistent antigen (Aloisi & Pujol-Borrell, 2006).

In human IBD, immunoglobulins are increased in IBD-afflicted tissues (Brandtzaeg *et al.*, 2006). Particularly, the percentage of immunoglobulin classes changes from predominantly IgA production in healthy individuals to an increase in pro-inflammatory IgG production in IBD patient (Dorn *et al.*, 2002). A reduction in IgG was noted in dogs afflicted with lymphocytic IBD (Jergens *et al.*, 1996), whilst IgG was increased in eosinophilic and unspecified IBD (Kleinschmidt *et al.*, 2007; German *et al.*, 2001). In the present material, IgA, IgG and IgM positive cells in the jejunal lamina propria were reduced, significantly for IgM in the EGE group. In serum, reduced levels of IgG and IgM in IBD-afflicted horses were primarily thought to be due to losses through the inflamed intestine, and not a true deficiency in production (Lindberg *et al.*, 1985). The limited involvement of B cells and immunoglobulins in the present IBD necropsy material would suggest a down-regulation of T_H2 response in the IBD lesions. This may be in favour of a T_H1 and/or T_H17 cell response. A T_H1 response would correspond well to the granulomatous lesions noted in these horses. There was no possibility to evaluate cytokine expression in the severe lesions as the material was formalin-fixed. Therefore analysis of inflamed rectal biopsies obtained from horses where clinical evaluation was suggestive of IBD was undertaken (Paper IV). In diseased rectal tissue, IL-17A was increased in chronic active proctitis. Even if IL-17 can be produced by other cells, T_H17 cells are the main producers. Thus, T_H17 are most likely involved in the more acute phases of the proctitis. As T cells show plasticity, T_H17 cells might convert to T_H1 in the tissue, stimulating macrophages and granuloma formation. However, in chronic proctitis, IL-12 was reduced, which could contradict the role of T_H1.

A wide range of possibilities to induce T cell mediated chronic disease in the horse has been described. The role of T helper cell subsets in other equine chronic diseases are varying; T_H2 in insect bite hypersensitivity (Heimann *et*

al., 2011), T_H1 and T_H17 in ERU (Regan *et al.*, 2012; Zipplies *et al.*, 2012; Gilger *et al.*, 1999) and for RAO and IAD the subset varies between T_H1, T_H2 and T_H17 (Debrue *et al.*, 2005; Cordeau *et al.*, 2004; Ainsworth *et al.*, 2003; Beadle *et al.*, 2002). Possibly, the T helper subset varies in equine IBD as well, but to clarify such plasticity in T cell subset further studies of these cells in known stages of disease are needed. Extended studies of cytokine gene expression in the rectal biopsies including those indicative of e.g. T_H2 cells are warranted. For an evaluation of T cell plasticity in disease development to be possible, afflicted horses need to be monitored over time and sampled in different stages of disease development. Such an approach is challenging, but necessary to understand more fully the immune mechanisms of equine IBD.

6 Future perspectives

Diagnosis of an idiopathic disease is challenging, and in equine IBD the diagnosis is complicated by the inaccessibility of the primary lesions. Lesions most certainly precede the clinical signs and improved diagnostics are crucial as the clinical signs are far from unique. Analysis of accessible tissues, such as the rectum, blood and faeces is the method of choice. Here, diagnostic tools for human and canine IBD could be applicable, such as visualisation of the mucosa by video capsule enteroscopy and measuring faecal markers of inflammation (e.g. lactoferrin and calprotectin). Earlier detection of diseased animals may increase the chance of finding initiating factors for the disease.

As environmental factors have been shown to participate in the development of IBD in other species, these need to be considered for horses as well. Both geographical and seasonal factor have been suggested previously, and the possibility that changes in diet might influence IBD progression should not be excluded. Today newer tools for microbial identification facilitate the detection of non-culturable pathogens and can discern differences in the microbiota. It is known that feed alters the composition of microbiota in horses. Combining research on seasonal, geographical and feed alterations with observations on their effect on the microbiota and immune cells in the host could contribute to an understanding of the whole disease process.

The histopathology of equine IBD indicates a delayed hypersensitivity reaction. Several other diseases in the horse share this feature and combining the research on several chronic equine diseases would be beneficial for the overall understanding of equine immunology. Especially, it would be of great interest to study further the contribution of dendritic cells, macrophages and T cells, especially T_{REGS}, as these cells seemingly are important in the continuation of inflammation in several equine diseases. In the work of the present thesis, collected intestinal material was snap-frozen and stored at -80°C. This stock of frozen material gives the opportunity to study sub-populations of immune cells, using methods not applicable to formalin-fixed

tissue and the investigation of this frozen material would complement the present results. Additionally, the bank of frozen samples can be used for future research on *in vitro* cultured cells and cellular responses to microbial antigens. Established methods exist regarding the retrieval of frozen cells and the methods established in the present thesis can subsequently be used to detect altered gene expression in *in vitro*-stimulated cells.

The evaluation of genetic alterations that affect intestinal immune cells could give insight into the pathogenesis of different chronic inflammations in horses. Both TLR4 and MHC II gene alleles are of major interest to study in equine IBD, as these genes are associated with other chronic equine diseases or IBD in other species. In addition to TLR4 and MHC II, mucus production also plays an important role in the intestinal barrier function. Environmental factors that disrupt the mucus layer have been found to increase the risk of murine intestinal inflammation (Chassaing *et al.*, 2015). Genetic studies of goblet cells in horses have been limited to the airways where *MUC5AC*, but not *MUC2* is expressed (Gerber *et al.*, 2003). A change in mucins and a break in the barrier function could also be an important pathogenetic feature in equine IBD.

In conclusion, equine IBD is a complex disorder and warrants further studies to discern its pathogenesis. To increase this understanding, a comprehensive approach must be adopted that combines several research areas and includes translational as well as comparative studies across species.

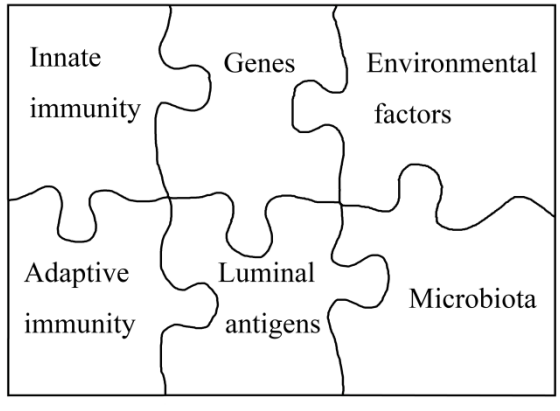


Figure 1. An illustration of the complexity in the suggested development of IBD.

7 Populärvetenskaplig sammanfattning

Kronisk idiopatisk inflammatorisk tarmsjukdom hos häst, även kallat equine inflammatory bowel disease (eqIBD), är en ovanlig, men allvarlig sjukdom som drabbar vuxna hästar oavsett kön och ras. Symtomen är framförallt avmagring, diarré och/ eller kolik. Kliniskt kan proteinförlust (hypoproteinemi och/eller hypoalbuminemi), malabsorption (försämrat upptag vid D-xylostest) och leverpåverkan ses. Ovanligare symtom är dermatit, anemi och subkutana ödem. Ibland kan sjukdomen gå i skov följda av episoder utan klinisk sjukdom. IBD är en uteslutningsdiagnos där klinisk utredning och patologanatomisk bedömning av affekterad tarm ligger till grunden för diagnos. Tre olika histologiska typer av eqIBD finns beskrivna i litteraturen; ekvin eosinofil gastroenterit (EEG), ekvin granulomatös enterit (EGE) och lymfoplasmacytär enterit (LPE). Prognosen för drabbade hästar bedömdes länge vara dålig, men har under senare år förbättrats med hjälp av långtidsbehandlingar med glukokortikoider samt kirurgi i utvalda fall. Sjukdomen saknar kända utlösande faktorer, men ett överreaktivt immunförsvar misstänks vara grundläggande i sjukdomsutvecklingen. Syftet med avhandlingen var att förbättra kunskapen om immunologisk reaktivitet i tarmen hos såväl friska hästar som hos hästar med kroniska inflammatoriska tarmsjukdomar. Teorin har varit att ett felaktigt reglerat immunförsvar ligger till grund för den kroniska tarminflammationen som ses vid eqIBD.

I den friska tarmen återfinns normalt immunceller av varierande typ, så som dendritceller, makrofager, lymfocyter och plasmaceller. Immunförsvaret har många sätt att känna igen främmande antigen. Ett av dem är receptorn TLR4 som främst uttrycks av dendritceller och makrofager. För att upprätthålla en balans där födoämnen och normalfloran inte initierar ett immunsvar finns nedreglerande mekanismer, så kallade regulatoriska celler. En av dessa celler är regulatoriska T celler (Tregs) och de hämmar andra T celler från att driva inflammatoriska processer. När ett för immunförsvaret okänt agens når tarmen startar en inflammation med rekrytering av granulocyter (neutrofiler,

eosinofiler), fagocyter (makrofager) och lymfocyter (T celler, B celler). Beroende på vilka signalsubstanser (cytokiner) som frisätts attraheras olika celltyper, däribland olika typer av T hjälpar (T_H) celler. Tidigare har man trott att det funnits två typer av T_H-celler; T_H1 som via IL-12 gett cell-medierade immunsvar och T_H2 som inducerat ett antikroppssvar (immunoglobuliner). På senare år har T_H17 upptäckts, en T_H-cell som producerar IL-17A och som är överuttryck i IBD hos människor. Hos människa har man även sett att IL-23 stiger i vävnaden hos IBD-patienter, ett cytokin som stabiliserar T_H17-celler så att de fortsätter driva inflammationen.

I avhandlingen påvisades i tarmpreparat från både EEG- och EGE-formen av eqIBD ett tydligt T-cellsdrivet immunsvar, med involvering av MHC II-positiva celler (makrofager och dendritceller). De regulatoriska T-cellerna följde inget tydligt mönster hos de sjuka hästarna, utan den individuella skillnaden var stor. Troligen beror det på att materialet kommer från kliniska fall, där hästarna befinner sig i olika stadier av sjukdomen. Få B-celler återfanns i det sjuka materialet, något som var mest påtagligt i de granulomatösa (EGE) fallen. Mängden immunoglobulinbildande celler skilde sig inte åt mellan EEG-drabbade hästar och friska hästar, men för EGE-hästarna var plasmaceller som bildade IgM mycket få.

Vid vidare studier av T-celler hos hästar med kronisk tarmsjukdom undersöktes rektumbiopsier. Biopsierna från friska hästar samt biopsier som uppvisade kronisk inflammation eller kronisk akutiserad inflammation ingick i studien. Uttrycket av cytokinerna IL-12, IL-17A och IL-23, samt receptorn TLR4 studerades med hjälp av kvantitativ PCR. Hos hästar med kronisk inflammation var uttrycket av IL-12 lägre än hos friska hästar och de med kronisk akutiserad inflammation. I den senare gruppen av hästar var uttrycker av IL-17A ökat. Detta indikerar att Th1-svaret är lågt vid kronisk inflammation och T_H17-celler bidrar till inflammationen i den akuta, kroniska sjukdomen.

Sammantaget kan resultaten i avhandlingen påvisa en dominans av T-celler i både EEG och EGE. Den samtida infiltrationen av makrofager tyder på att det är en fördröjd överkänslighetsreaktion som driver den kroniska inflammation, där ett antikroppssvar är av mindre vikt. De regulatoriska cellernas roll är fortfarande ej helt klarlagd och kräver vidare studier av cellernas funktion hos hästar. Även fortsatta studier för att se hur T_H17-celler driver eqIBD är av intresse då dessa celler troligen medverkar i de aktiva faserna av sjukdomen.

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